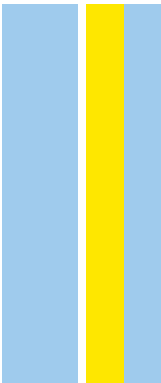


DOUTORAMENTO
BIOLOGIA MOLECULAR E CELULAR

UNDERSTANDING TUBERCULOSIS INFECTION AND
DISEASE PROGRESSION USING *MYCOBACTERIUM*
AFRICANUM AS A MODEL

Marta Lisete Santos da Silva

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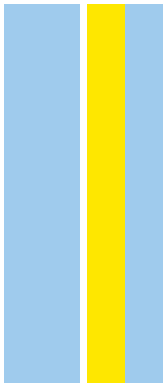
Marta Lisete Santos Silva. Understanding tuberculosis infection and disease
progression using *Mycobacterium Africanum* as model



D.ICBAS 2025

Understanding tuberculosis infection and disease progression using
Mycobacterium africanum as a model

Marta Lisete Santos da Silva



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**Understanding tuberculosis infection and disease progression
using *Mycobacterium africanum* as a model**

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PUBLICATIONS

- **Silva, M. L.**, Osório, N. S., Saraiva, M. (2025) Immunoinformatic Predictions on Variable *Mycobacterium tuberculosis* Lineage 6 T Cell Epitopes and HLA Interactions in West Africa. **Microorganisms**, 13, 1032.
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- “Host-pathogen interactions in tuberculosis: the immune response to *Mycobacterium africanum* during infection”. 03-04th October 2022, Porto, Portugal. Poster presentation at 5th PhDay | Best poster award;
- “Whole blood transcriptional analysis of *M.tuberculosis* vs *M.africanum* infected patients reveals the nuclear receptor pathway as a potential disease severity marker.” 29-31st, March 2023, Aveiro, Portugal. Poster presentation at XLVIII Annual Meeting of the Portuguese Society for Immunology;
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*“If you know you are on the right track, if you have this inner knowledge, then
nobody can turn you off...no matter what they say”*

Barbara McClintock

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TABLE OF CONTENTS

Publications _____	iii
Communications _____	iv
Agradecimientos _____	vii
Table of contents _____	viii
List of figures _____	x
List of tables _____	xii
List of abbreviations _____	xiii
Resumo _____	xvi
Abstract _____	xviii
CHAPTER I - Introduction _____	1
CHAPTER II - Aims _____	59
CHAPTER III - Immunoinformatic predictions on variable <i>Mycobacterium tuberculosis</i> Lineage 6 T cell epitopes and HLA interactions in west africa _____	62
CHAPTER IV - Initial characterization of <i>M. tuberculosis</i> L4 and L6 mixed infections _____	84
CHAPTER V - The blood transcriptome of tuberculosis patients reveals timely activation of the LXR pathway as a promising host-directed therapy _____	119
CHAPTER VI - General discussion _____	159

LIST OF FIGURES

CHAPTER I - Introduction

Figure 1. TB incidence rates worldwide in 2023.	4
Figure 2. Schematic representation of the clinical and immunological spectrum of tuberculosis infection and disease progression.	9
Figure 3. Early immune response to <i>M. tuberculosis</i> infection.	10
Figure 4. Antigen processing and presentation via the MHC class I and MHC Class II pathway.	17
Figure 5. Cellular composition and structure of a <i>M. tuberculosis</i> granuloma.	20
Figure 6. The phylogenetic structure of the MTBC.	25
Figure 7. Geographic restriction and prevalence of <i>M. africanum</i> .	26
Figure 8. Genetic identity of <i>M. africanum</i> lineages.	28

CHAPTER III - Immunoinformatic predictions on variable *Mycobacterium tuberculosis* lineage 6 T cell epitopes and HLA interactions in West Africa.

Figure 1. Schematic representation of the study.	70
Figure 2. Expression and selection of variant antigens.	72
Figure 3. Prediction of IFN- γ -inducing epitopes for the selected proteins.	73
Figure 4. Predicted number of high-binding affinity epitopes across lineages and HLA alleles.	78

CHAPTER IV - Initial characterization of *M. tuberculosis* L4 and L6 mixed infections.

Figure 1. Characterization of <i>M. tuberculosis</i> L4 and L6 isolates: in vitro and in vivo analysis.	92
Figure 2. Schematic representation of single and mixed infections with <i>M. tuberculosis</i> isolates L4 and L6 in different in vitro models and their readouts.	94
Figure 3. The presence of an L6 pathogen in L6/L4 mixed infections reduces TNF- α levels by human macrophages and PBMCs.	95
Figure 4. The presence of <i>M. tuberculosis</i> L6 in L6/L4 mixed infections reduces TNF- α levels across different cell types.	97

Figure 5. The presence of an L6 pathogen in L6/L4 mixed infections	98
reduces the production of multiple cytokines by infected mouse macrophages.	
Figure 6. The macrophage modulation by L6 pathogen in mixed infections	100
is independent of the other stimulus.	
Figure 7. The reduction of TNF- α levels is not linked to cell viability.	101
Figure 8. The presence of an inactivated L6 pathogen in a L6/L4 infection	102
does not affect the TNF- α levels by the L4-infected macrophage.	
Figure 9. Transcriptional profiles of <i>M. tuberculosis</i> L4 or L6 clinical	103
isolates in single and mixed infections are similar.	

CHAPTER V - The blood transcriptome of tuberculosis patients reveals timely activation of the LXR pathway as a promising host-directed therapy.

Figure 1. The signalling by nuclear receptors pathway associates with	126
disease severity.	
Figure 2. The LXR pathway is activated during TB.	129
Figure 3. Timely liver-X-receptor activation limits the growth of <i>M.</i>	
<i>tuberculosis</i> in the lungs of TB-resistant mice.	135
Figure 4. LXR activation reduces lung bacterial burdens and prolongs	138
survival of infected TB-susceptible mice.	
Supplementary Figure 1	152
Supplementary Figure 2	154
Supplementary Figure 3	156

CHAPTER VI - General discussion

Figure 1. PhD project main findings.	163
---	-----

LIST OF TABLES

CHAPTER I - Introduction

Table 1. Main differences discriminating <i>M. africanum</i> and <i>M. tuberculosis</i> .	29
--	----

CHAPTER III - Immunoinformatic predictions on variable *Mycobacterium tuberculosis* lineage 6 T cell epitopes and HLA interactions in West Africa.

Table 1. HLA class I allele frequencies across five West African countries.	75
--	----

Table 2. HLA class II allele frequencies across five West African countries.	76
---	----

Table 3. Predicted Differences in High-Affinity HLA Class I and II Binding Peptides for Mtb L6 and L4 Amino Acid Variants vs. Ancestral Strain, using NetMHCpan and NetMHCIIpan, in West African HLA Frequency Context.	77
--	----

CHAPTER IV - Initial characterization of *M. tuberculosis* L4 and *M. tuberculosis* L6 mixed infections.

Supplementary Table 1. List of primer-probes and oligonucleotides used in this study.	118
--	-----

CHAPTER V - The blood transcriptome of tuberculosis patients reveals timely activation of the LXR pathway as a promising host-directed therapy.

Supplementary Table 1. List of flow cytometry antibodies used in this study.	157
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Supplementary Table 2. List of Taqman probes used in this study.	158
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LIST OF ABBREVIATIONS

AEC	Alveolar epithelial cells
AM	Alveolar macrophages
APC	Antigen-presenting cells
ATRA	All trans-retinoic acid
BCG	Bacillus Calmette-Guérin
BMDM	Bone marrow derived macrophage
CCL2	Chemokine C-C motif ligand 2
CFU	Colony forming unit
CPM	Counts per million
CT	Computed tomography
DC	Dendritic cell
DEG	Differentially expressed genes
dLN	Draining lymph nodes
FACS	Fluorescent activated cell sorting
GSEA	Gene set enrichment analysis
H&E	Hematoxylin & eosin
HDT	Host directed therapy
HIV	Human immunodeficiency virus
HLA	Human Leukocyte antigen
IC	Inhibitory concentration
IFN	Interferon
IFN-I	Type I interferon
IFN-γ	Interferon gamma
IGRA	Interferon- γ release assay
IGRM	Immunity-related GTPase M

IL	Interleukin
IM	Interstitial macrophages
L4	Lineage 4
L6	Lineage 6
LTBI	Latent TB infection
LXR	Liver-X-receptor
MBL	Mannose binding lectin
MDR-TB	Multi-drug resistant TB
MHC	Major histocompatibility complex
Mtb	<i>Mycobacterium tuberculosis</i>
MTBC	<i>Mycobacterium tuberculosis</i> Complex
NAAT	Nucleic acid amplification tests
NET	Neutrophil extracellular traps
NI	Non-infected
NK	Natural killer
NOD	Nucleotide oligomerization domain
OD	Optical density
ORA	Over-representation analysis
PMA	Phorbol 12-myristate 13-acetate
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cells
PDIM	Phthiocerol dimycocerosate
PET	Positron emission tomography
PPD	Purified protein derivative
PRR	Pattern recognition receptor
RD	Region of difference

RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
SNPs	Single Nucleotide polymorphisms
SNR	Signalling by nuclear receptors
SVM	Support Vector Machine
TB	Tuberculosis
TCR	T cell receptor
Th	T helper
TNF-α	Tumor necrosis factor- α
TST	Tuberculin skin test
VAF	Variant allele frequency
WGS	Whole genome sequencing
WHO	World Health Organization
XDR-TB	Extensively drug-resistant TB

RESUMO

Palavras-chave: tuberculose, linhagem, resposta imune, co-adaptação, progressão da doença.

A tuberculose (TB), causada por *Mycobacterium tuberculosis*, tem-se mantido ao longo dos últimos anos um desafio à saúde global. De acordo com a Organização Mundial da Saúde (OMS), em 2023 contabilizaram-se um total de 10,8 milhões de novos casos e 1,25 milhões de mortes por TB. Dada a relevância destes números, torna-se cada vez mais urgente encontrar novas terapias para a TB. Este trabalho teve como principais objetivos compreender mecanismos imunológicos subjacentes, e as respectivas interações com o hospedeiro, de infeções por duas linhagens filogenéticas de *M. tuberculosis*: a linhagem 4 (L4), de distribuição global, e a linhagem 6 (L6), restrita à África Ocidental e associada a uma progressão mais lenta da doença.

Numa primeira abordagem, recorreu-se a ferramentas de imunoinformática para investigar possíveis estratégias de evasão imune por *M. tuberculosis* L6. Usando substituições não sinónimas presentes na L6, previamente identificadas em antígenos funcionais, concluiu-se que estas reduzem a afinidade de ligação a alelos de antígeno leucocitário humano de classe I e II prevalentes nas populações da África Ocidental, onde *M. tuberculosis* L6 é geograficamente restrita. Esta adaptação poderá constituir uma limitação à apresentação antigénica e consequentemente diminuir as respostas das células T, favorecendo a persistência desta bactéria nesta região, apesar da sua menor virulência em humanos e modelos animais.

No segundo estudo, analisou-se um grupo de pacientes de TB na Guiné-Bissau, onde demonstramos uma prevalência semelhante de infeções por *M. tuberculosis* L4 e L6, bem como a ocorrência de casos de infeções mistas. Dada a coexistência das duas bactérias na mesma região, a sua caracterização, assim como a dinâmica da infeção pelas duas linhagens em simultâneo é de elevado interesse. Através do uso de bactérias isoladas de pacientes, foi possível demonstrar que infeções por *M. tuberculosis* L4 induzem uma produção de citocinas em células mielóides significativamente superior à observada nas infeções por *M. tuberculosis* L6. Contudo, em infeções mistas, a *M. tuberculosis* L6 tem a capacidade de suprimir a resposta imune desencadeada por *M. tuberculosis* L4. Estas interações sugerem mecanismos distintos de modulação imune entre as linhagens.

No terceiro estudo, utilizou-se transcriptómica de sangue total de doentes para explorar as respostas imunológicas em TB. A via de sinalização por recetores nucleares (SNR) destacou-se como um regulador-chave da severidade da doença. Validações funcionais em

modelos animais demonstraram que a ativação farmacológica do recetor X hepático (LXR), componente da SNR, em fases tardias da infeção reduziu significativamente a carga bacteriana, associando-se a um maior efluxo de colesterol, com impacto mínimo no perfil celular imunológico. Estes resultados posicionam a SNR como um biomarcador de severidade e a ativação do LXR como uma promissora terapia dirigida ao hospedeiro.

Em suma, com este trabalho foi possível elaborar novas hipóteses sobre a restrição geográfica de *M. tuberculosis* L6, abrindo novas linhas de investigação focadas na diversidade do patogéneo. Adicionalmente, tirando partido de uma região com uma grande diversidade de linhagens, elucidamos interações imunológicas distintas entre *M. tuberculosis* L4 e L6. Por fim, demonstramos o potencial terapêutico da ativação tardia do LXR, e a sua possível classificação como biomarcador da severidade da doença. Estas descobertas abrem caminho a investigações futuras sobre o impacto destas interações no diagnóstico, tratamento e prognóstico da TB.

ABSTRACT

Keywords: Tuberculosis, lineage, immune responses, host-directed therapies, disease progression.

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a critical global health challenge. According to the WHO, in 2023, over 10.8 million people developed TB, and more than 1.25 million lost their lives to this disease. Tackling TB demands innovative preventive, diagnostic, and therapeutic strategies. Achieving this requires a deeper understanding and redefinition of the cellular and molecular mechanisms that drive TB protection. *M. tuberculosis* comprises ten phylogenetic lineages with distinct global distributions and clinical outcomes. Lineage 4 (L4) is globally widespread, while lineage 6 (L6) is geographically restricted to West Africa and associated with slower disease progression. My PhD was dedicated to the study of the immune mechanisms underlying infections by pathogens belonging to these two lineages.

In a first study, we used immunoinformatic tools to investigate possible immune evasion strategies employed by *M. tuberculosis* L6 that contribute to this restriction. In a set of functionally validated antigens, we identified *M. tuberculosis* L6-specific nonsynonymous substitutions that were predicted to reduce binding affinity of the peptide only to human leukocyte antigen (HLA) Class I and Class II alleles prevalent in West African populations. This feature may limit antigen presentation and T cell responses in a lineage-specific manner, and may therefore constitute an adaptation underlying the persistence of *M. tuberculosis* strains of L6 in West Africa despite their lower virulence in mice and humans.

In a second study, we resorted to a novel TB cohort constructed in Guinea-Bissau. We firstly revealed a similar prevalence of TB caused by infections with *M. tuberculosis* of L4 and L6, underscoring the shared contribution of these lineages to the regional TB burden. In these cohort we also identified patients who were simultaneously infected with *M. tuberculosis* of L4 and L6. Subsequently, we found that the cytokine production by myeloid cells upon *M. tuberculosis* L4 infections is much higher than that observed for *M. tuberculosis* L6 infections. Strikingly, in mixed infection experiments, we discovered that *M. tuberculosis* L6 overcomes the strong myeloid responses induced by *M. tuberculosis* L4 infections. Interestingly, we observed that live *M. tuberculosis* L6 is required to interfere with *M. tuberculosis* L4 infections. Thus, different mechanisms operate in *M. tuberculosis* L4 or L6 infected myeloid cells, conditioning their response to infection.

Finally, in a third study, we leveraged the strain diversity in our Guinea-Bissau cohort and used whole blood transcriptomics to investigate immune responses in TB patients. While shared TB immune signatures were identified, the "signalling by nuclear receptors" (SNR) pathway emerged as a key modulator, correlating with disease severity. Functional validation in mouse models demonstrated that pharmacological activation of the SNR component liver-X-receptor (LXR) at late infection stages significantly reduced lung bacterial loads and enhanced cholesterol efflux, with minimal impact on immune remodelling. These findings suggest that the SNR pathway may serve as a biomarker for TB severity, and offer LXR activation as promising host-directed therapy. By integrating transcriptomic, disease severity and experimental models, this study advances our understanding of TB pathology and highlights the therapeutic potential of targeting metabolic pathways in TB treatment.

Overall, my PhD generated new hypotheses for the geographic restriction of *M. tuberculosis* complex L6; highlighted the distinct immune interactions between *M. tuberculosis* L4; and demonstrated the use of LXR activation as a promising host directed therapy (HDT) for TB; and L6, opening new avenues for research into how these interactions may affect TB outcomes, diagnosis and treatment.

CHAPTER I - INTRODUCTION

Part of this chapter has been published in:

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1. TUBERCULOSIS: AN ANCIENT DISEASE FACING MODERN CHALLENGES

Tuberculosis (TB) remains one of the most ancient and deadly infectious diseases afflicting humankind^{1,2}. Evidence of TB has been identified in archaeological remains dating back over 5000 years³, emphasizing its long-standing impact on human health. Despite the remarkable advances in medical sciences, including the development of vaccines and antibiotics, the global burden of TB persists. According to the World Health Organization (WHO), in 2023 alone, an estimated 10.8 million new cases of TB were reported worldwide, resulting in approximately 1.25 million deaths⁴, making TB the leading cause of death from a single infectious agent. Throughout the XIX century, TB was a major public health threat, exacerbated by poor living conditions and rapid urbanization. The identification of *Mycobacterium tuberculosis* by Robert Koch in 1882 was an important hallmark in TB research, revolutionizing the understanding of the disease as an infectious entity^{3,5}. Subsequent developments, including the Bacillus Calmette-Guérin (BCG) vaccine, led to a gradual decline in incidence rates in many parts of the world⁶, and the discovery of effective antimicrobials brought hope for TB control⁷. However, despite these breakthroughs, challenges such as the spread of drug-resistant strains, the co-epidemic with the human immunodeficiency virus (HIV), and socioeconomic factors like poverty and malnutrition have stymied efforts to eliminate the disease⁸⁻¹¹.

The global challenge has been exacerbated by the emergence of multidrug-resistant (MDR-TB) and extensively drug-resistant TB (XDR-TB), with resource-limited settings being particularly affected^{12,13}. The interplay between TB and other associated comorbidities, such as diabetes and HIV, has further complicated the management of the disease^{14,15}. More recently, the COVID-19 pandemic has complicated the landscape of TB management, reducing the number of diagnostics and delaying the beginning of appropriate treatment, leading to an alarming resurgence in incidence and mortality rates⁴. The pandemic also revealed the fragile nature of TB control programs and the need for robust, integrated healthcare systems capable of managing concurrent global health threats^{11,16,17}. Advances in diagnostic technologies, such as the Xpert *M. tuberculosis*/RIF assay, discussed further in the next section, have improved the speed and accuracy of TB detection¹⁸. Nevertheless, despite these innovations, TB continues to disproportionately affect vulnerable populations in low- and middle-income countries, where barriers to healthcare remain substantial¹⁹. In the face of these enduring challenges, TB remains a critical focus of global public health efforts. Addressing the persistent burden of disease requires not only continued research into novel therapeutics and vaccines, but also sustained political and financial commitment to improve healthcare access, reduce transmission, reinforce TB research, and ultimately, end the TB epidemic^{12,13,19,20}.

1.1. EPIDEMIOLOGY OF TUBERCULOSIS

In 2023, an estimated 10.8 million people developed TB, with a global incidence rate of 133 cases per 100,000 individuals. This marked an increase from 10.6 million cases in 2022, 10.3 million cases in 2021, and 10.0 million in 2020, which reinforces the consequences of the decrease in diagnostics during the COVID-19 pandemic. TB incidence rose by 1.9% between both 2020-2021 and 2021-2022, largely due to disruptions in diagnosis and treatment during the COVID-19 pandemic, which saw a significant drop in newly diagnosed TB cases from 7.1 million in 2019 to 5.8 million in 2020 and 6.4 million in 2021. Of note, one cannot exclude a certain drop in actual TB cases, due to the COVID-19 lockdowns⁴.

TB cases were primarily concentrated in the regions of South-East Asia (45%), Africa (24%), and the Western Pacific (17%), with eight countries - India, Indonesia, China, the Philippines, Pakistan, Nigeria, Bangladesh, and the Democratic Republic of the Congo - accounting for over two-thirds of global cases (Fig.1).

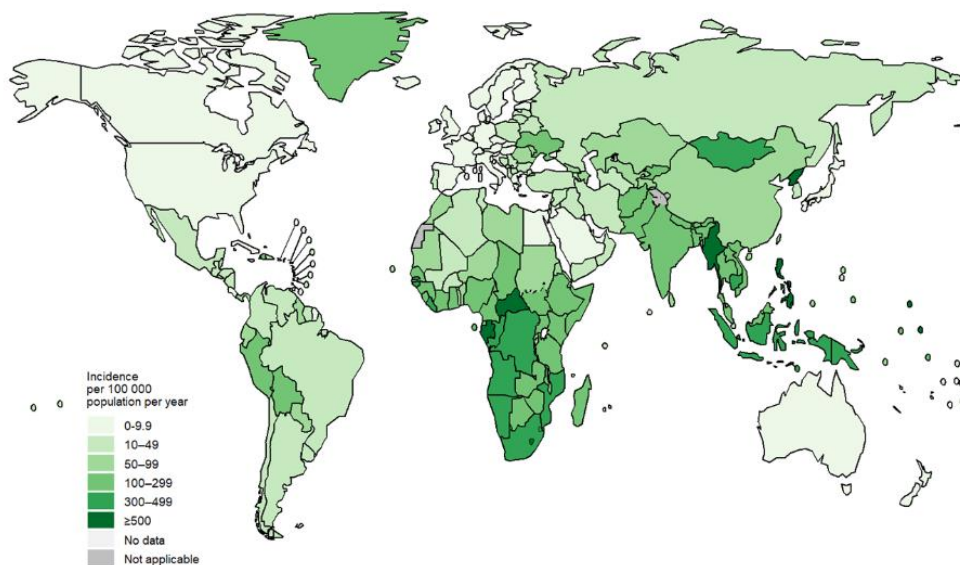


Figure 1. Tuberculosis incidence rates worldwide in 2023. The map depicts the estimated incidence rates of tuberculosis (TB) across the globe, highlighting significant regional disparities. Countries with the highest TB incidence, exceeding 500 cases per 100,000 population per year, are concentrated in Sub-Saharan Africa and parts of Southeast Asia. Moderate incidence rates (100–299 cases per 100,000) are observed in South Asia, Eastern Europe, and parts of the Western Pacific. Low-incidence regions (0–49 cases per 100,000) include North America, Western Europe, and Oceania. Areas with no data or inapplicability are marked in grey. The distribution emphasizes the disproportionate burden of TB in resource-limited settings. From¹.

Adult men represented the highest burden of disease in 2023, comprising 55% of cases, compared to 33% in women and 12% in children. Furthermore, TB-HIV coinfection was most prevalent in the African region. Although case notifications increased in 2022, only 75% of estimated cases were diagnosed, with lower detection rates among children under five years old (48%). Addressing the global rise in TB incidence will require time, with projections indicating that reductions may begin to occur by 2024 or 2025.⁴

1.2. PREVENTION, DIAGNOSIS AND TREATMENT OF TUBERCULOSIS

Over the years, numerous efforts have been made to improve both diagnostic and treatment strategies of TB. Multiple diagnostic tools have undergone development and refinement to improve TB case identification and optimize treatment strategies. The outcomes following infection are diverse, with some individuals successfully clearing the infection, others developing latent TB infection (LTBI), and a fraction progressing to active TB²¹.

The diagnosis of active TB relies on a combination of radiographic, microbiological, and molecular methods, such as chest X-ray, sputum microscopy, culture-based diagnostics, and nucleic acid amplification tests (NAATs)²². In cases of active TB, bacteriological methods such as sputum smear microscopy and culture remain the leading diagnostic methods, but molecular diagnostics, particularly the Xpert *M. tuberculosis*/RIF assay, have significantly improved the speed and accuracy of detection. This assay not only identifies *M. tuberculosis* DNA but also detects mutations conferring resistance to rifampicin⁴. Newer versions of the Xpert test can now detect resistance to additional drugs, including isoniazid and fluoroquinolones, which are critical in the treatment of MDR-TB²³. Advances in whole-genome sequencing (WGS) also offer promising potential for identifying drug resistance mutations and improving our understanding of TB transmission and outbreak dynamics^{24,25}. Furthermore, the integration of digital chest X-rays with AI-based software endorsed by the WHO transforms TB screening by bypassing the need for sputum samples, this approach is revolutionary²⁶. Researchers are developing affordable and portable digital X-ray systems, with the aim of expanding accessibility in primary care settings²⁷. Additionally, potential biomarkers are being evaluated through the use of emerging tools such as cough and lung sound recordings and AI-driven analysis to further improve TB screening and diagnosis by implementing non-invasive and scalable methods^{27,28}.

LTBI is characterized by a lack of clinical symptoms despite immunological evidence of infection²⁹. The detection of LTBI is commonly based on immune-based tests such as the tuberculin skin test (TST) and interferon- γ (IFN- γ) release assays (IGRAs). Both methods rely on memory T cell responses to *M. tuberculosis* antigens, however they present

limitations, such as low sensitivity in immunocompromised individuals, as in HIV-positive individuals, and an inability to distinguish latent from active TB^{30,31}. Additionally, these tests fail to indicate the viability of *M. tuberculosis* bacilli, as T cell responses can persist long after successful treatment and bacilli elimination^{32,33}. Comparisons between TST and IGRA in predicting LTBI progression to active TB have been inconsistent³⁴, highlighting the need for more reliable diagnostic methods²⁹. Thus, more accurate diagnostic tools are urgently needed to identify individuals at high risk of progression, particularly those transitioning from latent or subclinical TB to active disease³⁵. Looking ahead, host transcriptional profiling holds promise as a future diagnostic tool, allowing for the identification of immune signatures specific to active TB. Early studies have shown that transcriptomic signatures, particularly those based on interferon-related genes, can reliably distinguish active TB from LTBI and healthy controls³⁶⁻³⁸. Although this approach requires further validation and refinement, it represents a promising avenue for the development of more precise diagnostic tools^{37,39}.

Despite these diagnostic advancements, TB treatment remains a long and complex process. The standard treatment for drug-susceptible TB recommends a six-month antibiotic regimen, starting with an intensive phase of isoniazid, rifampicin, pyrazinamide, and ethambutol (or streptomycin), followed by a continuation phase of isoniazid and rifampicin⁴⁰. However, patient non-compliance due to adverse effects such as hepatotoxicity and renal dysfunction represents a significant challenge, leading to treatment failure, relapse, and the emergence of drug-resistant strains⁴. The increasing incidence of MDR-TB, which is resistant to at least isoniazid and rifampicin, further complicates treatment, often requiring extended regimens of 9-24 months, using more toxic and costly drugs. The rise of XDR-TB, resistant to both first-line and second-line drugs, exacerbates the public health burden⁴. To complement these diagnostic advances, the development of novel antitubercular agents such as bedaquiline, delamanid and pretomanid has begun to address the challenges of prolonged, toxic multidrug regimens. Bedaquiline, a diarylquinoline that inhibits mycobacterial ATP synthase, demonstrated accelerated sputum-culture conversion and improved cure rates when added to optimized background therapy in a pivotal phase II trial⁴¹. Delamanid, a nitroimidazole targeting mycolic acid synthesis, significantly increased two-month culture conversion rates in patients with multidrug-resistant TB in randomized studies⁴². Pretomanid, a nitroimidazooxazine effective against both replicating and nonreplicating bacilli, achieved high favorable outcome rates in highly drug-resistant TB when combined with bedaquiline and linezolid in the Nix-TB regimen⁴³. Collectively, these all-oral, shorter-course therapies offer improved tolerability

and better compliance, representing a paradigm shift in the management of drug-resistant TB.

Vaccination remains one of the main strategies for TB prevention. However, BCG, the only vaccine currently available, has been in use for nearly a century and offers variable protection against pulmonary TB in adults (0-80%), though it is highly effective in preventing severe forms such as TB meningitis and miliary TB in infants^{44,45}. Given the limited efficacy of BCG in adults, intensive research is ongoing to develop novel vaccines. Candidates include live-attenuated strains, inactivated whole-cell vaccines, protein subunits, and viral vector-based platforms, many of which are currently in clinical trials^{38,46}. However, so far no TB vaccine candidate has proven more efficacious than BCG, highlighting the gap in knowledge of what actually composes TB protective responses⁴⁷.

1.3. THE SPECTRUM OF TUBERCULOSIS DISEASE

Transmission of *M. tuberculosis* occurs via inhalation of aerosol droplets containing infectious bacilli, which, once in the lungs, establish infection and may lead to varying clinical outcomes^{21,48}. Commonly, coughing has been considered the primary mode of transmission, as it expels large amounts of infectious particles⁴⁹. However, recent evidence indicates that *M. tuberculosis* can also be detected in smaller particles exhaled during normal breathing, raising concerns that the risk of transmission may have been underestimated⁴⁹. Classically, following infection, individuals would be classified as active TB patients, presenting symptoms, for instance, a productive cough, haemoptysis, fevers, night sweats, and significant weight loss or as LTBI (asymptomatic but with evidence of infection)⁴⁸. Notably, productive cough, particularly in cases of cavitary lung disease, serves as a hallmark of pulmonary TB⁴⁸. In severe cases, destruction of bronchial arteries can result in massive haemoptysis, a serious complication that can lead to life-threatening outcomes⁵⁰. Although TB is primarily a pulmonary disease, it can also manifest in extrapulmonary forms, involving virtually any organ^{22,51}.

Growing evidence challenges the traditional binary classification of TB into active and latent forms, instead supporting a dynamic spectrum of disease from initial infection clearance to severe, symptomatic disease^{50,52}. In a cohort of HIV-1-infected individuals previously classified as having LTBI, subclinical forms of TB have been identified. These individuals, while asymptomatic, exhibited lung lesions indicative of early TB disease and were more likely to progress to active disease⁴⁹. This suggests that the clinical classification of TB may underestimate the extent of disease, as many individuals likely progress through subclinical stages before manifesting symptoms³². Furthermore, historical autopsy studies support this

hypothesis, showing signs of active TB in individuals who died from non-TB causes, without any record of TB symptoms during life^{53,54}. More recently imaging studies using advanced techniques such as computed tomography (CT) and positron emission tomography (PET) scans have detected metabolically active granulomas in asymptomatic individuals, further supporting the concept of a TB spectrum^{53,54}.

Based on these evidence, it is now accepted that there are several outcomes following *M. tuberculosis* infection (Fig.2). After infection, certain individuals may resist the establishment of the pathogen or even clear it due to their immune system. A second outcome of the infection is when the individuals become infected but remain asymptomatic, harbouring the bacteria in a latent, dormant state. Nonetheless, these individuals are thought to have the potential to progress to active disease at some point in their lifetime⁵². In both scenarios, whether the infection is cleared or concealed, there are no clinical symptoms, nor are there positive findings from bacteriological, radiological, or molecular diagnostic tests. In both cases (clearance or latent), TST or IGRA results can be either negative or positive. When positive, these cases are classified as LTBI. However, the implications for disease progression and transmission risk differ significantly⁵⁰. This highlights the importance of looking at TB as a spectrum and to develop advanced diagnostics that could indicate whether someone could be in progression from latent or sub-clinical disease to active manifestation^{35,52}. The progression from LTBI to active TB is influenced by several host-related factors, most notably co-infections such as HIV, as well as conditions like diabetes and immunosuppression^{55,56}. On a molecular level, whole blood transcriptomic studies have identified immune pathways associated with TB pathogenesis, including a dominant IFN signature that correlates with the severity of radiological findings⁵⁰. This signature, primarily driven by neutrophils, has been detected in both active TB patients and in a subset of LTBI individuals, suggesting that it may serve as a biomarker for progression to active disease^{36,37}. Serological studies have also uncovered distinct glycosylation patterns of anti-*M. tuberculosis* antibodies between LTBI and active TB patients, further indicating a potential for refining diagnostic tools⁵². Collectively, these findings underscore the need for novel biomarkers capable of distinguishing between the different stages of TB and identifying individuals at risk of disease progression^{35,50}. Moreover, TB progression often includes a subclinical phase, where individuals are sputum-culture positive but asymptomatic. During this phase, standard diagnostic tools, such as chest radiographs, may fail to detect any abnormalities, making subclinical TB underreported^{52,57}. This highlights the critical need for more sensitive diagnostic methods that can identify TB at earlier stages, ideally before transmission occurs. Understanding the

molecular and immunological mechanisms that govern the transitions along this spectrum is crucial for improving TB diagnostics and treatment strategies.

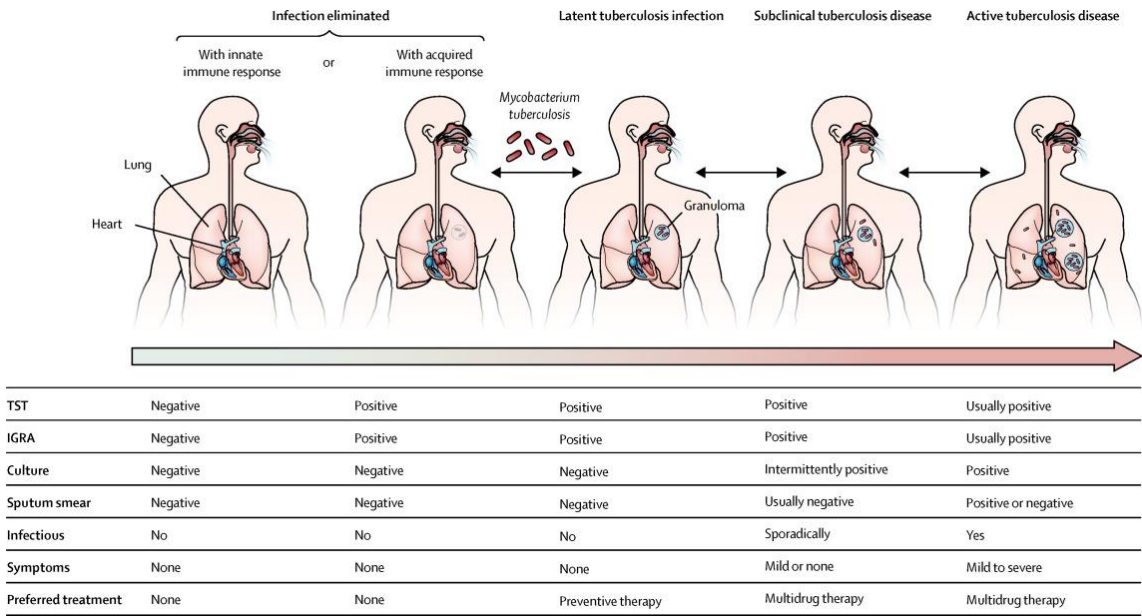


Figure 2. Schematic representation of the clinical and immunological spectrum of tuberculosis infection and disease progression. The stages range from infection elimination (via innate or acquired immune responses) to latent tuberculosis infection (LTBI), subclinical tuberculosis, and active tuberculosis disease. Diagnostic outcomes and clinical characteristics are summarized, including TST, IGRA, culture, sputum smear, infectiousness, symptoms, and recommended treatments. Each stage differs in diagnostic profiles and potential infectiousness, highlighting the complexity of tuberculosis management and progression. **Abbreviations:** TST, tuberculin skin test; IGRA, interferon- γ release assay. From⁹.

2. HOST IMMUNE RESPONSES TO TUBERCULOSIS

The immune response to TB is highly complex (Fig.3). Research in humans and experimental models has highlighted the importance of specific molecular and cellular mediators in shaping both protective immunity and the pathogenesis of TB. The following sections will focus on the key cellular players and mechanisms explored throughout this project, providing an overview of the innate and adaptive immune responses involved.

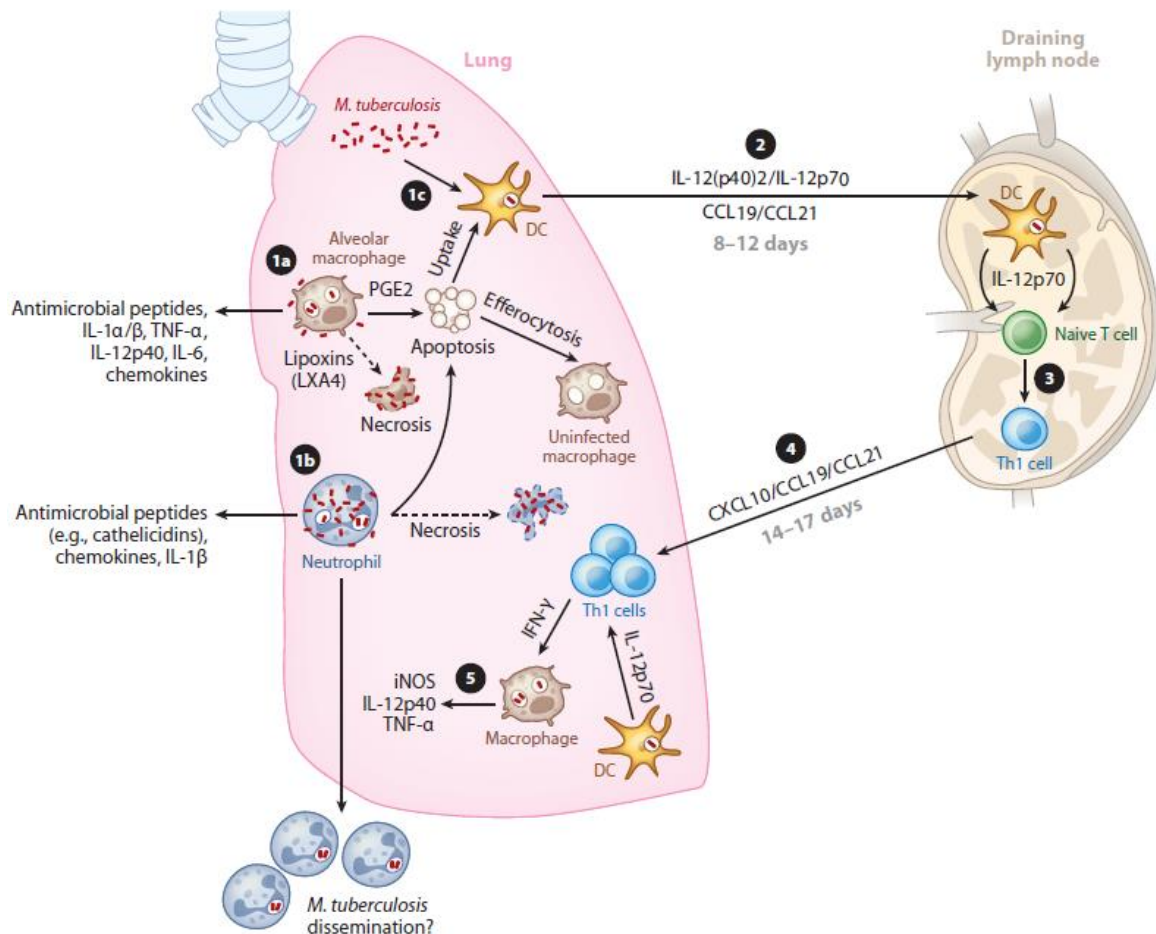


Figure 3. Early immune response to *Mycobacterium tuberculosis* infection. Following inhalation, *M. tuberculosis* is phagocytosed by alveolar macrophages, triggering the release of cytokines and antimicrobial peptides. Infected macrophages may undergo apoptosis or necrosis, the latter promoting bacterial spread. Neutrophils are recruited and contribute to the inflammatory response. Dendritic cells (DCs) migrate to the draining lymph node to activate CD4⁺ T cells into Th1 cells (8–12 days), which then return to the lung (14–17 days) and produce IFN- γ to activate macrophages and enhance bacterial control. Ineffective responses may lead to bacterial dissemination. **Abbreviations:** IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; From⁹⁰.

2.1. EARLY INNATE IMMUNE RESPONSES TO TUBERCULOSIS

M. tuberculosis is mainly transmitted via aerosol droplets, which are expelled when an individual with active TB coughs. These droplets, containing *M. tuberculosis* bacilli, are inhaled into the airways, where they encounter the alveolar space and initiate infection⁵⁸⁻⁶⁰. Alveolar macrophages (AMs) play a key role in the host immune responses to TB as the first host cells to interact with the bacteria in the lungs. AMs initiate a cascade of immune events through the recognition of pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors. This interaction triggers a coordinated network of signalling pathways that leads to the recruitment of other immune cells and establishes the early immune environment⁶¹. *M. tuberculosis* enters AMs through a variety of receptors, including scavenger receptors, complement receptors, and the mannose receptor, which collectively mediate bacterial uptake⁶². Upon internalization, the bacterium resides in the phagosome, where it employs mechanisms to avoid elimination⁶³. While non-viable bacilli are rapidly degraded⁶³, viable *M. tuberculosis* can inhibit phagosome-lysosome fusion, preventing phagosome acidification and allowing the bacteria to survive within the macrophage⁶⁴. Interestingly, when the bacteria are opsonized before infection, this blockade is overcome, enabling normal phagosome maturation and improved bacterial clearance⁶⁴. Additionally, autophagy, a process in which cellular components and pathogens are sequestered into autophagosomes and degraded in lysosomes, further contributes to bacterial elimination. Autophagy can be activated, enhancing phagosome acidification and increasing the host ability to kill *M. tuberculosis*^{65,66}.

Upon phagocytosing *M. tuberculosis*, AMs migrate from the alveoli into the lung interstitium, contributing to the progression of the host immune response^{59,63}. This early interaction between *M. tuberculosis* and AMs sets the stage for either bacterial containment or disease progression⁵⁹. In addition to AMs, alveolar epithelial cells (AECs) also contribute to the lung's initial immune defence. AECs produce surfactant proteins that have been shown to play a role in controlling *M. tuberculosis* growth in vitro⁶⁷. These early events highlight the multifaceted cellular responses in the lung during *M. tuberculosis* infection, underscoring the significance of both classical and non-classical immune cells in modulating the course of the disease^{60,68}.

As infection progresses, other immune cells are recruited to the lungs. Neutrophils, eosinophils, and dendritic cells (DCs) are essential components of the innate immune response to *M. tuberculosis*^{69,70}. Neutrophils are among the first immune cells recruited to the site of infection and have been described as having both protective and detrimental

roles during TB⁶⁹⁻⁷¹. On one hand, neutrophils are capable of phagocytosing *M. tuberculosis*; however, they are relatively inefficient in killing the bacteria and may instead serve as permissive hosts for *M. tuberculosis* replication^{70,71}. This dual nature is further exemplified in experimental models, where excessive neutrophil infiltration leads to exacerbated lung pathology, increased tissue damage, and higher mortality rates^{72,73}. Interestingly, lung neutrophils can facilitate the activation of antigen-specific CD4⁺ T cells by delivering *M. tuberculosis* to DCs, which subsequently initiate the adaptive immune response⁷⁴. However, some *M. tuberculosis* strains, such as H37Rv, inhibit neutrophil apoptosis, leading to delayed activation of naive CD4⁺ T cells in lung-draining lymph nodes⁷⁵. In contrast, a pro-apoptotic *M. tuberculosis* mutant (nuoG) accelerates bacterial uptake by DCs, promoting faster CD4⁺ T cell priming⁷⁵. These observations suggest that the influence of neutrophils in TB outcomes is shaped by both host and bacterial genetics, as well as the stage of infection^{69,75}. Expanding beyond experimental models, in humans, the dual nature of neutrophils has also been further described in TB pathogenesis through various transcriptomic analyses. A major study identified a 390-transcript signature dominated by IFN-inducible genes, linked to active TB and related to disease extent and inflammation. This signature was significantly driven by neutrophils, highlighting their importance in TB pathogenesis⁷⁶. These results established the prominence of neutrophils in the immune landscape of TB. Subsequent studies supported this finding, showing that neutrophils not only predominate in the peripheral blood of TB patients⁷⁷ but also exhibit distinct transcriptomic profiles, contributing to a signature associated with TB severity⁷⁶. Furthermore, it has been demonstrated that upregulated pathways in IFN signalling have been linked to neutrophil responses, and further correlated with TB severity and clinical outcomes⁷⁸.

The complex interplay between neutrophils and type I interferon (IFN-I) signalling in TB has been extensively studied due to its potential to significantly influence disease progression and immune regulation. Neutrophils can release DNA-containing structures known as neutrophil extracellular traps (NETs), which play a critical role in triggering immune responses. NETs activate plasmacytoid dendritic cells (pDCs), promoting the production of IFN-I, which then contributes to further inflammatory signalling in the lungs, facilitating an environment supportive of TB infection⁷⁹⁻⁸¹. IFN-I signalling, in turn, intensifies neutrophil recruitment to the lungs and induces a shift in haematopoiesis toward granulocyte production, which prioritizes neutrophil generation over lymphocyte (especially T cells) production. This increase in neutrophils driven by IFN-I leads to an excessive formation of NETs, which has been associated with higher bacterial loads and more severe outcomes of the disease in animal models⁸². Furthermore, this granulocyte-biased haematopoiesis

suppresses effective T-cell responses, weakening the adaptive immunity against *M. tuberculosis* and allowing the bacteria to evade immune control⁸². Thus, a feedback loop where neutrophils and IFN- γ amplify each other's activity operates in TB, exacerbating lung damage and worsening TB pathogenesis, emphasizing how an overactive IFN- γ response can inadvertently support the disease rather than containing it. Collectively, these findings support the notion that monitoring neutrophil-driven signatures could refine diagnostic and therapeutic approaches, offering a clearer picture of TB progression and recovery. Tight control over neutrophil function could allow these cells to contribute to protection without exacerbating disease⁷³.

Regarding eosinophils, it has been described that these cells are also part of the early immune response to *M. tuberculosis*. Although their precise function in TB remains unclear, studies have shown that mice lacking eosinophils are more susceptible to infection, and that there is an inverse correlation between eosinophil numbers and TB severity in patients^{70,83,84}. This suggests a protective function for eosinophils in TB, in contrast to neutrophils, whose elevated numbers are often associated with worse clinical outcomes⁷⁰.

Macrophage populations in the lung are heterogeneous, with distinct subsets contributing to different aspects of the immune response against *M. tuberculosis*. AMs and recruited interstitial macrophages (IMs) differ notably in their functional roles, metabolic states, and response to *M. tuberculosis* infection. AMs, which primarily rely on fatty acid oxidation, tend to be more permissive to *M. tuberculosis* survival, creating a niche within the alveolar space that supports bacterial persistence^{61,85,86}. In contrast, monocyte-derived IMs and other recruited macrophages are characterized by a highly glycolytic metabolic profile, which is associated with enhanced antimicrobial activity and a greater ability to limit *M. tuberculosis* growth than AMs^{61,85-87}. The recruitment of these monocyte-derived macrophages, primarily driven by chemokines like chemokine C-C motif ligand 2 (CCL2)^{88,89}, highlights the dynamic and adaptable nature of the macrophage response during TB infection^{54,78,81}. Additionally, recent studies have revealed transcriptional diversity within lung macrophage populations, identifying specific subpopulations that can differentially support or restrict *M. tuberculosis* replication, with some macrophages facilitating granulomas formation that can either contain or exacerbate infection⁸⁷. Furthermore regarding metabolic regulation in macrophages, it was recently demonstrated that glycolysis-associated pathways in IMs enhance reactive oxygen species (ROS) production and autophagy, mechanisms essential for *M. tuberculosis* control⁸⁷. By modulating their metabolic states, these macrophages shift from a permissive to a restrictive state, supporting an immune environment less favourable to *M. tuberculosis* survival^{90,91}.

Together, these early innate immune responses are critical in shaping the host's ability to control or succumb to *M. tuberculosis* infection. The interplay between different immune cell populations and the metabolic and transcriptional changes they undergo in response to infection are key determinants of the outcome, influencing whether *M. tuberculosis* is cleared, contained, or allowed to proliferate, leading to active disease^{35,86}.

Furthermore, DCs act as antigen-presenting cells (APCs) forming the crucial bridge between the innate and adaptive immune responses. DCs phagocytose *M. tuberculosis*, migrate to the draining lymph nodes (dLNs), and present antigens to T cells, thereby initiating the adaptive immune response⁹², that will be discussed in more detail in the following section.

2.2. ADAPTIVE IMMUNE RESPONSES TO TUBERCULOSIS

The initiation of the adaptive immune response in TB is deeply influenced by early innate immune events. APCs, DCs and blood monocytes, migrate from the site of infection to the dLNs, where they prime naïve T cells for a subsequent adaptive response⁹³. This migration occurs about 2 to 3 weeks post-infection in the mouse model⁹⁴. Importantly, *M. tuberculosis* can manipulate APC responses and maturation, which may influence the type of adaptive immunity elicited, potentially contributing to disease progression⁹⁵. APCs prime both CD4⁺ and CD8⁺ T cells in response to *M. tuberculosis* antigens, initiating their activation and subsequent immune functions⁹⁴. T lymphocytes are central to immune defence against TB, as demonstrated by the susceptibility of T cell-deficient individuals, including humans, nonhuman primates, and mice, to rapidly progressive TB disease⁹⁶⁻⁹⁸. Among these, CD4⁺ T cells are critical for protective immunity: the depletion of CD4⁺ T cells in HIV-positive individuals is associated with an increase susceptibility to TB, while antiretroviral therapy that restores CD4⁺ T cells counts reduces this susceptibility⁹⁶.

APCs drive the differentiation of T helper (Th)1 and Th17 cells, with interleukin (IL)-12 promoting Th1 responses and IL-6, IL-1 β , and IL-23 supporting Th17 development. Both subsets are essential for generating an effective immune response against *M. tuberculosis*^{99,100}. Th1 cells produce IFN- γ , a key cytokine that activates macrophages and enhances their bactericidal mechanisms, such as phagolysosome fusion and nitric oxide production^{65,101,102}. CD4⁺ T cells are a primary source of IFN- γ during infection, that has a crucial role of controlling bacterial growth, ensuring the survival of the host¹⁰³. In mice lacking CD4⁺ T cells, infection advances uncontrolled, emphasizing the essential role of IFN- γ in T cell-mediated immunity¹⁰³. Previous studies have been demonstrating that deficiencies in the Th1-IFN- γ axis, such as in Mendelian susceptibility to mycobacterial

disease or HIV infection, result in an increased vulnerability to TB¹⁰⁴⁻¹⁰⁶. Moreover, in the mouse model of infection, it has been demonstrated that IFN- γ is essential for *M. tuberculosis* control, once mice lacking IFN- γ exhibit uncontrolled bacterial growth and severe tissue damage, emphasizing the role of this cytokine for an effective immune responses^{105,107}. Furthermore, IFN- γ also plays an essential role in modulating immune responses, including inhibiting Th17 responses and regulating the exacerbation of neutrophil activity to minimize tissue damage¹⁰⁸⁻¹¹⁰. IL-12 is another major contributor in the immune response to *M. tuberculosis*, as it drives the differentiation of naive CD4⁺ T cells into Th1 cells, a subset that is vital for protective immunity in TB^{111,112}. IL-12 increases the production of IFN- γ by T cells and NK cells, establishing a positive feedback loop that sustains the Th1 response that is critical for controlling *M. tuberculosis* replication¹¹³. In mouse models, IL-12 deficiency leads to impaired IFN- γ production and an inability to control bacterial growth, highlighting its importance in anti-TB immunity¹¹⁴. In patients with active disease high levels of IL-12 have been correlated with disease severity and inflammation^{115,116}. Moreover, mutations in IL-12 receptor β 1 (IL-12R β 1), that are one of the most frequent causes of Mendelian Susceptibility to Mycobacterial Disease, is associated with a high risk of several infections by *M. tuberculosis*, highlighting the importance of IL-12 in this disease¹¹⁷⁻¹¹⁹. Another cytokine, IL-17, produced predominantly by Th17 cells, also play a key role in the immune response to *M. tuberculosis*¹²⁰. IL-17 induces the production of chemokines that recruit neutrophils to the site of infection, consequently contributing to the containment of *M. tuberculosis* in the initial stages of infection^{121,122}. Nevertheless, the role of IL-17 in TB is complex, as excessive neutrophil recruitment can also contribute to tissue damage and disease, as discussed above¹²³. In summary, the interplay between Th1 and Th17 responses, mediated by cytokines such as IL-12, IFN- γ , and IL-17, is critical for the effective control of *M. tuberculosis* infection. Understanding these dynamics offers valuable insights into potential therapeutic strategies aimed at modulating immune responses to combat tuberculosis.

Even though the role of CD8⁺ T cells is less prominent than that of CD4⁺ T cells, they also contribute to TB immunity. CD8⁺ T cells induce the production of fundamental cytokines such as IFN- γ , tumour necrosis factor- α (TNF- α), and IL-2, that have critical roles in the immune response to TB including the activation of macrophages and control of infection^{124,125}. Additionally, CD8⁺ T cells possess cytotoxic functions, allowing them to kill infected cells directly¹²⁴. Interestingly, CD8⁺ T cells may be more essential during the chronic phase of infection, as their depletion has been linked to increased bacterial burdens in mice¹²⁴. The role of CD8⁺ T cells in TB extends beyond cytotoxicity once they also play a role in antigen cross-presentation, which enhances the activation of CD4⁺ T cells and

contributes to a more robust immune response. This interaction between CD8⁺ and CD4⁺ T cells is essential for the development of effective adaptive immunity against TB, influencing the outcome of the infection¹²⁶. However, despite robust CD4⁺ and CD8⁺ T cell responses to antigens, the pathogen often persists within the host for life, allowing for reactivation, progression, and further transmission of TB. While the full spectrum of mechanisms underlying *M. tuberculosis* persistence and immune evasion remains unclear, they include modulation of antigen expression, suboptimal antigen presentation, and interference with T cell effector functions¹²⁷.

The role of B cells in TB immunity is still not fully understood, although recent studies suggest a more complex involvement than previously thought. B cells produce a variety of immune mediators and appear to play a modulating role in immune responses during TB infection¹²⁸. Their involvement seems to be more relevant during the acute phase of infection, and there is emerging evidence of their importance in granuloma integrity and bacterial containment within this structure¹²⁸⁻¹³³. Antibodies from individuals with LTBI have been shown to promote macrophage-mediated killing of intracellular *M. tuberculosis*, suggesting a potentially protective humoral response¹³⁴.

2.3. T CELL-EPILOPE RECOGNITION - THE DETAILED CASE OF TUBERCULOSIS INFECTIONS

For T cells to produce an effective response, they must recognize specific epitope peptides presented by major histocompatibility complex (MHC) molecules on the surface of APCs. CD4⁺ T cells recognize peptides bound to MHC class II molecules, whereas CD8⁺ T cells recognize those bound to MHC class I molecules (Fig.4). For an epitope to be immunogenic, it must bind with sufficient affinity to one or more MHC proteins and be recognized by the T cell receptor (TCR), a critical point in the process of adaptive immune response¹³⁵. Variations in epitope sequences can disrupt MHC binding or TCR recognition, facilitating immune escape^{136,137}.

On the other side, microbial pathogens must adapt to the host environments to successfully replicate and evade the host's innate and adaptive immune defences. In pathogens that encounter adaptive immune responses, one well-known adaptation mechanism is antigenic variation. This process involves the alteration of pathogen targets, typically recognized by antibodies and T lymphocytes, through mutations that enable the pathogen to escape immune detection and subsequent clearance¹³⁸⁻¹⁴¹. As pathogens evolve to escape the immune system through changes in their antigens, the host's immune system reciprocates

by generating antibodies and T cells specific to these newly selected antigens, perpetuating a continuous cycle of immune evasion and adaptation^{142,143}.

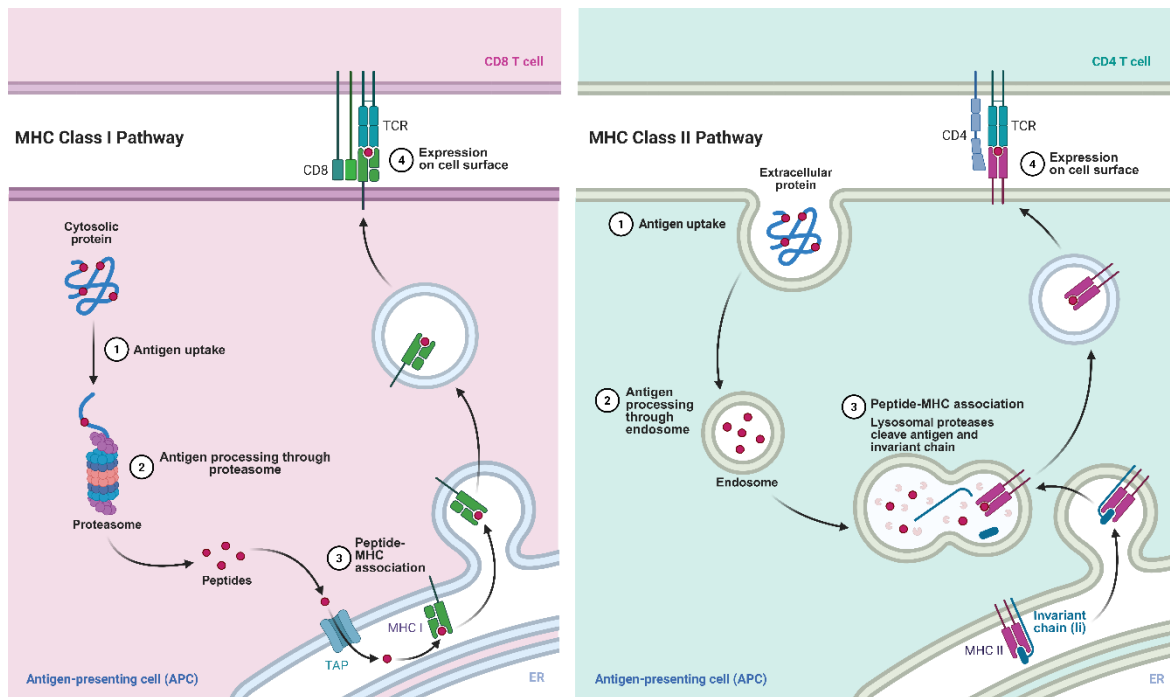


Figure 4. Antigen processing and presentation via the MHC class I and MHC Class II pathway - Left panel - MHC class I pathway: Endogenous (cytosolic) proteins are degraded into peptides by the proteasome. These peptides are translocated into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP). Peptides are loaded onto major histocompatibility complex (MHC) class I molecules in the ER. The peptide–MHC class I complexes are then transported to the cell surface for presentation to CD8⁺ T cells via the T cell receptor (TCR), triggering cytotoxic immune responses. Right panel - MHC class II pathway: Exogenous (extracellular) proteins are internalized and degraded into peptides within endosomal compartments. MHC class II molecules, synthesized in the ER and stabilized by the invariant chain (Ii), are transported to endosomes where the invariant chain is degraded. Peptides associate with MHC class II molecules, and the resulting peptide–MHC class II complexes are transported to the cell surface for presentation to CD4⁺ T cells via the TCR, initiating helper T cell responses. **Abbreviations:** TCR, T cell receptor; MHC, major histocompatibility complex; ER, endoplasmic reticulum; TAP, transporter associated with antigen processing. Illustration downloaded from BioRender.

The dynamic process of immune evasion and adaptation can result in persistent infections, including viral¹⁴⁴, parasitic, or bacterial infections^{138,141}. Moreover, antigenic variation plays a crucial role in pandemics, as seen in influenza¹⁴⁵, and can lead to reinfection after recovery from previous disease¹⁴⁶. The implications for vaccine development are substantial, often requiring multivalent formulations, such as those used for poliovirus and *Streptococcus pneumoniae*, or necessitating regular updates, as seen with seasonal influenza vaccines. In extreme cases, such as with HIV, antigenic diversity presents one of

the greatest challenges for vaccine development, as achieving protection against a wide array of viral strains remains elusive¹⁴⁷.

M. tuberculosis, an obligate human pathogen, has coevolved with its human host for millennia¹⁴⁸. Its success as a pathogen is based on its efficient transmission and its remarkable capacity to evade the host's adaptive immune responses¹²⁷. In contrast to the aforementioned pathogens, in *M. tuberculosis*, T cell epitopes exhibit a significant degree of evolutionary hyperconservation, a feature that is crucial for the pathogen's survival and immune evasion. This conservation enables efficient recognition by the human immune system, contributing to the persistence of the pathogen¹⁴⁹. A comprehensive study identified 491 validated human T cell *M. tuberculosis* epitopes, revealing minimal sequence variation and a significantly reduced nonsynonymous-to-synonymous mutation ratio when compared to both essential and nonessential genes¹⁴⁹. These findings highlight strong purifying selection exerting pressure on these epitopes. Subsequent analysis of 16 antigens across 99 strains further confirmed the hyperconservation of these epitopes, suggesting that *M. tuberculosis* takes advantage of their preservation as a strategy for immune evasion¹⁴⁹. The hyperconserved epitopes elicit robust T cell responses, especially in LTBI individuals. T cells that efficiently bound to these conserved epitopes demonstrate similar phenotypic characteristics across diverse mycobacterial species, implying a shared mechanism of immune recognition¹⁵⁰.

The hyperconservation of T cell epitopes has been observed in other species, including various bacteria and viruses. Notable examples include RNA viruses such as measles, rubella, and influenza type C¹⁵¹. One proposed explanation for this phenomenon is the structural constraint of viral proteins or the detrimental effects of mutations, which result in reduced infectivity and transmission. However, infections by these RNA viruses typically lead to acute disease, with rapid immune clearance, particularly through adaptive immunity. In contrast, *M. tuberculosis* infections often result in chronic disease, where adaptive immunity fails to eliminate the pathogen. Moreover, while infections by RNA viruses confer some degree of protection against reinfection, TB does not follow this pattern, as reinfections and mixed infections have been increasingly reported in recent years¹⁵¹. Several hypotheses have been proposed to explain the selective pressure exerted by *M. tuberculosis* to hyperconserve these epitopes. First, T cell responses are essential for the establishment of latent infection, as evidenced by the rapid progression to active disease in CD4+ T cell-deficient HIV-positive individuals following *M. tuberculosis* infection^{152,153}. Second, growing evidence suggests that T cell responses may play a role in human-to-human transmission of *M. tuberculosis*, supporting the idea that the preservation of T cell epitopes may benefit the pathogen¹⁵³.

2.4. THE TUBERCULOSIS GRANULOMA

The granuloma has long been recognized as the hallmark pathological feature of TB, a distinction that has been acknowledged by pathologists for over a century^{154,155}. This structure forms around the infecting *M. tuberculosis*, the causative agent of TB, effectively isolating the bacteria from the host immune response¹⁵⁴. These structures consist of a necrotic core filled with bacteria and surrounded by cells aggregates (Fig.5). Initially these structures serve as a defence mechanism, however *M. tuberculosis* may exploit them to its own advantage¹⁵⁵. A failure to establish a well-formed granuloma can result in the dissemination of bacteria, perpetuating infection among individuals¹⁵⁵.

Granulomas are fundamentally described as compact and organized aggregates of macrophages that are formed in response to persistent stimuli, providing a structural framework for the immune response^{154,156-160}. In the context of TB, granulomas are initially form as aggregates of macrophages recruited to the site of infection¹⁶¹. These macrophages undergo differentiation into various phenotypes, including mature macrophages, epithelioid macrophages, foamy macrophages, and multinucleated giant cells, which together create a macrophage-dominated core¹⁶². A distinctive feature of TB granulomas is the formation of necrotic regions, resulting from the death of participating cells, particularly macrophages. These necrotic areas, referred to as caseum when observed in gross pathology, are a hallmark of the disease^{163,164}.

As the infection progresses, the granuloma architecture evolves with the recruitment of additional immune and stromal cell populations. These include DC's, neutrophils, natural killer (NK) cells, T and B lymphocytes, fibroblasts, extracellular matrix-secreting cells, and epithelial cells, which contribute to the maintenance of the structural organization of the granuloma¹⁵⁵. In its mature state, the granuloma is typically characterized by a necrotic, caseous core enriched with *M. tuberculosis* bacilli, encircled by infected macrophages and lymphocytes¹⁵⁵. While granulomas serve as a containment strategy, limiting bacterial dissemination, they simultaneously provide a protected niche that facilitates pathogen persistence. This dual role underscores the complex interplay between host defence mechanisms and pathogen survival strategies¹⁶⁵.

However, *M. tuberculosis* can remain viable within the hypoxic and nutrient-limited environment of aging pulmonary granulomas, a phenomenon widely associated with LTBI³⁵.

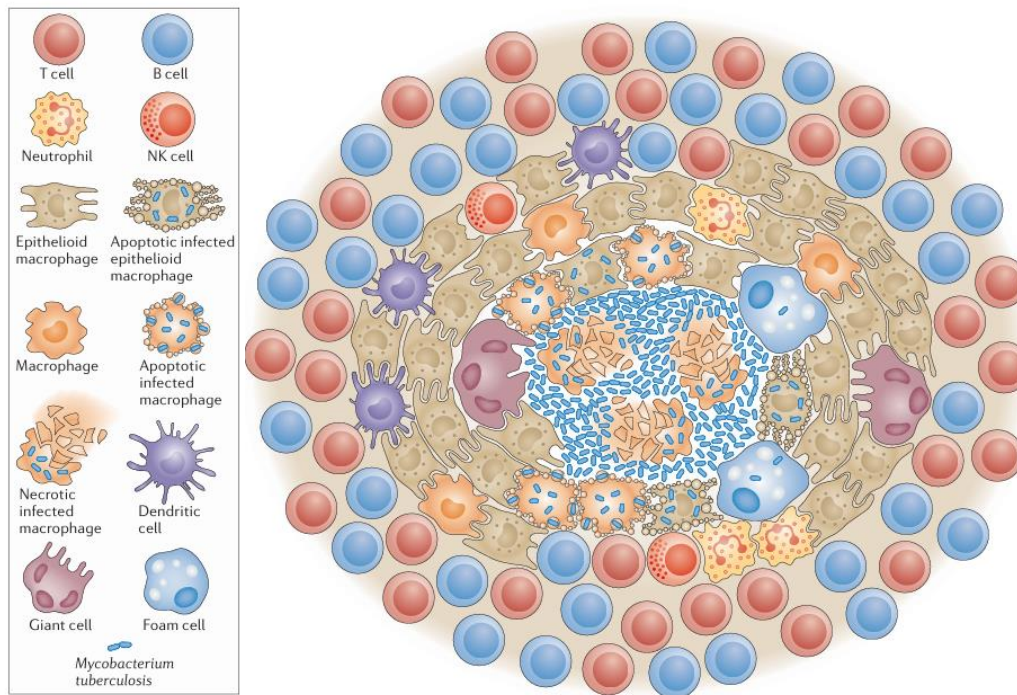


Figure 5. Cellular composition and structure of a *M. tuberculosis* granuloma. The diagram illustrates the intricate cellular organization within a *M. tuberculosis* granuloma, a hallmark of tuberculosis pathology. The central core contains infected and necrotic macrophages, laden with bacilli (blue), surrounded by layers of macrophages, epithelioid cells, and immune cells. Outer layers comprise lymphocytes, including T cells (red) and B cells (blue), along with NK cells (red with a dotted nucleus) and dendritic cells (purple). Foam cells and giant cells are also present in the structure, contributing to granuloma stability and immune modulation. This complex microenvironment reflects the dynamic interplay between pathogen persistence and host immune response. **Abbreviations:** NK, natural killer cells. From¹²⁰.

Furthermore, studies using zebrafish models have revealed that *M. tuberculosis* leverages granuloma formation to promote its proliferation^{166,167}. Through the RD1 locus, the bacteria promote the recruitment and chemotaxis of macrophages, driving the death of infected macrophages, which are subsequently phagocytosed by newly recruited macrophages. This cycle enables the spread of infection within the granuloma^{166,168}. These findings challenge the traditional view of granulomas as static barriers to infection, instead highlighting their role as dynamic hubs that facilitate bacterial replication and dissemination. This paradigm shift highlights the intricate interplay between host immune structures and pathogen strategies¹⁶⁹.

The granuloma can also have an impact in the *M. tuberculosis* transmission since immune imbalances can lead to granuloma disruption, resulting in the formation of cavities within the lungs, which in turn facilitates the escape of bacteria and subsequent dissemination through respiratory droplets, thus promoting disease transmission¹⁶⁵. For instance, the

suppression of TNF- α is associated with granuloma dysfunction and the reactivation of LTBI¹⁷⁰.

Experimental studies have been raising the importance of the complex, dynamic and heterogeneous nature of the granuloma¹⁷¹. The heterogeneity of granulomas has been observed in humans through proteomic analyses of individual granulomas¹⁷². The detailed molecular profiling has revealed that distinct proteome signatures exist within the granuloma, organized into separate compartments that may change as the granuloma progresses¹⁷². Recognizing this heterogeneity is essential for advancing our understanding of TB transmission dynamics and improving models of antibiotic distribution and efficacy during treatment¹⁷³. A finely balanced immune response is vital throughout the infection process to establish protective immunity, enabling pathogen clearance while minimizing tissue damage¹⁷⁴.

2.5. CYTOKINES IN THE IMMUNE RESPONSE AGAINST TUBERCULOSIS

Cytokines act as fundamental drivers in the host's immune response to *M. tuberculosis*, balancing the activation of immune cells and simultaneously preventing excessive inflammation that could lead to tissue damage.

Among the most essential cytokines in TB is TNF- α , that promotes macrophage activation, enhancing the production of reactive nitrogen intermediates (RNIs), which are crucial for the intracellular killing of *M. tuberculosis*^{175,176}. Moreover, TNF- α drives the production of chemokines that recruit immune cells to the site of infection, and promotes apoptosis in infected macrophages, preventing *M. tuberculosis* replication¹⁷⁶⁻¹⁷⁸. Furthermore, TNF- α is essential for the formation and maintenance of granulomas that are crucial to contain the infection and prevent bacterial dissemination^{179,180}. In the mouse model, deficiencies in TNF- α lead to impaired immune responses, resulting in higher susceptibility to TB¹⁸¹⁻¹⁸³. In humans, the same phenotype is recapitulated, as anti-TNF therapies, while effective for autoimmune diseases, can reactivate latent TB due to reduced immune surveillance¹⁸².

IFNs constitute a critical axis of host immune regulation during *M. tuberculosis* infection, with type I (IFN- α and IFN- β) and type II (IFN- γ) interferons exerting opposing effects. While IFN- γ is indispensable for host survival, orchestrating macrophage activation and promoting bacterial clearance, type I IFNs are recognized for their detrimental influence on disease outcome¹⁸⁴. Notably, type I IFN signalling has been implicated in facilitating *M. tuberculosis* pathogenesis, potentially by subverting host immunity to favour bacterial persistence. This is particularly evident in infections with the hypervirulent *M. tuberculosis* strain HN878,

where elevated type I IFN activity correlates with suppression of protective cytokines, contributing to increased pathogenicity¹⁸⁴. In addition, type I IFNs have been shown to antagonize IFN- γ -mediated antimicrobial responses¹⁸⁵, further compromising host defence. A prominent neutrophil transcriptional signature, enriched in type I IFN related genes, has also been observed in the peripheral blood of patients with active TB⁷⁶, supporting a role for this cytokine family in immunopathology rather than protection. Furthermore, in mouse models of infection, it was demonstrated that a type I IFN dominated response is associated with disease progression similarly to the transcriptional signatures described in humans¹⁸⁶.

IL-10 plays a dual role in the immune response to *M. tuberculosis*. Primarily, this cytokine has an immunosuppressive role in TB, modulating the inflammatory response to prevent excessive tissue damage. While this regulation is necessary for maintaining tissue integrity, IL-10 can also inhibit macrophage activation and reduce TNF- α and IFN- γ production, thus impairing the host's ability to clear the infection¹⁸⁷⁻¹⁹¹. Higher levels of IL-10 have been associated with an increase in susceptibility to *M. tuberculosis* infection due to the decrease in the production of some key cytokines as TNF- α and IFN- γ ^{115,116}. Furthermore, early IL-10 exposure leads to CD4⁺ T cells with reduced expression of CXCR3, limiting their migration to infected tissues, which is critical for effective immune responses against pathogens¹⁹². Elevated levels of IL-10 are often associated with chronic infection and may contribute to the persistence of *M. tuberculosis* in the host¹⁹⁰. In the mouse model, it was demonstrated that in the absence of IL-10, *M. tuberculosis* infection resulted in less bacterial burden in the lungs¹⁹³.

The pro-inflammatory cytokines IL-1 α and IL-1 β , which belong to the IL-1 family, are predominantly secreted by monocytes, macrophages, and DCs in response to *M. tuberculosis* infection. In response to *M. tuberculosis*, these cytokines engage with the IL-1 receptor (IL-1R), widely expressed across various cell types, triggering a cascade of signalling events that activate downstream regulatory pathways, leading to a robust inflammatory response that is protective for the host¹⁹⁴. In the context of TB, both IL-1 α and IL-1 β have been shown to be essential for maintaining host resistance against *M. tuberculosis*¹⁹⁴. Notably, mice lacking either IL-1 α or IL-1 β , as well as those deficient in IL-1R, exhibit higher susceptibility to infection and increased bacterial loads within the lungs^{195,196}. Recent investigations have elucidated that the function of IL-1 β extends beyond mere bacterial burden control; it is also crucial for initiating early protective mechanisms against *M. tuberculosis*¹⁹⁷ and facilitating the dissemination of bacilli from the alveolar region to the lung interstitium⁵⁹. Additionally, recent evidence highlights the importance of IL-1R signalling in constraining the number of infected cells and in preventing further dissemination, thereby enhancing host defence¹⁹⁸. However, it is essential to note that

excessive production of IL-1 β may be detrimental, being associated with severe forms of the disease and increased pulmonary damage¹⁹⁸. The production of IL-1 β in response to *M. tuberculosis* is associated with the pathogen's ability to evade host cytosolic surveillance systems, particularly in macrophages. Research indicates that genetic diversity among *M. tuberculosis* isolates significantly influences IL-1 β secretion and the severity of tuberculosis TB outcomes³¹.

These cytokines and others, TNF- α , IFN- γ , IL-12, IL-10, IL-1, and IL-17, form a tightly regulated network that influences the outcome of *M. tuberculosis* infection. Understanding their interactions is crucial for developing new immunotherapeutic strategies attempting to increase the protective immunity while minimizing tissue damage during TB infection.

3. *MYCOBACTERIUM TUBERCULOSIS*: THE CAUSATIVE AGENT OF TUBERCULOSIS

In 1882, Robert Koch identified *M. tuberculosis* as the causative agent of TB, marking a pivotal moment in the understanding of this disease. *M. tuberculosis* is a member of the genus *Mycobacterium* and the family *Mycobacteriaceae*, characterized as an aerobic pathogen that thrives at 37°C and typically forms visible colonies with rough morphology after 3-6 weeks of growth^{199,200}. Like other mycobacteria, *M. tuberculosis* is a gram-positive, acid-fast bacillus that can be identified using the Ziehl-Neelsen stain. Its lipid-rich cell wall and mycolic acid content contribute to its acid-fast properties and resistance to traditional gram staining^{199,200}.

The first *M. tuberculosis* whole genome sequence was obtained for the strain H37Rv and revealed a genome comprising 4,411,529 base pairs, rich in guanine and cytosine^{201,202}. Notably, the *M. tuberculosis* genome contains a significant number of genes encoding Pro-Glu (PE) and Pro-Pro-Glu (PPE) proteins, which, while not yet fully understood, are believed to play an important role in the pathogen's ability to infect hosts given their abundance, comprising 7-10% of the genome^{201,202}. WGS of *M. tuberculosis* has rapidly transitioned from a research tool to a clinical and public health application for the diagnostic and managing of TB, enabled by decreases in sequencing costs and advances in technology²⁰³. Molecular approaches like WGS can provide rapid diagnosis, drug susceptibility profiling, and an understanding of *M. tuberculosis* transmission clusters^{203,204}.

M. tuberculosis belongs to the *M. tuberculosis* complex (MTBC) and is the most prominent member of this complex in terms of health. The animal and human strains within the MTBC are divided in lineages according to distinct genetic classifications based on WGS^{203,205}. This classification is important for the identification of specific strains and its characteristics as drug resistance profiles and transmission dynamics allowing for a more effective management of the impact in public health¹⁴⁸. The MTBC includes closely related bacteria with relatively little genomic variation²⁰⁶. This group comprises several human-adapted pathogens, such as *M. tuberculosis sensu stricto* and *Mycobacterium tuberculosis* var. *africanum*, as well as animal-adapted strains like *M. bovis* (infecting cattle), *Mycobacterium caprae* (goats and sheep), *Mycobacterium pinnipedii* (seals and sea lions), *Mycobacterium microti* (voles), and *Mycobacterium orygis* (oryxes)^{207,208}. Other notable members of the genus *Mycobacterium* that cause human diseases include *Mycobacterium ulcerans* (the causative agent of Buruli ulcer) and *Mycobacterium leprae* (responsible for leprosy)³⁵. Currently, 10 distinct lineages of the MTBC have been identified (Fig.6), emerging as a result of human migration and evolution during the Neolithic period^{207,208}. These lineages

show strong phylogeographical structures, with lineages 2, 3, and 4 distributed globally, while lineages 1, 5, 6, 7 and 8 present more geographically restricted distributions²⁰⁹.

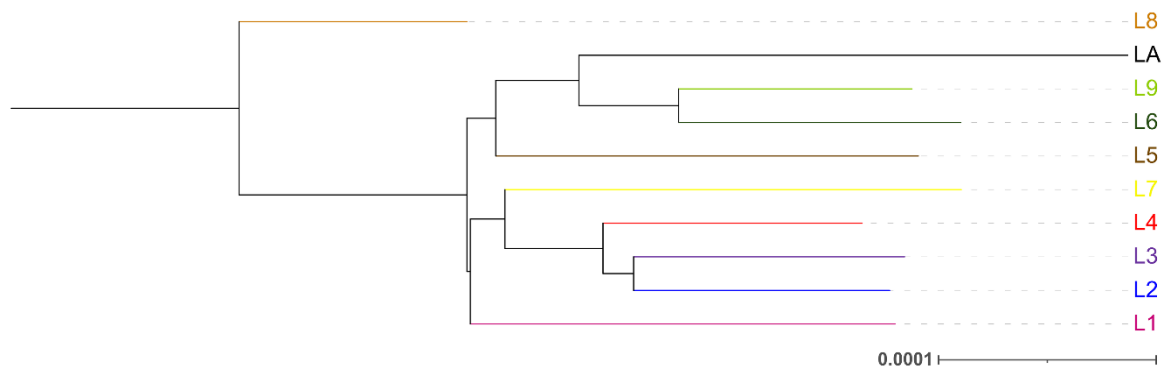


Figure 6. The phylogenetic structure of the MTBC. Phylogenetic analysis of 10 MTBC genomes selected to include 1 genome from each of the known MTBC lineages (accession numbers: SRR1162469, ERR2704812, ERR181314, SRR10828835, ERR1193734, SRR8237291, ERR3470572, ERR3470655, ERR756344, and ERR015582). A maximum likelihood tree was created with IQ-TREE v2.1.2 using TVM+F+I (the best-fit model of substitution according to AIC). The tree was coloured using iTOL 6.5.2 with the commonly used colour scheme for the different MTBC lineages: Lineage 1 (L1) in pink; Lineage 2 (L2) in blue; Lineage 3 (L3) in purple; Lineage 4 (L4) in red; Lineage 5 (L5) in brown; Lineage 6 (L6) in green; Lineage 7 (L7) in yellow; Lineage 8 (L8) in light brown; Lineage 9 (L9) in light green; and animal-associated lineages (LA) in black. Strains from L1–L4 and L8 are considered *M. tuberculosis sensu stricto* and L5, L6, and L9 *M. africanum*. The scale bar indicates the number of nucleotide substitutions per site. **Abbreviations:** AIC, Akaike information criterion; MTBC, *Mycobacterium tuberculosis* complex. From²⁷².

Recent investigations into whole-genome sequencing and genotyping databases have led to the identification of a new lineage of the MTBC, designated as L10, found in central Africa. This lineage is distinguished by a new region of deletion, IS6110 insertions, and 243 SNPs, including notable mutations in *gyrA*, *recN*, and *dnaG*. L10 is closely related to lineages L6 and L9, found in western and eastern Africa, respectively, and provides a potentially critical insight into the evolutionary history and migration patterns of *M. tuberculosis* var. *africanum*, thereby enhancing our understanding of its diversity across the continent²¹⁰.

These lineage characteristics play a key role in TB transmission and evolution and will be further elaborated in subsequent sections. The scope of this thesis is centred on *Mycobacterium tuberculosis* var. *africanum*, a distinctive pathogen within the *M. tuberculosis* complex. The following sections will delve into the biology, epidemiology and immune response to this member of MTBC.

3.1. TUBERCULOSIS CAUSED BY *M. TUBERCULOSIS* VAR. *AFRICANUM*

As discussed above TB remains one of the most significant global health threats, primarily caused by the closely related bacteria. While *M. tuberculosis sensu stricto* has a worldwide presence, *M. tuberculosis var. africanum* also formerly known as *M. africanum* is predominantly found in West Africa (Fig.7), where it poses a major health challenge. Despite the identification of several differences between these pathogens, *M. tuberculosis var. africanum* is considerably less studied than its counterpart. In the following review, we delve into the genetic, phenotypic, and clinical distinctions and similarities between the two pathogens. We also explore the current understanding of the immune response to *M. tuberculosis var. africanum*, including its implications for disease progression and geographical distribution, raising important questions that remains unanswered.

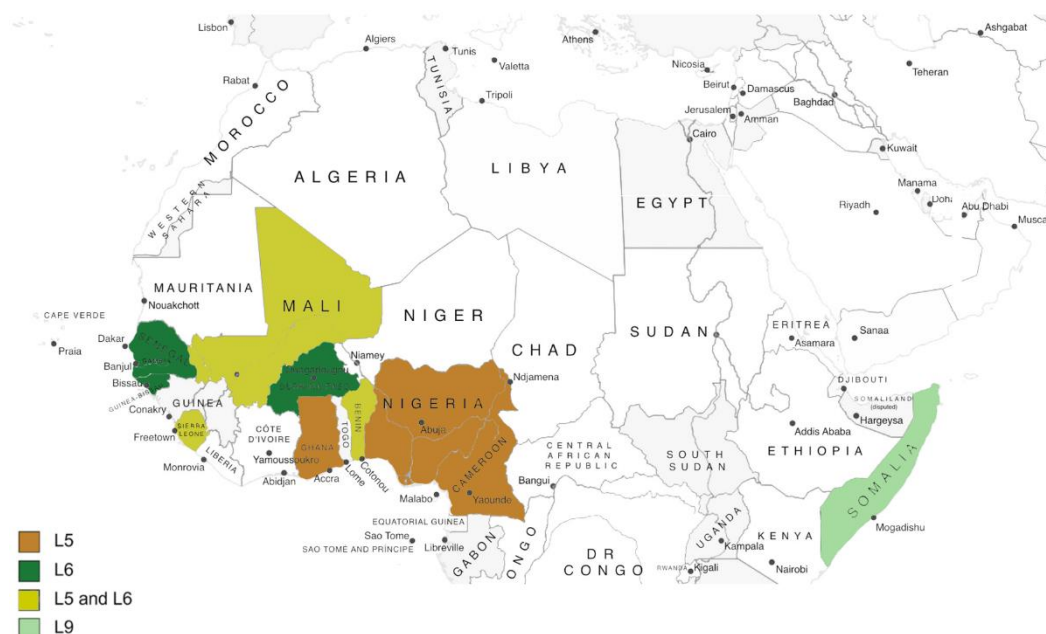


Figure 7. Geographic restriction and prevalence of *M. africanum*. Geographic distribution of *M. africanum* lineages 5, 6, and 9 across Africa. Created with <https://mapswire.com/africa/political-maps/>.

This part of the introduction is based on the following publication:

PLOS PATHOGENS

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REVIEW

Tuberculosis caused by *Mycobacterium africanum*: Knowns and unknowns

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3.2. THE IDENTITY OF *M. TUBERCULOSIS* VAR. *AFRICANUM*

M. africanum strains were initially divided into two main subtypes based on their geographic origin: *M. africanum* subtype I, for strains originating from West Africa and *M. africanum* subtype II for strains from East Africa²¹¹. Subsequent genetic studies placed strains of *M. africanum* subtype II, also known as the “Uganda genotype”, as belonging to a sublineage of L4, thus leaving *M. africanum* strains restricted to West Africa^{211,212}. More recently, comparative genomics approaches established the presence or absence of specific genomic regions from the common ancestor in *M. tuberculosis* and *M. africanum*, allowing *M. africanum* strains to be subdivided into two genetically distinct lineages: L5 and L6 (previously known as Maf1 and Maf2, respectively)^{156,213} (Fig.8). *M. tuberculosis* and *M. africanum* strains are readily distinguishable by the deletion of the region of difference (RD) 9 in the genome of *M. africanum* strains. The genome of strains within L5 has a further deletion in RD711 while genomes of strains within L6 are characterized by a deletion of RD702²¹³. Through spoligotyping, L5 and L6 can be respectively identified by the loss of spacers 8-12 and 37-39 (L5), and 7-9 and 39 (L6) in their genomes^{213,214}. Together, these genomic deletions can be used as phylogenetic markers distinguishing *M. africanum* from *M. tuberculosis* strains and allowing the genomic identification of L5 and L6 strains (Figure 3). More recently, with the increasing availability of multi-locus and whole genome sequencing and analyses, several specific SNPs in the genome of strains belonging to L5 and L6 have been identified, which are the basis of SNP assays for rapid identification of strains belonging to L5/L6 *M. africanum* lineages^{215,216}. Whole genome analyses also revealed the existence of genetic diversity within *M. africanum* L5 and L6 strains²¹⁷⁻²²⁰ and allowed the construction of detailed MTBC phylogeny trees. It is interesting to note that

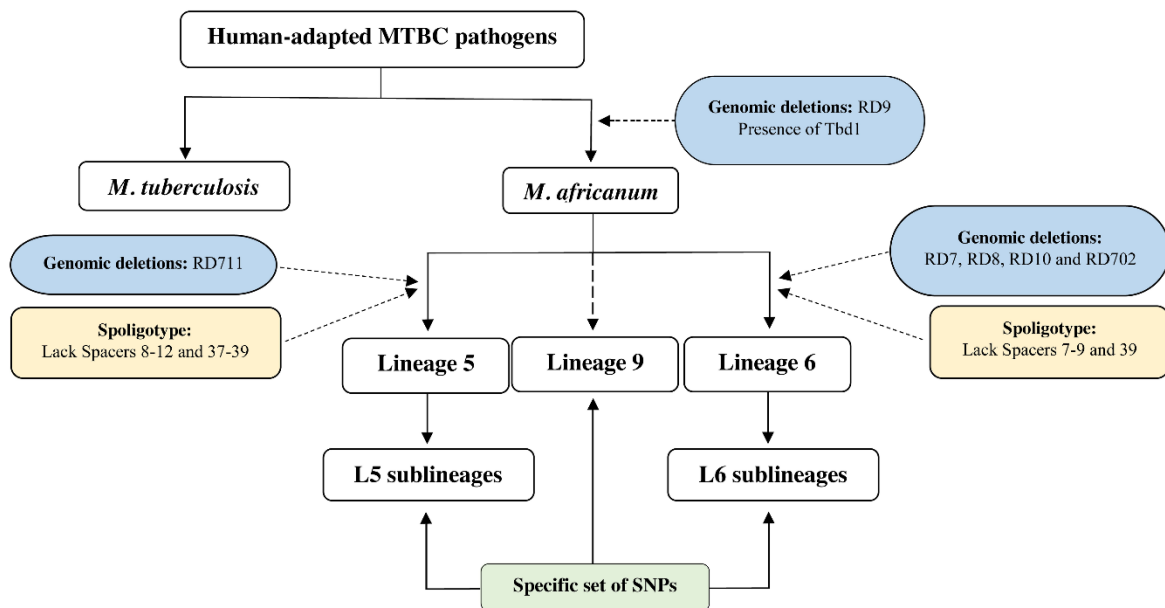


Figure 8. Genetic identity of *M. africanum* lineages. Represented are the genetic markers that distinguish *M. africanum* from *M. tuberculosis* and lineages 5, 6, and 9 within *M. africanum*. Of note, since no robust genetic deletions allowing the classification of L9 have been reported, the identification of this lineage is based in specific SNPs. **Abbreviations:** L, lineage; MTBC, *Mycobacterium tuberculosis* complex; RD, region of difference; SNP, single nucleotide polymorphism.

strains of the *M. africanum* L6 are separated from the other lineages of human-adapted members of the MTBC and instead placed among the animal lineages of the MTBC (Fig.7)²²¹. Furthermore, several studies support the idea that the dassie bacillus shared a common ancestor with *M. africanum* L6^{221,222}. In all, data are compatible with an evolutionary scenario in which the ancestor of L6 strains was a generalist pathogen that subsequently adapted to different host species, and with the hypothesis that L6 strains may originate from an animal reservoir, as further discussed below.

The most recently proposed *M. africanum* lineage, L9, resulted from the analysis of 675 *M. africanum* genomes, of which five could not be classified into any of the known MTBC lineages²²⁰. L9 appears as a sister clade of L6, being placed between L6 and the animal-adapted lineages. Genomes of strains belonging to L9 share some genomic deletions with those of strains belonging to L6, as RD702, but not others also present in genomes of strains of animal-associated lineages, such as RD1 and RD5. Owing to its distinct geographic location and the still significant genetic separation from L5/L6 strains genomes, a new *M. africanum* lineage was thus proposed²²⁰. Of note, phylogenetic markers to identify L9 based on genomic deletions have yet not been described (Fig.8). Instead, a list of SNPs common to all L9 genomes and absent from other lineages was proposed as

phylogenetic markers for L9²²⁰. This can now be used as a framework for the discovery of other strains within L9, as well as for further functional studies of this lineage.

Given the close phylogenomic proximity of *M. tuberculosis* and *M. africanum* strains these two pathogens may be considered as belonging to the same species^{223,224}, which is still a matter of debate. Of note, the phylogeographic distribution and the host preference of the different members of the MTBC suggest that this complex could represent host-adapted ecotypes²²⁵. Also, there are important molecular and phenotypical differences between *M. tuberculosis sensu stricto* and *M. africanum* (Table 1). Initial studies showed that unlike *M. tuberculosis*, *M. africanum* strains are unable to use glycerol as a sole carbon source, a characteristic similar to *M. bovis* which implies the need to supplement the culture medium with pyruvate in laboratory cultures²²⁶. A recent study confirmed the preference of *M. africanum* L5 and L6 strains for pyruvate over glycerol as a carbon source, even though L5 also grows in media with glycerol²²⁷. Furthermore, several studies show that, as compared to *M. tuberculosis*, *M. africanum* strains present a slower growth rate in axenic medium, which is particularly visible in the case of L6²²⁸⁻²³¹. A lower growth rate for both L5 and L6 strains as compared to L4 ones was also documented in 7H11 solid medium and in 7H9 liquid medium²²⁷.

Table 1. Main differences discriminating *M. africanum* and *M. tuberculosis*.

	<i>M. africanum</i> [*] L5	<i>M. africanum</i> L6	<i>M. tuberculosis</i>
Growth characteristics			
Carbon source	Glycerol/pyruvate	Pyruvate	Glycerol
Growth rate	Slow	Slowest	Fastest
Depth of growth	Microaerophilic	Microaerophilic	Aerophilic
Colony morphology	Dysgonic	Dysgonic	Eugonic
Biochemical characteristics			
Nitrate reductase	Negative/weak	Negative/weak	Positive
Urease activity	Positive (high)	Positive	Positive
Geographical distribution	Mainly east side of West Africa	Mainly west side of West Africa	Widespread
Clinical characteristics			
Sputum conversion		Slower**	
Reported associations		Elder, HIV+, and malnutrition	
Rates of transmission	Reduced**	Similar**	
Progression to active TB		Slower**	

**M. africanum* L9 is not included due to lack of data.

**As compared to *M. tuberculosis*.

Also remarkable is the evidence that *M. africanum* strains prefer minimal oxygen microenvironments (microaerophilic) and produce dysgonic colonies, contrary to *M. tuberculosis* strains that exhibit eugonic colonies²³². Moreover, *M. africanum* L6 strains are adapted to growth under hypoxic conditions²³³. Metabolic differences have also been described between *M. tuberculosis* and *M. africanum* strains. The nitrate reductase activity is strong in the case of *M. tuberculosis* strains, and weak to negative for both L5 and L6 strains²³⁴⁻²³⁶; and *M. tuberculosis* L4 and *M. africanum* L6 strains present a stronger average urease activity, which is directly linked to the bacteria nitrogen metabolism, than L5²²⁷. Therefore, globally, L5 and L6 strains present several characteristics that are distinctive of *M. tuberculosis*. No phenotypic data pertaining L9 strains exist thus far, and so it is as yet not possible to include L9 strains in these comparisons. Hence, the classification of TB-causing isolates into the different lineages of *M. tuberculosis* or *M. africanum* based in genotypic differences (Fig.8) is more accurate than one based on phenotypic assays, also because one cannot exclude phenotypic diversity among isolates of the same lineage or even sublineage.

At the genetic level, *M. africanum* strains present important variations in some “classical” virulence factors of *M. tuberculosis*. An example are the DosR regulon genes, that have been linked to virulence in the MTBC strains, and shown to be downregulated in the sputum of *M. africanum* L6-infected patients as compared to infections by *M. tuberculosis* L4²³³. *M. africanum* strains accumulate mutations in major DosR regulon genes, and the lower expression of the DosR regulon may explain why *M. africanum* strains present microaerobic growth and associates with extrapulmonary disease²³³. Strains of the *M. africanum* lineages were also described to accumulate mutations in genes encoding enzymes of the electron transport chain and central carbon metabolic pathways, as compared to *M. tuberculosis* strains, which has been suggested as another possible adaptation to ecological niches characterized by low oxygen tension²³⁷. Furthermore, *M. africanum* L6 strains were shown to harbour loss-of-function mutations in the two-component virulence regulation system *phoP/R*^{238,239}. Because this system is involved in several pathogenic processes, as the secretion of the virulence factor ESAT-6, biosynthesis of acyltrehalose-based lipids, and the modulation of antigen export, such mutations were expected to render the pathogen avirulent. However, *M. africanum* L6 strains evolved to compensate the deleterious effects of the *phoP/R* mutations and so maintain their pathogenic ability. Indeed, the RD8 specific deletion in both animal-adapted and *M. africanum* L6 strains restores ESAT-6 secretion by a *phoP/R*-independent mechanism, by ensuring high levels of expression of the operon *espACD*, which is required for ESAT-6 secretion^{238,239}. How this occurs is not fully understood, but it is possible that polymorphisms upstream the *espA* gene might increase

the affinity of PhoP or EspR for this promoter region, which results in *espA* expression in the absence of a fully functional phoP/R system²³⁸. In a different study, *M. africanum* L5 isolates were also shown to efficiently secrete and induce immune responses against ESX-1 substrates²¹⁷.

3.3. TUBERCULOSIS CAUSED BY *M. AFRICANUM* VS *M. TUBERCULOSIS*

Several studies have been performed with the aim of unveiling associations between clinical and epidemiologic data and the infecting bacteria, ie, *M. tuberculosis* or *M. africanum*. It is important to note that these studies have been performed in different countries, at different times and using different methodologies. Therefore, multicentric studies are still in need and it is somehow not surprising that some discrepant results are seen across different reports, as further discussed below.

No marked differences were found in the chest X-ray presentation of TB caused by *M. africanum* or *M. tuberculosis*²⁴⁰. Furthermore, both pathogens were shown to respond similarly to the standard 4 first-line drugs in TB treatment, although patients diseased with *M. tuberculosis* L4 strains responded faster to TB treatment than those with *M. africanum* L6 strains²⁴¹. The slow clinical recovery of *M. africanum*-infected patients as compared to *M. tuberculosis*-infected ones may result from a higher content of persister-like *M. africanum* bacilli in sputum at diagnosis²⁴². Despite the similar clinical presentation of TB, several studies associated infections with *M. africanum* strains with more vulnerable hosts. Studies conducted in The Gambia found *M. africanum* infections to be more common in HIV co-infected patients, as well as in older individuals and individuals presenting severe malnutrition²⁴⁰. The association between *M. africanum* infection and elder patients was also reported in Ghana²⁴³ and with lower body mass index individuals in Mali²⁴⁴. However, a clear association between *M. africanum* and HIV coinfection is still controversial. A recent study in Ghana showed no significant differences between the prevalence of *M. tuberculosis* or *M. africanum* infections in individuals with diabetes, another important comorbidity in TB²⁴⁵. Furthermore, it is possible that *M. africanum* infections correlate with slower progression to active TB. A study from The Gambia showed that despite similar rates of transmission, individuals exposed to *M. tuberculosis* strains were more likely to progress to active TB disease than those infected with *M. africanum* ones²⁴⁶. This was supported by another study in Mali associating a longer time between symptom onset and TB diagnosis in *M. africanum* infections²⁴⁴. In line with these findings, infection of mouse models with *M. africanum* strains showed a slower progression of the disease^{231,247,248} with mild lung

pathology even in mice lacking IFN- γ , which are highly susceptible to *M. tuberculosis* infection²³¹. Of note, whether transmission rates are equivalent between *M. tuberculosis* and *M. africanum* is not fully set, as a study in Ghana associated *M. africanum* strains with reduced recent transmission rates²⁴⁹. Importantly, whereas in The Gambia the prevalent *M. africanum* lineage is L6²⁴⁶, in Ghana it is L5²⁴⁹, and so differences in transmission rates may reflect the specific characteristics of L5 or L6 strains. Thus, all in all, *M. africanum* strains present several differences when compared to *M. tuberculosis* ones (Table 1) and is generally viewed as a less virulent pathogen than *M. tuberculosis*. As mentioned before, it is as yet not possible to establish comparisons between the recently identified L9 strains and those of L5/L6 or *M. tuberculosis*.

3.4. HOST IMMUNE RESPONSES TO *M. AFRICANUM*

3.4.1. INNATE IMMUNE RESPONSES

Infection of human monocyte-derived macrophages with distinct strains of the MTBC showed variation of the induced cytokine responses including between the two isolates of *M. africanum* tested, with one inducing strong cytokine release and another inducing a weak response²⁵⁰. Interestingly, in the same study, both *M. africanum* isolates seemed to grow less inside resting macrophages than their *M. tuberculosis* counterparts²⁵⁰. Another study, focusing on the pathogen transcriptional adaptation upon macrophage infection, reported distinct MTBC lineage signatures, including the failure of *M. africanum* strains to induce the phthiocerol dimycocerosate (PDIM) locus, a complex cell wall lipid unique to mycobacteria associated with its virulence²⁵¹. More recently, an isolate of *M. africanum* L6 was shown to induce considerably less IFN- β by infected bone marrow-derived macrophages than *M. tuberculosis* strains from L2 or L4²⁵². Although the *M. africanum* isolate also triggered cGAS and STING, infections of macrophages by this pathogen induced less mitochondrial stress, thus decreased production of mitochondrial reactive oxygen species which contributed to less type I IFN being produced²⁵². The *in vivo* effect of IFN- $\alpha\beta$ signalling during infection by *M. africanum* strains was subsequently studied in mouse models. In agreement with the detrimental role of type I IFN in TB²⁵³ lack of type I IFN receptor signalling led to reduced lung bacterial burdens and less severe histopathological findings upon *M. africanum* infection²⁴⁷. These results highlight that even the lowest levels of IFN- $\alpha\beta$ induced during chronic *M. africanum* infection are potentially pathogenic²⁴⁷. Collectively, these studies and others²³¹ demonstrate that *M. africanum* strains infect macrophages, inducing a cytokine response, whilst adapting to the host cell. The molecular mechanisms underlying these responses, such as the recognition of *M. africanum* strains by pattern recognition receptors,

remain however elusive. *M. africanum* strains were shown to bind recombinant human mannose-binding lectin (MBL), a plasma opsonin, more efficiently than *M. tuberculosis* strains and a protective association between TB and the human MBL2 G57E variant, associated with lower MBL levels, was described, only in TB caused by *M. africanum*²⁵⁴. It is possible that the stronger binding of *M. africanum* strains to MBL may favour the bacteria uptake by macrophages, promoting the establishment of infection *in vivo*, and thus the protective MBL deficiency may have been selected in the human population in regions endemic for *M. africanum*. Another study has identified increased levels of *TLR9* expression in unstimulated blood of patients infected with *M. africanum* isolates as compared to other MTBC strains infections²⁵⁵, which may suggest a role for TLR9 in innate immune responses to *M. africanum* strains. Of note, the levels of IL-12p70 and *IL12A* were also significantly higher in *M. africanum*-infected patients, while those of IL-15, IL8 and MIP-1 α were higher in *M. tuberculosis*-infected patients²⁵⁵. A broader study comparing peripheral blood gene expression profiles between *M. africanum* - and *M. tuberculosis*-infected patients showed no differences at diagnosis, although there were distinct signatures associated with each infection post-treatment, predominantly associated with immune responses and metabolic diseases²⁵⁶.

3.4.2. ADAPTIVE IMMUNE RESPONSES

Comparison of T cell responses from *M. tuberculosis*- or *M. africanum*-infected TB patients before chemotherapy and following overnight stimulation of whole blood with ESAT-6/CFP-10 or with purified protein derivative (PPD) showed higher single-TNF- α -producing CD4 and CD8 T cells and lower single-IL-2-producing T cells in the case of *M. africanum* infections²⁵⁷. Additionally, a persistently high proportion of activated T cells was reported in *M. africanum*-infected individuals post-treatment²⁵⁷. However, the frequencies of PPD-specific polyfunctional CD4 T cells did not differ between the two infections²⁵⁷, both before and after treatment, suggesting an overall uniform immune response triggered by either pathogen. This is in line with the abovementioned studies on peripheral blood transcriptomic and metabolic profiles obtained at diagnosis²⁵⁶. Interestingly, stimulation of whole blood with ESAT-6/CFP-10 stimulation after treatment induced significantly higher production of pro-inflammatory markers, such as IFN- γ , in the case of *M. tuberculosis*-infected TB patients^{255,256}. In the mouse model of infection, a modest immune response has been reported upon infection with a *M. africanum* isolate, also associated with restricted lung pathology²³¹. Taken all this together, it is possible that a lower immune response takes place upon *M. africanum* infection, which although precluding the clearance of the

pathogen, may protect the host from tissue immune pathology. This hypothesis is compatible with a slower progressing infection and may be explained by pathogen-associated factors. Pathogens belonging to the MTBC are known to have remarkably hyperconserved T cell epitopes, suggesting that ensuring T cell responses is more important to these agents than evading them²⁵⁸. Interestingly, a study showed that the L6 strains of *M. africanum* were significantly more genetically diverse than the L5 ones, including in predicted T cell epitopes²⁵⁹. Additionally, even though the majority of the T cell epitopes were conserved between the two lineages, a higher ratio of non-synonymous to synonymous single nucleotide variation was detected in the epitopes from L6 strains relatively to L5 ones²¹⁸. Thus, it is tempting to speculate that the evolutive pressure to hyperconserve T cell epitopes may be weaker in the case of L6 strains, leading to lower T cell responses and favouring the persistence of the pathogen in its host population. Further studies are required to address these hypotheses and investigate the contribution of T cell responses to TB caused by *M. africanum* strains.

3.5. GEOGRAPHIC RESTRICTION OF *M. AFRICANUM*: A CASE OF IMMUNE ADAPTATION?

A specific adaptation of *M. africanum* to the host population, particularly to the host immune response, is a conceivable hypothesis to explain the geographic restriction of this pathogen. Previous studies provide compelling evidence towards this hypothesis in the case of *M. africanum* L5 strains. In a study in Ghana, *M. africanum* was significantly more common in TB patients belonging to the Ewe ethnic group an association that was mainly driven by L5 strains²⁶⁰. Possible interactions between *M. africanum* infection and human genetic diversity were also described in other studies. A polymorphism in the exonic allele (g.760A) of the ALOX5 gene (which encodes for 5-lipoxygenase, an important regulator of the immune response in TB^{261,262}) was associated with higher risk of TB in Ghana, an association that was stronger in infections caused by *M. africanum* L6 strains²⁶³. Furthermore, another study identified a highly frequent variant of the human immunity-related GTPase M (IGRM), a regulator of the autophagic process, in the Ghanaian population and associated it with protection against *M. tuberculosis* L4 strains, but not against *M. africanum* isolates²⁶⁴. Thus, higher frequencies of genetic variants conferring increased susceptibility to *M. africanum* strains in West African individuals may at least partially explain the geographical restriction of this pathogen to this region. Still, more studies linking human and pathogen genetic diversities are needed to validate this hypothesis. In this line, specific HLA genetic associations may be of potential interest to

explain the geographic distribution of *M. africanum* vs *M. tuberculosis* infections. A study conducted in Mali identified various class I HLA-A and HLA-B alleles associated with active TB disease caused by either pathogen. However, several class II HLA-DR variants were found to be associated with *M. tuberculosis* but not *M. africanum* strains, with only the variant DRB1*03:01 being associated with both groups²⁶⁵. It is tempting to speculate that specific associations between HLA variants and *M. africanum* strains may reflect variations affecting T cell epitopes in *M. africanum*, which as described above are not as hyperconserved as in *M. tuberculosis*.

More recently, the hypothesis that differences in the intestinal microbiota of patients infected with *M. africanum* isolates could contribute to the high susceptibility of West African individuals to infections with this pathogen has been proposed²⁶⁶. Patients infected with *M. africanum* strains presented less microbiome diversity than individuals infected with *M. tuberculosis* isolates or healthy controls, and were enriched in bacteria from the Enterobacteriaceae phylum Proteobacteria as compared to healthy controls²⁶⁶. Since a positive correlation between the abundance of Enterobacteriaceae and an inflammatory gene expression profile was reported, differences in the intestinal microbiome may contribute as host-associated factors predisposing to infections by *M. africanum*.

Other two hypotheses may explain the geographic restriction of *M. africanum* infections, which are less related to the host immune response. It is possible that *M. africanum* is an attenuated member of the human-adapted TB-causing bacteria, being therefore outcompeted by *M. tuberculosis*. This hypothesis is supported by the reduction of TB cases caused by *M. africanum* strains over time for some countries²⁶⁷⁻²⁶⁹, although it is not observed in other countries^{269,270} as discussed above. Finally, *M. africanum* L6 strains share a common ancestor with animal (non-human) adapted strains (Fig.6). Animal-adapted lineages are composed of Mycobacteria that infect different species of animals as preferential hosts, including non-human primates and other mammals. The ancestry of *M. africanum* L6 allows raising the hypothesis that, despite having become a human pathogen, strains of this lineage may still be adapted to an animal that could function as a reservoir in West Africa. Although *M. africanum* strains have been isolated from several animal species, including pigs and cows²⁷¹, an animal reservoir has never been identified. Of note, the animal reservoir hypothesis is less likely to prove valid in the case of L5 strains since this lineage is phylogenetically less related to animal adapted members of MTBC²²¹ (Fig.6).

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CHAPTER II - AIMS

AIMS

As previously discussed, the immune response to TB is shaped by a complex interplay between the pathogen's genetic diversity and the host's immunogenetic background. In West Africa, the coexistence of distinct *M. tuberculosis* lineages, such as the globally prevalent *M. tuberculosis* L4 and the regionally restricted *M. tuberculosis* L6, is linked to differences in disease progression and host immune activation. In this context, my thesis is dedicated to elucidating how lineage-specific antigenic variations, mixed infections, and host transcriptional responses converge to shape TB pathogenesis. My specific aims are to:

- Define the impact of non-synonymous substitutions in highly variable genes associated with *M. tuberculosis* L6 on epitope binding to HLA alleles prevalent in distinct geographical regions. My PhD work employed comprehensive immunoinformatic analyses to predict the binding affinities of T cell epitopes derived from *M. tuberculosis* L4 and L6 isolates to HLA molecules that are highly frequent in West African populations. In parallel, the immunogenicity of these lineage-specific peptides was evaluated by predicting their capacity to induce IFN- γ production. This integrated approach aims to elucidate the molecular determinants by which genetic variations in *M. tuberculosis* may influence antigen presentation and ultimately modulate host immune responses across diverse populations.
- Characterize the dynamics of infections by isolates from different lineages recovered from TB patients in Guinea-Bissau and to elucidate the impact of *M. tuberculosis* mixed infections on host immune responses and disease progression. For that, my PhD work employed an integrated approach using both clinical samples and experimental models. I compared the immune profiles elicited by single-strain infections with those triggered by co-infections of *M. tuberculosis* L4 and L6, thereby revealing how the interplay between these distinct *M. tuberculosis* lineages modulates key inflammatory responses and influences disease progression. This work aims to identify differential cytokine responses and cellular activation patterns that underlie the varied clinical outcomes observed in patients, ultimately advancing our understanding of the role of mixed infections in TB pathogenesis.
- Identify candidate host transcriptional biomarkers associated with TB disease severity in a setting of high strain diversity. In this part of my PhD, I focused on whole blood RNA sequencing of TB patients from Guinea-Bissau, where a high diversity of *M. tuberculosis* strains is observed- aiming to reveal distinct

transcriptional signatures that correlate with disease progression/severity. This work establishes a framework for the identification of novel host-derived biomarkers that could serve as indicators of TB severity and inform the development of immune-based interventions.

**CHAPTER III - IMMUNOINFORMATIC PREDICTIONS ON
VARIABLE *MYCOBACTERIUM TUBERCULOSIS* LINEAGE 6 T
CELL EPITOPES AND HLA INTERACTIONS IN WEST AFRICA**

This chapter is based on the following publication:

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Immunoinformatic predictions on variable *Mycobacterium tuberculosis* Lineage 6 T cell epitopes and HLA interactions in West Africa

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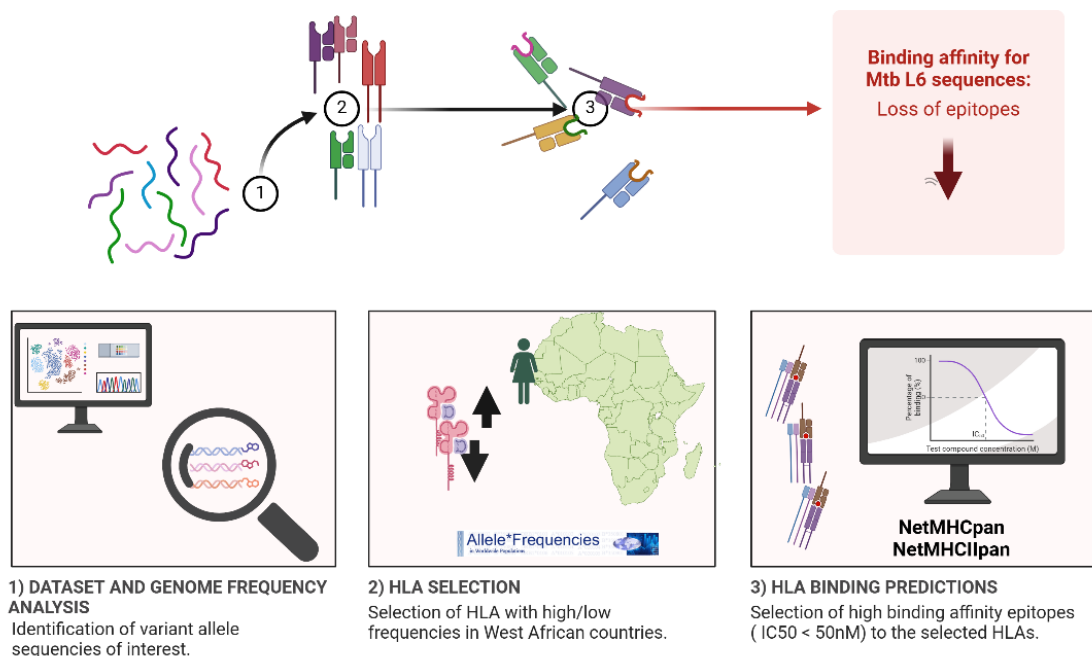
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Graphical abstract



Article

Immunoinformatics Predictions on Variable *Mycobacterium tuberculosis* Lineage 6 T Cell Epitopes and HLA Interactions in West Africa

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Abstract: Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), remains a global health challenge. The human-adapted TB-causing bacteria are distributed into ten lineages with distinct global distributions and clinical outcomes. Mtb lineages 4 (L4) and L6 are good prototypes of these differences, because L4 is globally prevalent, whereas L6 is geographically restricted to West Africa and associated with slower disease progression. Given the fundamental role of T cells for the control of TB, we questioned whether Mtb L4 or L6 antigens and HLA interactions would be disrupted in West African hosts. Here, we selected variable and validated antigens and demonstrate their expression during in vivo Mtb L4 or L6 infections. We then compared the predicted number of IFN- γ -inducing and HLA high-binding-affinity peptides in Mtb ancestral, L4, or L6 proteins, considering HLA alleles of high or low frequency in West Africa. Our immunoinformatics approach predicts that non-synonymous substitutions of high variance in Mtb L6 strains diminish binding affinities to HLA alleles prevalent in West African populations, suggesting specific adaptations of these strains to their preferred hosts. Future functional studies will advance our knowledge on lineage-specific evolution and inform strategies to enhance TB control in endemic regions.

Keywords: *Mycobacterium tuberculosis*; phylogeographic lineages; antigens; HLA binding affinity; host–pathogen interactions; co-evolution



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1. Introduction

Tuberculosis (TB) remains a health problem, with an estimated 10.8 million new cases and 1.25 million deaths reported in 2023 alone [1]. The causative agent of TB, *Mycobacterium tuberculosis* (Mtb), is an obligate human pathogen that likely originated thousands of years ago [2–4] and that belongs to the *Mycobacterium tuberculosis* complex (MTBC) [5]. Selective pressures from host immune responses and environmental factors have driven the diversification of Mtb into ten phylogenetic lineages (L1–L10) [5,6] that show a striking geographic structuring, even in the face of increased human mobility and globalization [3,5]. For example, MTBC L4 is globally distributed [7], displaying high incidence across Europe, Africa, and the Americas, while L2 dominates in East Asia [8]. In contrast, MTBC L5, L6, and L9 (formerly known as *Mycobacterium africanum* [9]) are largely restricted to specific regions

of West and East Africa [10–12]. In these regions, MTBC L5, L6, and L9 may co-exist with globally spread lineages, most notably L4, without evidence of being out-competed [11,12].

Host–Mtb sympatry highlights the adaptation of the pathogen to specific hosts, and may be explained by different mechanisms, including co-evolution [13], genetic drift [14], and host restriction [15–17]. Several studies reported specific association of Mtb L5 infections with the Ewe ethnic group [18–20], with an effect visible on macrophage responses [21,22]. Furthermore, Mtb strains of L6 displayed reduced infectivity in allopatriotic macrophages and reduced transmission in allopatriotic populations [15] despite similar transmission rates in its West African niche [23]. A major gap in knowledge relates to the impact of Mtb–host sympatric adaptations in T cell responses, which are key players for TB control in humans and experimental models [24,25]. Mtb T cell epitopes are generally hyperconserved, a feature that may however be disrupted in Mtb L6, as T cell epitopes in Mtb strains of this lineage are more variable, as shown by a significantly higher average nucleotide diversity and a greater number of single nucleotide polymorphisms (SNPs) between sequences [10,26,27]. Furthermore, T cells from TB patients infected with Mtb L6 strains showed reduced production of key cytokines, such as interferon (IFN)- γ and interleukin (IL)-2, in response to antigens like ESAT-6 and CFP-10 [28], which are critical for robust T cell-mediated immunity [29–31]. This reduced immune activation may potentially explain the ability of Mtb L6 to cause TB disease despite being a slow-growing pathogen [32,33]. On the other hand, it could also lead to decreased inflammation and less tissue damage, allowing the pathogen to persist in the host while minimizing detection.

We hypothesized that the higher antigen variability in Mtb L6 may reflect an evolutionary adaptation of this lineage to evade immune recognition by human leukocyte antigen (HLA) molecules predominant in West African populations. This hypothesis would help to explain the geographic restriction of Mtb L6, whilst also fitting with the high sympatry of Mtb L6 pathogens and their West African host niche [11,12]. Leveraging the potential of immunoinformatics as a transformative tool in immunology [34–36], we focused on a set of seven experimentally validated antigens [27], previously described as variable in Mtb L6 genomic sequences, and conducted *in silico* analyses of HLA binding affinity and predictions of functional immunogenicity. The Mtb L6 variant proteins were predicted to associate with a reduction in the number of high-binding-affinity peptides to both HLA class I and class II alleles of high frequency in West African populations. In contrast, no predicted trend for increased or reduced binding affinity was detected for the Mtb L4 version of the candidate proteins, irrespective of the tested HLA. These *in silico* findings warrant future experimental validation so that they may potentially impact the development of more efficient TB vaccines, informed by host–pathogen interactions.

2. Materials and Methods

2.1. Ethics Statement

Animal experiments complied with the ARRIVE guidelines, followed the 2010/63/EU Directive and were approved by the i3S Animal Ethics Committee and the Portuguese National Authority for Animal Health (DGAV; #018413/2021-11-24).

2.2. Animal Housing and Infection

C57BL/6 mice were bred and housed at the i3S animal facility. Males and females of 8 to 11 weeks old were used for infections, and maintained under contention conditions in the animal biosafety level 3 facility at i3S, at a controlled temperature (20–24 °C) and humidity (45–65%), and a light cycle of 12 h (light/dark). Water and food were provided *ad libitum*. Mice were infected with Mtb (clinical isolates from L4 or L6) through the aerosol route using an inhalation exposure system (Glas-Col) [37,38]. To determine the dose of

infection, the bacterial load in the lungs of 3 mice was quantified 3 days post-infection. Infected mice were weighed every week. Mice were euthanized by CO₂ inhalation at the indicated time points.

2.3. Lung Processing, CFU Determination, and mRNA Analysis

Lungs were aseptically excised and processed as previously described [37,38]. Briefly, lungs were digested with Collagenase D (Roche, Basel, Switzerland, Cat. #11088882001) followed by physical disruption and filtration through 70 µm cell strainers (Falcon, Corning, NY, USA Cat. #352350.0). Cell suspensions were used for colony forming unit (CFU) determination and mRNA analysis. Lung cell suspensions were lysed with saponin serial dilutions prepared and plated in Middlebrook 7H11 agar (BD Biosciences, Franklin Lakes, NJ, USA, Cat. #212203.0) supplemented with OADC and PANTA (BD BioSciences, Franklin Lakes, NJ, USA, Cat. #245114). CFUs were enumerated after 21–28 days of incubation at 37 °C. Total RNA was extracted from infected mouse lungs using TRIzol reagent (GRI SP, Porto, Portugal, Cat. #GB23.0100) according to the manufacturer's instructions. Briefly, samples were stored in TRIzol at −80 °C, homogenized, and subjected to phase separation using chloroform. RNA was precipitated with isopropanol, washed in 70% ethanol, and resuspended in RNase/DNase-free water. Glycogen was used as a carrier to enhance RNA yield. RNA concentration and purity were assessed by Nanodrop spectrophotometry. For qPCR analysis, cDNA was synthesized with the SuperScript First-Strand Synthesis System using Random Primer Mix (60 µM) for RT-PCR (ThermoScientific, Waltham, MA, USA, Cat. #E6300L). Target gene mRNA expression was quantified by real-time PCR, using SYBR green (Biorad, Hercules, CA, USA, Cat. #1725121). Gene expression was normalized to SigA. Oligonucleotide sequences are listed in Supplementary Table S1. Data from the qPCR (Figure 2) were analyzed using GraphPad Prism software v10.1.1. Differences were considered significant at $p \leq 0.05$ and represented as follows: * $p \leq 0.05$; ** $p \leq 0.01$; and *** $p \leq 0.001$.

2.4. Dataset and Genome Sequence Analysis

Publicly available MTBC genome sequences from Napier et al. were utilized [39]. A subset of 247 genomes, comprehensively representing Mtb L6 and diverse MTBC lineages, was selected. Raw sequencing reads were downloaded from the NCBI SRA and processed using a standardized bioinformatic pipeline. This pipeline included quality control with BBDuk (version 39.01); mapping to the inferred Mtb ancestral reference genome sequence (MTB_anc.fasta) derived from Comas et al. [2] using BWA-MEM (version 0.7.17); duplicate marking with samtools markdup (version 1.17); and targeted variant calling focused on specific regions of interest using bcftools mpileup (parameters --min-MQ 30, --min-BQ 20) and bcftools call (version 1.17). Variant filtering was applied using bcftools filter (version 1.17) to remove variants flagged as “LowQual” (criteria: QUAL < 15). Functional annotation of variants was performed using bcftools csq (version 1.17) with the GFF3 annotation file corresponding to the Mtb H37Rv reference genome (NC_000962.3).

2.5. Variant Allele Frequency Analysis and Visualization

Variants identified within the target genes of interest were extracted from the annotated VCF files. For each variant position in each sample, the variant allele frequency (VAF) was calculated using the DP4 (depth per allele) field from the VCF output of bcftools mpileup. This VAF represents the proportion of reads supporting the alternative allele at a given variant position. VAF values were analyzed using Python (version 3.12.2) with the pandas (version 2.2.3) and numpy (version 2.2.4) libraries. To identify variants differing significantly in frequency between lineages, the mean VAF per genomic position was calculated separately for Mtb L6 and L4 isolates, utilizing the lineage classifications

provided in [40]. The difference between the mean VAF in Mtb L6 and the mean VAF in Mtb L4 was computed for each variant position. Variants were then ranked based on this VAF difference (VAF_Difference_L6_L4) to identify positions with the largest frequency discrepancies. Distributions of VAF values for selected high-difference variants across specific lineages were visualized using heatmaps generated with the seaborn (version 0.13.2) and matplotlib (version 3.10.1) libraries in Python. These heatmaps displayed the mean VAF per variant position (columns) for each lineage (rows).

2.6. IFNepitope Analysis

The IFNepitope server (<https://webs.iitd.edu.in>, IFNepitope, module “Scan”, accessed on 23 March 2025) [40] was utilized to predict IFN- γ inducing epitopes for Mtb L6 and L4 candidate gene variants and the corresponding ancestral sequences, considering a length of 15 residues. This tool enables the identification of IFN- γ -inducing epitopes from a set of peptide sequences or a peptide library [41]. The search model employed was the support vector machine (SVM) motif hybrid, which integrates the SVM-based model with motif contributions relevant to IFN- γ induction.

2.7. HLA Selection for T Cell Epitope Prediction

Allele frequency data were retrieved from the publicly available database allelefrequencies.net to identify HLA alleles of high or low frequency in West Africa, as compared to other regions (Europe, North America, and Asia). In parallel, data from the Immune Epitope Database (IEDB; www.iedb.org) were consulted to confirm the availability of experimental binding data and to aid in the verification of allele representation in the target populations.

2.8. T Cell Epitope Prediction

Peptides of different lengths (8 to 14 mers for HLA class I and 9 to 18 mers for HLA class II) were generated for each candidate antigen. NetMHCpan-4.1b and NetMHCIIpan-4.0 were used to predict the binding potential of ancestral, Mtb L4, and Mtb L6 peptide variants to the selected HLAs. These machine learning-based tools were trained with a comprehensive number of mass spectrometry eluted ligands and have been considered the best overall predictors [42–44], namely in the HLA-DR locus [44]. HLA-binders were selected based on the half maximal inhibitory concentrations (IC₅₀). IC₅₀ < 50 nM was used as the cutoff for high-binding-affinity peptides.

3. Results and Discussion

3.1. Selection of Highly Variable Antigens in Mtb L6 Strains

Given the sympatry between Mtb isolates belonging to L6 and their West African hosts [11,12,15], we hypothesized that specific host–pathogen interactions may be in place and mediated by alterations in epitope recognition by HLAs. To investigate this hypothesis, we followed the approach summarized in Figure 1.

We reasoned that particular epitope–HLA interactions would be more likely for the rare variable Mtb antigens than for the more common hyperconserved ones. Thus, we selected for our study seven proteins previously identified as rare variant epitopes across 216 Mtb strains and experimentally validated as immunogenic in samples from individuals with active TB [27]. Although epitopes for the selected proteins had been tested for their immunogenicity in human samples [27], whether the corresponding genes were expressed during Mtb L6 infections had not been tested. We aerosol-infected C57BL/6 mice with a Mtb clinical isolate belonging to L6 [33] or with one belonging to L4 [45]. Analyses were performed at day 120 or day 30 post-infection for the Mtb L6 or Mtb L4 infection,

respectively (Figure 2A). At these time points, the infections reach a chronic phase with stabilized bacterial burdens. As expected from previous studies [33], the bacterial burden in the slow-progressing Mtb L6-infected mice was lower than that observed in Mtb L4-infected mice (Figure 2B). The transcriptional analysis of the candidate genes by qRT-PCR showed their *in vivo* expression, irrespective of the Mtb isolate used (Figure 2C). The pattern of expression was similar for all tested genes, with the exception of Rv0990c, which at the indicated time points displayed higher expression in the lungs of mice infected with the Mtb L6 isolate as compared to Mtb L4 infections (Figure 2C). While B6 mice may not be the best models to study TB pathogenesis, our findings show that the selected genes are expressed in the lungs of Mtb-infected mice in the presence of a protective immune response, which positions them as possible natural antigens.

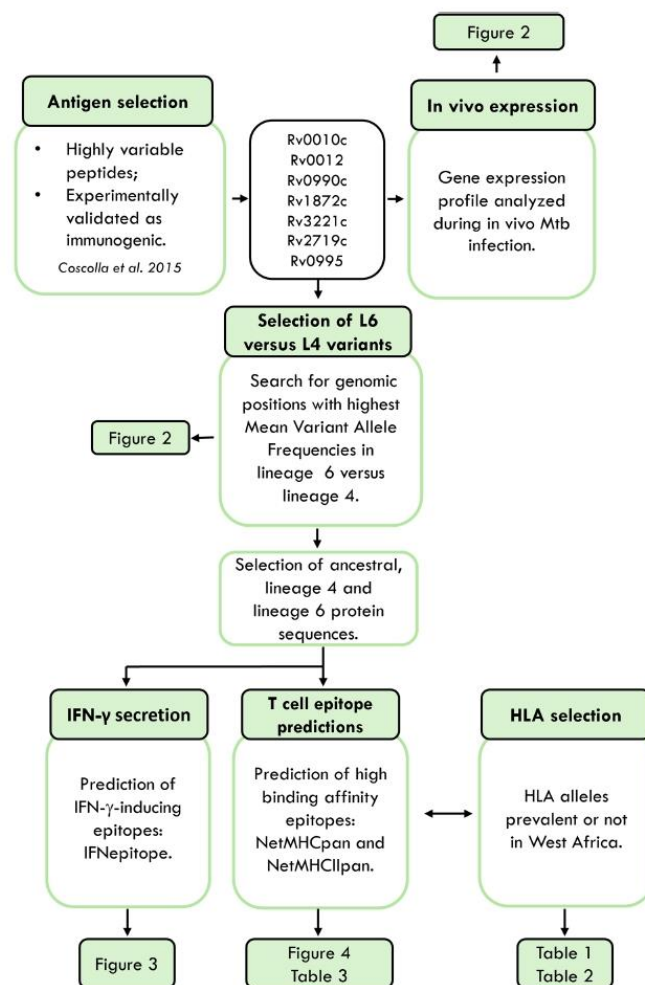


Figure 1. Schematic representation of the study. Mtb, *Mycobacterium tuberculosis*. HLA, human leucocyte antigen. Antigens were retrieved from [27].

The genes encoding these seven proteins were inspected for genomic variants using a collection of 247 MTBC genomes from [39] that comprehensively represent L6 and diverse MTBC lineages. For each sample and genomic position within these genes, we

determined the presence of variants and calculated their VAF. Mean VAF analysis across all lineages revealed a predominantly low VAF across the majority of positions (Supplementary Table S2), consistent with the expected conservation of T cell epitopes in Mtb [26,27]. To specifically highlight variants with differential prevalence between L6 and L4, we ranked genomic positions based on the difference in mean VAF between these two lineages. To visualize the top 10 L6 versus L4 VAF patterns, we generated a heatmap (Figure 2D) displaying mean VAF for each genomic position (columns) across different MTBC lineage level 3 classifications (rows). The color intensity in each cell corresponds to the mean VAF for that position within that lineage group, ranging from dark blue for low VAF to bright yellow for high VAF (Figure 2D). Four genomic mutations identified as high mean VAF in L6 versus L4 strains, or vice versa, led to non-synonymous amino acid substitutions in the corresponding proteins (Figure 2D). Genomic positions 1107897 (containing the SNP resulting in the A68V substitution in protein Rv0990c), 2122380 (containing the SNP resulting in the L258V substitution in protein LldD2/Rv1872c), and 3597581 (containing the SNP resulting in the Q62H substitution in protein Rv3221c) exhibited markedly elevated mean VAF values in several L6 sub-lineages (e.g., 6, 6.1, 6.2, 6.3) compared to other lineages shown in the heatmap (Figure 2D). On the other hand, the non-synonymous substitutions in Rv0010c, Rv0012, RimJ, and P8L in Rv00990c exhibited elevated mean VAF values in only one of the L6 sub-lineages (Figure 2D). Thus, while the selected proteins are generally conserved across MTBC, specific genomic positions within these candidate genes display increased variability specifically within L6 strains. These positions, characterized by higher VAF in L6, may represent sites under positive selection within this lineage, potentially influencing epitope presentation and subsequent T cell recognition within West African populations. Regarding the mutations detected as high mean VAF in L4 versus L6, we also identified some that are L4-specific and others that also span other lineages, most commonly L2 and L3 (Figure 2D).

3.2. Immunogenicity Predictions for the Variant Epitopes

We then assessed the predicted potential of the ancestral, Mtb L4, and Mtb L6 variant proteins to elicit IFN- γ responses. We used the IFNepitope tool to predict IFN- γ -inducing peptides (15 amino acid residues) across the 10 highest or lowest VAFs in Mtb L6 strains, causing non-synonymous substitutions (Figure 2D). Of the top 10 L6 VAFs, Rv0010c and TB7.3 displayed no predicted IFN- γ -inducing epitopes that included the mutated sites. The pattern for the other candidate L6 variants varied, with a loss of predicted IFN- γ -inducing epitopes in RimJ (Figure 3A) and LldD2 (Figure 3B), a gain of epitopes in the case of Rv0012 (Figure 3C), and no change for Rv0990c (Figure 3D). In the case of the variants with a low VAF in Mtb L6, a mixed pattern of increase and decrease was detected for LldD2, depending on the amino acid substitution (Figure 3B), while Rv0012 showed a predicted decrease (Figure 3C), and an increase in the number of predicted IFN- γ -inducing peptides was detected for the Mtb L4 variants of Rv0990c (Figure 3D) and Rv2719c (Figure 3E). Therefore, no specific pattern of gain or loss of predicted IFN- γ -inducing epitopes was detected as being common across each protein or for each lineage. This suggests that any potential functional impact of the antigen variants may be primarily dependent on the binding of the variable peptide to the host HLA. Importantly, HLA genes are highly polymorphic in human populations and several alleles have been previously associated with TB susceptibility in different populations [46–54]. Thus, it is possible that the binding affinity of the variable antigens to the matching host HLAs may vary, resulting in altered T cell responses.

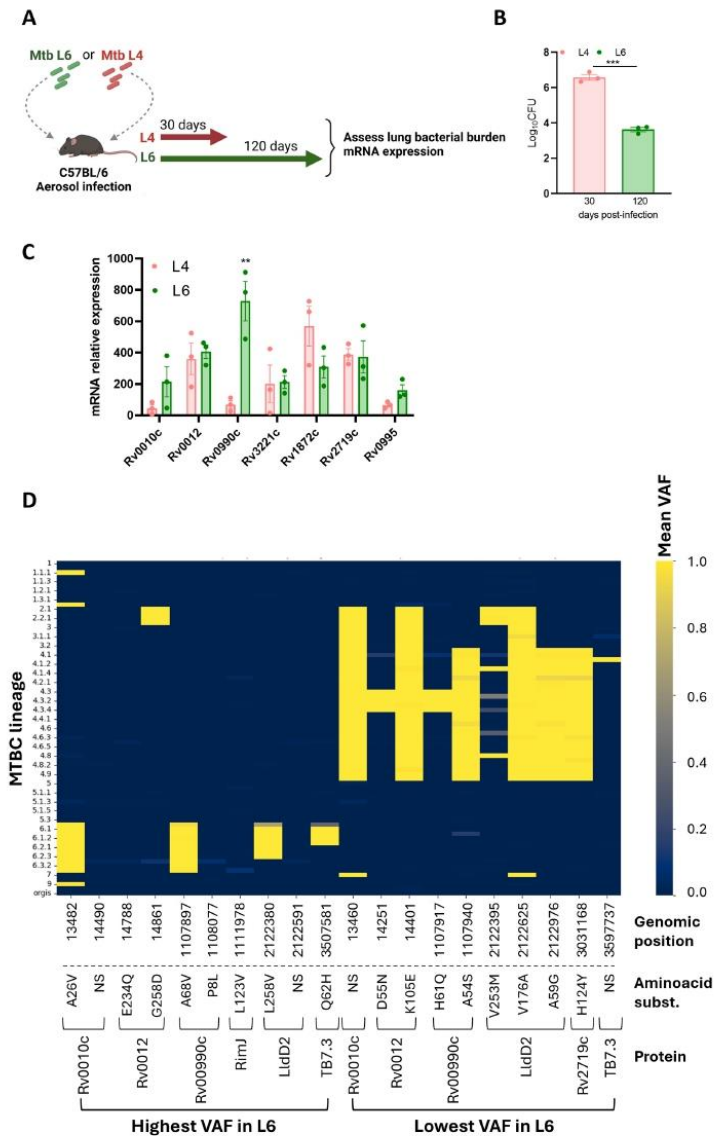


Figure 2. Expression and selection of variant antigens. (A) Experimental infection model. C57BL/6 mice were infected via aerosol with *Mtb* lineage 4 or lineage 6, and at the indicated time points, lung bacterial burdens (B) and candidate gene expression (C) were assessed. Data are represented as mean $\pm$ SEM. Each dot represents an individual mouse. Statistical analysis was performed using 2-tailed unpaired Student's *t* test. (D) Heat map representing the genomic regions with the highest and lowest mean variant allele frequencies (VAFs) in *Mtb* L6 compared to *Mtb* L4. L, lineage. *Mtb*, *Mycobacterium tuberculosis*. MTBC, *Mycobacterium tuberculosis* complex. Ns represents synonymous substitutions. Differences were considered significant at $p \leq 0.05$ and represented as follows: ** $p \leq 0.01$; and *** $p \leq 0.001$.

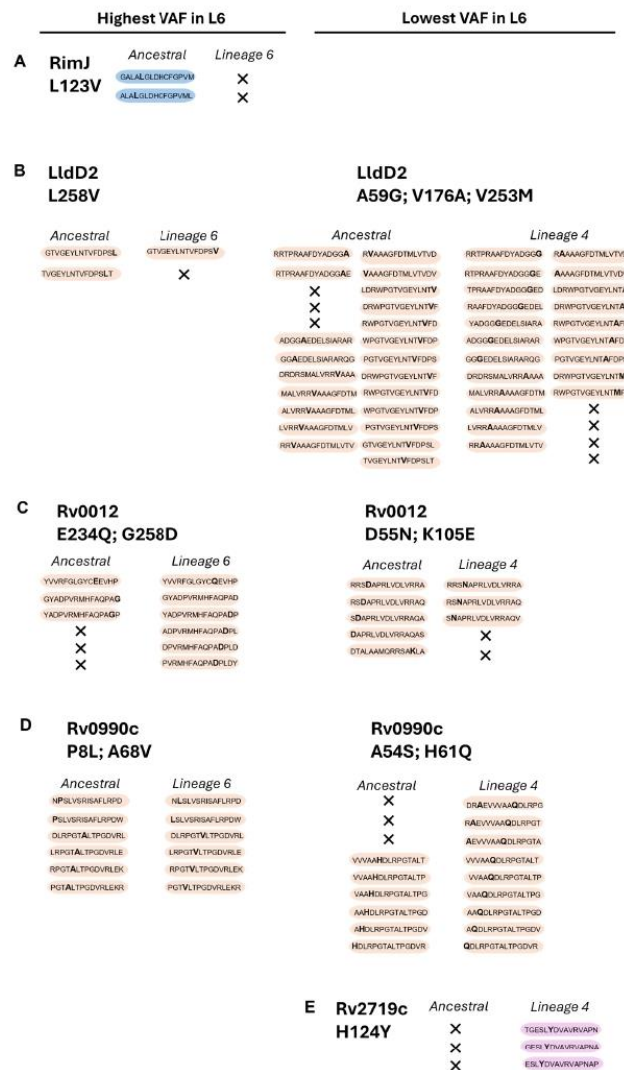


Figure 3. Prediction of IFN- γ -inducing epitopes for the selected proteins. Data represent computational predictions performed using IFNepitope (SVM) to discriminate IFN- γ -inducing epitopes. These are selected based on reaching a predicted positive score. Non-synonymous substitutions and predicted IFN- γ -inducing epitopes are indicated for the selected proteins: RimJ (A), LldD2 (B), Rv0012 (C), Rv0990c (D), and Rv2719c (E), across the two Mtb variants (L4 and L6) in comparison with the ancestral sequence. In each peptide, the non-synonymous amino acid substitution is represented in bold; X represents loss of IFN- γ -inducing epitopes. Peptides with non-synonymous substitutions only in Mtb L6 are shaded blue; those common to both Mtb L6 and L4 are shaded orange; and those only in Mtb L4 are shaded pink.

3.3. HLA Binding Affinity Predictions for Mtb L6 Epitopes

To investigate whether the peptide variants of highest or lowest prevalence in Mtb L6 versus L4 would impact their predicted interactions with host HLAs, we started by retrieving a comprehensive set of HLA alleles, based on the geographic restriction of Mtb L6 to West Africa [10–12] and the global prevalence of L4 [7]. From the 10387 HLA class I

alleles available in NetMHCpan-4.1, and the 660 HLA-DRB alleles (class II) available in NetMHCIIpan-4.0, we selected those with

1. high frequency in at least one of the five West African countries (Guinea-Bissau, Mali, Senegal, The Gambia, and Burkina Faso), but not at a global level;
2. high frequency in Europe, North America, and Asia, and low frequency in West African countries.

We excluded HLA-C class I alleles due to their limited polymorphism and lower expression levels (about 90% less) compared to the other two loci [55–57]. Within the HLA class II -DR locus, only the DRB1 alleles were selected, as they bind most Mtb epitopes and were previously predicted as displaying a five-times-higher expression in comparison to other alleles [58]. HLA-DP and HLA-DQ class II loci were not considered for the study due to the lack of experimental binding data available [59]. Thus, we selected five HLA-A and five HLA-B class I alleles (Table 1), and six HLA-DRB1 class II alleles (Table 2) of high or low frequency in West Africa.

Using computational predictions from NetMHCpan-4.1 and NetMHCIIpan-4.0, we assessed the number of high-binding-affinity epitopes ($IC_{50} < 50$ nM) of the ancestral or variant protein for each selected HLA. Different patterns of variation were detected between the Mtb L6 or L4 variants and the ancestral haplotype, depending on the HLA/peptide pair. Non-synonymous variants of Rv0010c, which were only detected for Mtb L6, did not impact the number of high-binding-affinity epitopes predicted for class I HLAs but reduced the predicted number of high-binding-affinity epitopes for HLA class II alleles, independently of them having high or low prevalence in West Africa (Table 3). In the case of RimJ, the identified non-synonymous variant of L6 did not impact the predicted number of high-binding-affinity epitopes to either class of HLA (Table 3). The last Mtb L6 unique non-synonymous mutation, in TB7.3, led to a loss in the predicted number of high-binding-affinity epitopes only to HLA class I and only for those highly prevalent in West Africa (Table 3). Regarding Rv0012, Rv0990c, and LldD2, the three proteins showing non-synonymous mutations in both Mtb L6 and Mtb L4 strains, a pattern of loss of the predicted number of high-binding-affinity epitopes for the Mtb L6 variants to HLA class I and II alleles of high prevalence in West Africa was detected (Table 3). However, this pattern was not recapitulated for HLAs alleles of low frequency in West Africa, nor for the Mtb L4 strain variants (Table 3). Finally, no difference in the number of high-binding-affinity epitopes was predicted for the Rv2719c variant identified for the Mtb L4 strains as compared to the ancestral protein (Table 3). In summary, the epitope substitutions with high VAF in Mtb L6 were predicted to display an overall reduced binding affinity to both HLA class I and II alleles of high prevalence in West Africa. This pattern was not visible in the case of HLA alleles of low prevalence in West Africa, nor for Mtb L4 variants independently of the HLA allele frequency.

Thus, the findings so far support the hypothesis that Mtb L6 strains may have evolved to persist within a specific host genetic landscape, potentially explaining its confinement to West Africa. Of note, although we did not detect a difference in the number of high-binding-affinity peptides for RimJ when comparing Mtb L6 and the ancestral sequences, our *in silico* analysis predicted a loss of IFN- γ -producing peptides in the case of the Mtb L6 protein (Figure 3A). Similarly, a loss of IFN- γ -producing peptides was predicted for the Mtb L6 variant of LldD2 (Figure 3B). However, this loss of IFN- γ -producing peptides was not seen for Rv0012 and Rv0990c (Figure 3C,D), despite the decrease in the number of predicted high-binding-affinity peptides (Table 3). It is possible that different levels of adaptation exist to modulate T cell responses and confer a selective advantage by reducing immune detection while maintaining sufficient infectivity to ensure transmission within sympatric populations.

Table 1. HLA class I allele frequencies across five West African countries. Values represent the total number of copies of each allele in the population sample (Alleles/2n) in decimal format. Alleles are categorized into those with high or low prevalence in West Africa.

		Allele Frequency *				
		Guinea-Bissau	Mali	Senegal	The Gambia	Burkina Faso
HLAs with high prevalence in West Africa	HLA-A * 23:01	0.1858	0.228	0.177	n.d.	n.d.
	HLA-A * 02:01	0.1084	0.083	0.081	n.d.	0.0945
	HLA-A * 33:03	0.0882	0.094	0.081	n.d.	n.d.
	HLA-A * 74:01	0.13175	0.036	0.022	n.d.	0.032333
	HLA-A * 02:02	0.0714	0.076	0.091	n.d.	0.053
	HLA-B * 15:03	0.108	0.069	n.d.	n.d.	0.1045
	HLA-B * 35:01	0.144	0.127	0.122	n.d.	n.d.
	HLA-B * 53:01	0.1	0.159	0.053	n.d.	0.171
	HLA-B * 58:01	0.078	0.022	0.069	n.d.	n.d.
	HLA-B * 08:01	0.077	0.007	0.048	n.d.	n.d.
HLAs with low prevalence in West Africa	HLA-A * 24:02	0.0224	0	0.005	n.d.	n.d.
	HLA-A * 01:01	0.051	0.007	0.027	n.d.	n.d.
	HLA-A * 02:07	n.d.	n.d.	n.d.	n.d.	0.005
	HLA-A * 24:07	n.d.	n.d.	n.d.	n.d.	n.d.
	HLA-A * 26:08	0	0	n.d.	n.d.	n.d.
	HLA-B * 07:02	0.023	0.058	0.058	n.d.	n.d.
	HLA-B * 18:01	n.d.	0.0070	0.0270	n.d.	n.d.
	HLA-B * 27:05	0	0	n.d.	n.d.	n.d.
	HLA-B * 40:01	0	0	n.d.	n.d.	0.014
	HLA-B * 15:01	0	0	n.d.	n.d.	0

n.d. indicates that the allele was not detected in the respective population; * data retrieved from allelefrequency.net.

Table 2. HLA class II allele frequencies across five West African countries. Values represent the total number of copies of each allele in the population sample (alleles/2n) in decimal format. Alleles are categorized into those with high or low prevalence in West Africa.

		Allele Frequency *				
		Guinea-Bissau	Mali	Senegal	The Gambia	Burkina Faso
HLAs with high prevalence in West Africa	HLA-DRB1 * 11:01	0.1080	n.d.	0.0330	0.0846	n.d.
	HLA-DRB1 * 10:01	0.100	n.d.	0.1890	0.0628	0.0900
	HLA-DRB1 * 04:05	0.0770	n.d.	n.d.	0.0475	n.d.
	HLA-DRB1 * 09:01	0.0770	n.d.	0.0330	0.0742	n.d.
	HLA-DRB1 * 11:02	0.0770	n.d.	0.1440	0.0753	n.d.
	HLA-DRB1 * 13:02	0.0770	n.d.	0.0440	0.1440	n.d.
	HLA-DRB1 * 15:03	0.0093	n.d.	0.0220	n.d.	n.d.
HLAs with low prevalence in West Africa	HLA-DRB1 * 04:01	0	n.d.	n.d.	0.0055	n.d.
	HLA-DRB1 * 07:01	n.d.	n.d.	0.0110	0.0557	n.d.
	HLA-DRB1 * 11:04	n.d.	n.d.	n.d.	0.0005	n.d.
	HLA-DRB1 * 14:02	n.d.	n.d.	n.d.	n.d.	n.d.
	HLA-DRB1 * 15:01	0.0080	n.d.	n.d.	n.d.	n.d.
	HLA-DRB1 * 01:01	0	n.d.	0.0110	n.d.	n.d.

n.d. indicates that the allele was not detected in the respective population; * data retrieved from allelefrequency.net.

Given our analysis suggesting a possible match between variants of Rv0012, Rv0990c, and LldD2 in Mtb L6 strains and the populational HLA alleles, we next inspected with more detail the predictions obtained for these proteins with respect to the individual HLAs tested. In the case of Rv0012 Mtb L6 variants, the loss of two HLA class I high-binding-affinity peptides was caused by the mutation E234Q, specifically on HLA-B*15:03 and 35:01, both of high prevalence in West Africa (Figure 4A). This mutation in Rv0012 was also predicted to decrease the binding of peptides to HLA class II alleles HLA-DRB1*11:01 and HLA-DRB1*11:02, of high frequency in West Africa, and HLA-DRB1*07:01, of low frequency in West Africa (Figure 4D). In this protein, the Mtb L4 variants affected several HLA class I and II alleles, of high or low frequency in West Africa, with a global gain of predicted high-affinity peptides (Figure 4A,D). With respect to LldD2, the Mtb L6 variant L258V was predicted to display less high-binding-affinity epitopes to the high-frequency HLA class I alleles HLA-A*02:01 and HLA-A*02:02 and the class II HLA-DRB1*11:02 allele, whereas it associated with an increase in the number of high-binding-affinity peptides for the HLA-B*58:01 (class I) allele (Figure 4B,E). Mtb L6 variants of LldD2 did not impact the predicted binding to HLA class I or II alleles of low frequency in West Africa (Figure 4B,E), whereas Mtb L4 variants of this protein impacted the binding predictions to several HLA alleles, again with no specific pattern between high- or low-prevalence alleles and with a net gain in predicted high-binding-affinity epitopes (Figure 4B,E). Finally, the mutations affecting the Mtb L6 protein Rv0990c had only a minor effect on HLA class I (HLA-A*02:02) binding predictions (Figure 4C). However, both Mtb L6 and L4 Rv0990c variants widely

impacted the predicted number of high-binding-affinity peptides in the context of HLA class II, specifically those of high prevalence in West Africa (Figure 4F). In both cases, a predicted loss in high-binding-affinity epitopes was detected (Figure 4F). Only the Mtb L6 variant of Rv0990c affected the binding to HLA class II alleles of low frequency in West Africa, in this case increasing the number of high-binding-affinity peptides (Figure 4F). Of note, the variants of Rv0012 (E234Q and G258D) and P8L of Rv0990c are represented in sub-lineage 6.2.3, whereas variants A68V of Rv0990c and L258V of LldD2 are broadly represented in L6 (Figure 2D). Therefore, it is tempting to speculate that the variant L258V of LldD2 might have the widest impact on the interaction of Mtb L6 strains with West African hosts. Future functional studies will need to take this potentially differential effect of antigen mutations in distinct sub-lineages into account.

Table 3. Predicted differences in high-affinity HLA class I and II binding peptides for Mtb L6 and L4 amino acid variants vs. ancestral strain, using NetMHCpan and NetMHCIIpan, in the West African HLA frequency context.

HLA Class I			Frequency in West Africa			
			High		Low	
Protein	L6 Variants	L4 Variants	L6–Anc.	L4–Anc.	L6–Anc.	L4–Anc.
Rv0010c	A26V	-	0	-	0	-
RimJ	L123V	-	0	-	0	-
TB7.3	Q62H	-	−2	-	*	-
Rv0012	E234Q; G258D	D55N; K105E	−2	+1	0	+1
Rv0990c	P8L; A68V	A54S; H61Q	−2	0	0	0
LldD2	L258V	A59G; V176A; V253M	−2	+4	0	+4
Rv2719c	-	H124Y	-	0	-	0

HLA Class II			Frequency in West Africa			
			High		Low	
Protein	L6 Variants	L4 Variants	L6–Anc.	L4–Anc.	L6–Anc.	L4–Anc.
Rv0010c	A26V	-	−4	-	−5	-
RimJ	L123V	-	0	-	0	-
TB7.3	Q62H	-	0	-	0	-
Rv0012	E234Q; G258D	D55N; K105E	−2	+4	−5	0
Rv0990c	P8L; A68V	A54S; H61Q	−7	−5	+3	0
LldD2	L258V	A59G; V176A; V253M	−1	+1	+3	0
Rv2719c	-	H124Y	-	0	-	0

Orange color indicates loss of predicted high-binding-affinity peptides, yellow no difference, and green a gain for the Mtb L6 or L4 variant. *, indicates that no high-binding-affinity epitopes were detected for the HLA/protein pair.

Globally, whereas variations of Mtb L6 proteins in the predicted number of HLA class I high-affinity peptides did not affect a particular allele (within those selected for the study), in the case of HLA class II, the Mtb L6 mutations analyzed for the three selected antigens commonly affected the HLA-DRB1*11:02 allele, being invariably associated with a reduction in the number of high-affinity predicted epitopes. It would be interesting in the future to investigate whether individuals bearing this HLA class II allele show differences in susceptibility to TB disease or in the type and quality of CD4 T cell responses, particularly upon infection by Mtb L6 strains. Interestingly, in a study performed in East Asia, the HLA-DRB1*11 gene polymorphism was suggested to be a protective factor for TB risk [60]. Despite the many studies investigating possible association of HLA genetic variants with TB susceptibility [46–54], the results remain inconsistent. Our data highlight the genetic

background of the infecting Mtb as a variable to consider in these studies. Supporting this view, a study conducted in 682 TB patients and 836 healthy controls in Thailand found an association of the HLA-DRB1*09:01 and HLA-DQB1*03:03 alleles with TB susceptibility in patients infected with modern Mtb strains and suggested strain specific susceptibility to TB [61]. Also relevant, only a few studies on HLA association with TB were performed in individuals of African ancestry, despite the high burden of TB in Africa [1].

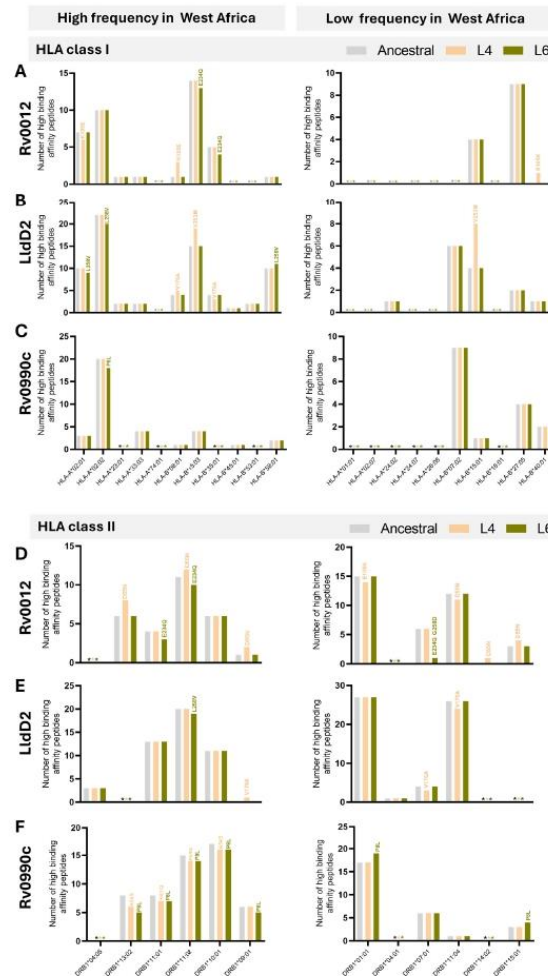


Figure 4. Predicted number of high-binding-affinity epitopes across lineages and HLA alleles. Number of high-binding-affinity epitopes predicted for Rv0012 (A,D), LldD2 (B,E), and Rv0990c (C,F) across the considered HLA class I (A–C) or HLA class II (D–F) alleles, as indicated on the x axis. Predictions for the ancestral sequences are represented in grey bars, for Mtb L4 variants in orange, and for Mtb L6 variants in green. Graphs on the left represent predictions for the indicated HLA alleles detected with high frequency in West Africa. Graphs on the right represent predictions for the HLA alleles detected with low frequency in West Africa. The non-synonymous substitutions that affect the number of high-binding-affinity predicted epitopes are indicated above each corresponding bar. Predictions were performed using NetMHCpan (HLA class I) and NetMHCIIpan (HLA class II), considering 8 to 14 mers for HLA class I and 9 to 18 mers for HLA class II. HLA frequencies were retrieved from allelefrequency.net. * (grey, orange, or green) indicates the absence of predicted high-binding-affinity peptides for the ancestral sequences, Mtb L4 variants, and Mtb L6 variants, respectively.

Although outside of the scope of this study, experimental validation of the reported predictions is needed to confirm their functional impact. Future studies employing in vitro assays with peripheral blood mononuclear cells from West African individuals typed for specific HLA alleles will be crucial to elucidate the extent to which the variant Mtb L6 proteins may modulate immune responses in a natural infection setting. Moreover, expanding our analysis to additional Mtb antigens under positive selection in L6 could reveal broader immune evasion strategies that contribute to its epidemiological niche. Our study is based on the extensively validated pan-specific tools NetMHCpan and NetMHCIIpan. Also important as a valuable avenue for future work will be the development of a dedicated QSAR model.

4. Conclusions

The immunoinformatics-driven analysis presented in this study offers insights into the potential host–pathogen interactions in place between Mtb L6 strains and their preferred hosts. Focusing on a rare subset of highly variant Mtb antigens, we revealed possible alterations in binding patterns of Mtb L6 antigen variants to host HLAs that may underlie the restricted geographic distribution of Mtb L6. Further understanding the functional impact of these findings may have important implications for TB research, from vaccine design to genetic susceptibility studies, specifically those involving TB caused by Mtb L6 strains. It will be important in the future to experimentally validate the predictions presented in this study. That will include testing the immunogenicity of the Mtb L4 and L6 variant antigens in cells from TB patients stratified for the infecting bacteria (i.e., Mtb L4 or L6) and for their HLA allele. Also important will be the application of other in silico tools, such as QSAR, to improve the power of our predictions. Furthermore, integrating in silico predictions with high-throughput in vitro epitope screens or single-cell TCR repertoire analyses will expand our understanding of how antigenic variation shapes T cell recognition across populations. Coupling HLA–epitope binding data with genome-wide association studies (GWAS) holds the potential to uncover host genetic markers of susceptibility or resistance to Mtb L6, guiding lineage- or region-specific vaccine design and precision medicine strategies. Together, these approaches underscore the need to account for both pathogen diversity and host genetic background when developing next-generation TB control measures. Combined, this knowledge can also guide researchers in identifying broad-spectrum antigens that promote appropriate immune responses in different populations, increasing the effectiveness of universal vaccines. Collectively, this will contribute to improve TB control efforts in endemic regions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microorganisms13051032/s1>, Table S1: List of oligonucleotides used in this study; Table S2: Mean VAF analysis across all lineages.

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Data Availability Statement: The data on variant allele frequencies are available in Supplementary Table S2. All genome sequences used for analyses were previously deposited online [39].

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Abbreviations

The following abbreviations are used in this manuscript:

CFU	Colony forming unit
HLA	Human leucocyte antigen
IC	Inhibitory concentration
IFN	Interferon
IL	Interleukin
L	Lineage
Mtb	<i>Mycobacterium tuberculosis</i>
MTBC	<i>Mycobacterium tuberculosis</i> complex
SNPs	Single nucleotide polymorphism
SVM	Support vector machine
TB	Tuberculosis
VAF	Variant allele frequency

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CHAPTER IV - INITIAL CHARACTERIZATION OF *M. TUBERCULOSIS* L4 AND L6 MIXED INFECTIONS

Initial characterization of *M. tuberculosis* L4 and L6 mixed infections

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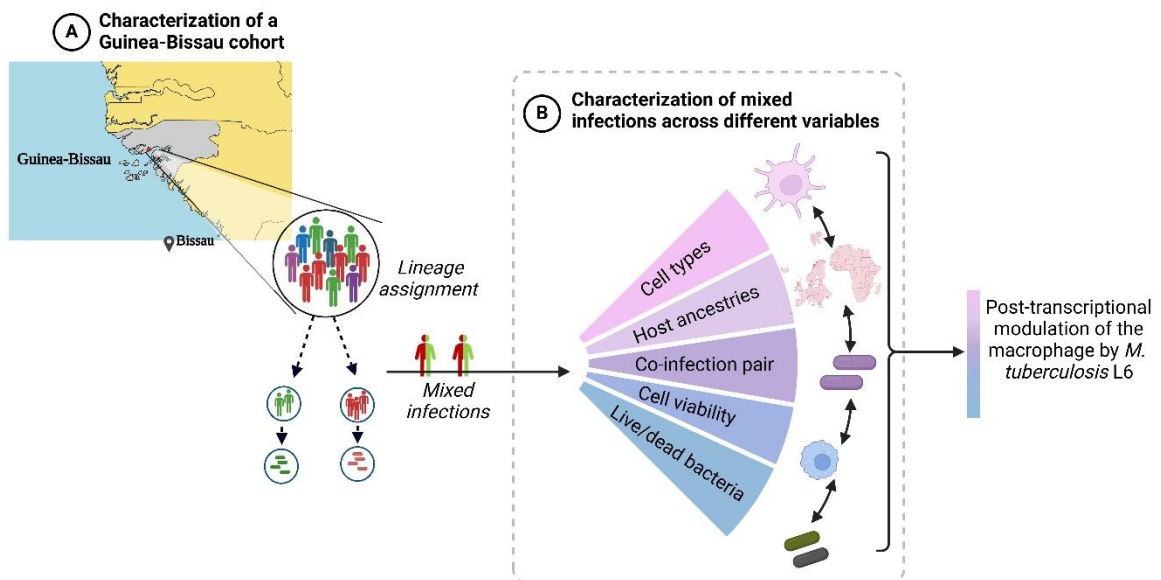
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Graphical abstract



This chapter is in preparation for publication.

Abstract

Tuberculosis (TB) remains one of the most persistent and lethal infectious diseases worldwide, causing over 1.25 million deaths annually. TB is caused by *Mycobacterium tuberculosis*. Ten human-adapted lineages (L) of *M. tuberculosis* have been described with different characteristics, namely their geographic distribution. *M. tuberculosis* L4 is globally prevalent and linked to severe disease, while *M. tuberculosis* L6 is confined to West Africa and associates with slower disease progression and reduced transmission. Interestingly, these two lineages co-exist in Guinea-Bissau, where mixed infections were also detected.

We investigated the immune response profiles in macrophages infected with clinical isolates of *M. tuberculosis* L4, L6, or a combination of both. Our findings indicate that the *M. tuberculosis* L6 strain modulates the macrophage response to *M. tuberculosis* L4, thereby reshaping the overall immune activation landscape. Collectively, these results underscore the critical need to further investigate mixed infections, as they may have profound implications for TB diagnostics, treatment strategies, and disease control.

Keywords: tuberculosis; mixed infections; disease severity; pathology; cytokines.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains one of the deadliest infectious diseases globally, despite decades of research and control efforts¹. Most studies focusing on TB are based on the assumption that a TB episode is caused by a single clonal *M. tuberculosis* strain. However, this concept has been challenged with the development of advanced molecular diagnostics, which revealed that a single TB patient can harbor multiple distinct *M. tuberculosis* strains simultaneously, a phenomenon known as mixed-strain infection²⁻⁵. The development of molecular detection techniques, including PCR-based assays targeting specific *M. tuberculosis* genotypes, has been essential in identifying mixed infections. These assays allow for strain-specific detection directly from clinical samples, uncovering mixed infections at significant rates, particularly in high TB-burden areas⁶⁻⁹. Interestingly, mixed infections have also been documented in regions with moderate and even low TB incidence, highlighting the global relevance of this phenomenon¹⁰⁻¹². Despite these advancements, documenting mixed infections remains challenging due to limitations in diagnostic sensitivity and the complexity of genotyping¹³.

Mixed-strain infections may have profound implications for TB management. Strain diversity within a patient complicates treatment selection and drug resistance profiling. Mixed infections have been associated with unfavourable treatment outcomes, including persistent infection, relapse, and treatment failure^{2,14}. This is particularly concerning in regions with high drug-resistant TB prevalence, where mixed infections with drug-sensitive and resistant strains can accelerate treatment resistance and transmission dynamics¹⁵. Importantly, the dynamics of TB disease progression and immune responses in the context of mixed infections remain underexplored. Some studies suggest that strain interactions within the host may influence disease severity, immune evasion, and transmission potential¹⁶. Understanding these interactions is crucial for improving TB treatment protocols and informing global TB control efforts. As the field advances, addressing the complexities of mixed infections is essential for developing effective public health interventions. Future research on elucidating the mechanisms driving mixed infections, their impact on treatment efficacy, and the evolutionary dynamics that shape strain coexistence within hosts is needed.

Our study focuses on the interaction between *M. tuberculosis* L4 and *M. tuberculosis* L6 in Guinea-Bissau, where these two lineages coexist, offering a unique system to study mixed infections. *M. tuberculosis* L4 is the most widely spread lineage, causing TB in all continents¹⁷⁻¹⁹. In contrast, *M. tuberculosis* L6 is geographically restricted to West Africa²⁰⁻²², although in this region it may cause up to 50% of the reported TB cases²³. Clinical

isolates of these lineages have been shown to behave differently in various models of virulence. *M. tuberculosis* L6 is described as having an overall reduced virulence, both in in vitro studies and in the mouse model of infection²⁴, being well controlled in the mouse lung²⁴⁻²⁶. On the other hand, studies in vitro and in vivo models with several L4 isolates²⁷⁻²⁹ have suggested that these pathogens are more virulent than *M. tuberculosis* L6 ones²⁴. Thus, *M. tuberculosis* L4 and L6 coexist in the same area, infecting similar hosts with different consequences, which makes their study a very interesting research topic. Given the distinct immunological profiles and ecological coexistence of *M. tuberculosis* lineages L4 and *M. tuberculosis* L6, we questioned whether and how these two *M. tuberculosis* lineages interact or influence each other during mixed infections. Through a comprehensive analysis of human and mouse immune cells infected with *M. tuberculosis* L4, L6 or in a mixed infection condition, we demonstrated that *M. tuberculosis* L6 suppresses pro-inflammatory cytokine production, particularly TNF- α , even in the presence of the *M. tuberculosis* L4 strain. This immune modulation is independent of the host's genetic background and persists across different immune cell types. These findings provide critical insights into how lineage-specific interactions shape the host immune response, potentially influencing TB disease progression.

Results

Characterization of *M. tuberculosis* L4 and L6 infections

Previous studies have shown that *M. tuberculosis* L4 and L6 coexist in West Africa³⁰⁻³³, where they contribute to the high burden of TB in this region. A cohort of 372 active TB patients (over 15 years old; 29.6% females; with GeneXpert and sputum positive results; at presentation) was recruited in Bissau, and the bacterial DNA extracted from each individual's sputum sample used to classify the infecting bacteria based on L1-L6 specific single nucleotide polymorphisms (SNPs) (Fig.1A). The majority of TB cases (93.3%; n = 347) were caused by *M. tuberculosis* of L1-L6, with *M. tuberculosis* L4 and L6 being the most prevalent *M. tuberculosis* lineages, representing respectively approx. 44% (164/372) and 40% (147/372) of the cases (Fig.1B). Interestingly, 4% (16/372) of the detected TB cases presented mixed infections with *M. tuberculosis* L4 and L6 strains (Fig.1B). Finally, 5.4% (20/372) of TB cases were caused by L1, L2, and L3 pathogens and 6.5% (25/372) likely by non-human-adapted members of the *Mycobacterium tuberculosis* complex (MTBC) (Fig.1B). No *M. tuberculosis* L5 infections were identified. Building on these epidemiological insights, we started by characterizing *M. tuberculosis* L4 and L6 isolates. (Fig.1A). We started by focusing in axenic culture, where the isolates exhibited distinct growth kinetics. Optical density (OD) measurements revealed that *M. tuberculosis* L4 replicated at a higher rate than *M. tuberculosis* L6 (Fig.1C), consistent with previous findings³⁴.

Building on these findings, we next focused on understanding how *M. tuberculosis* L4 and *M. tuberculosis* L6 infected host cells, using bone marrow derived macrophages (BMDMs), given their ability to closely mimic in vivo conditions. Our primary goals were to determine the infected rate in each condition and further assess the intracellular bacterial growth. To begin, we measured the percentage of infected cells in each condition by leveraging the use of fluorescently labelled bacteria. For this, we used *M. tuberculosis* L6 that has been tagged with GFP and *M. tuberculosis* L4, tagged with mCherry. The percentage of infected cells was quantified 4 hours post-infection using flow cytometry technique in the two single infections of *M. tuberculosis* L6 and L4. As shown in the graph (Fig.1D), the infection rate of BMDMs with *M. tuberculosis* L6 was approximately 30%, which was significantly lower than that of *M. tuberculosis* L4 (approximately 50%).

While the percentage of infected cells provides insight into the initial infection dynamics, it does not fully capture the intracellular growth of *M. tuberculosis* strains. For that, we evaluated the intracellular bacterial replication to determine whether the reduced bacterial growth of *M. tuberculosis* L6 infections seen in axenic medium was also reflected in the

bacterial growth within macrophages. For that, we quantified the bacterial load 4 hours and 4 days post-infection using colony-forming units (CFUs). The results show that *M. tuberculosis* L4 exhibited a higher bacterial load within BMDMs, in both time-points (Fig.1E, F).

We then extended this characterization to in vivo models by infecting the TB-susceptible C3HeB/FeJ mice with either isolate. The results revealed striking differences in disease progression and severity (Fig.1G, H). Mice infected with *M. tuberculosis* L4 showed rapid disease progression illustrated by the increase in the CFU burden in the lungs (Fig.1G) and severe lung pathology (Fig.1H), reaching the humane endpoint approximately 26 days post-infection. In contrast, mice infected with *M. tuberculosis* L6 exhibited much slower disease progression, with significantly lower CFU burden (Fig.1G) with almost no lung pathology observed (Fig.1H). Collectively, these findings demonstrate that the clinical isolates of *M. tuberculosis* L4 and L6 display distinct infection outcomes across the experimental models tested, potentially underscoring the divergent pathogenic potential of these lineages.

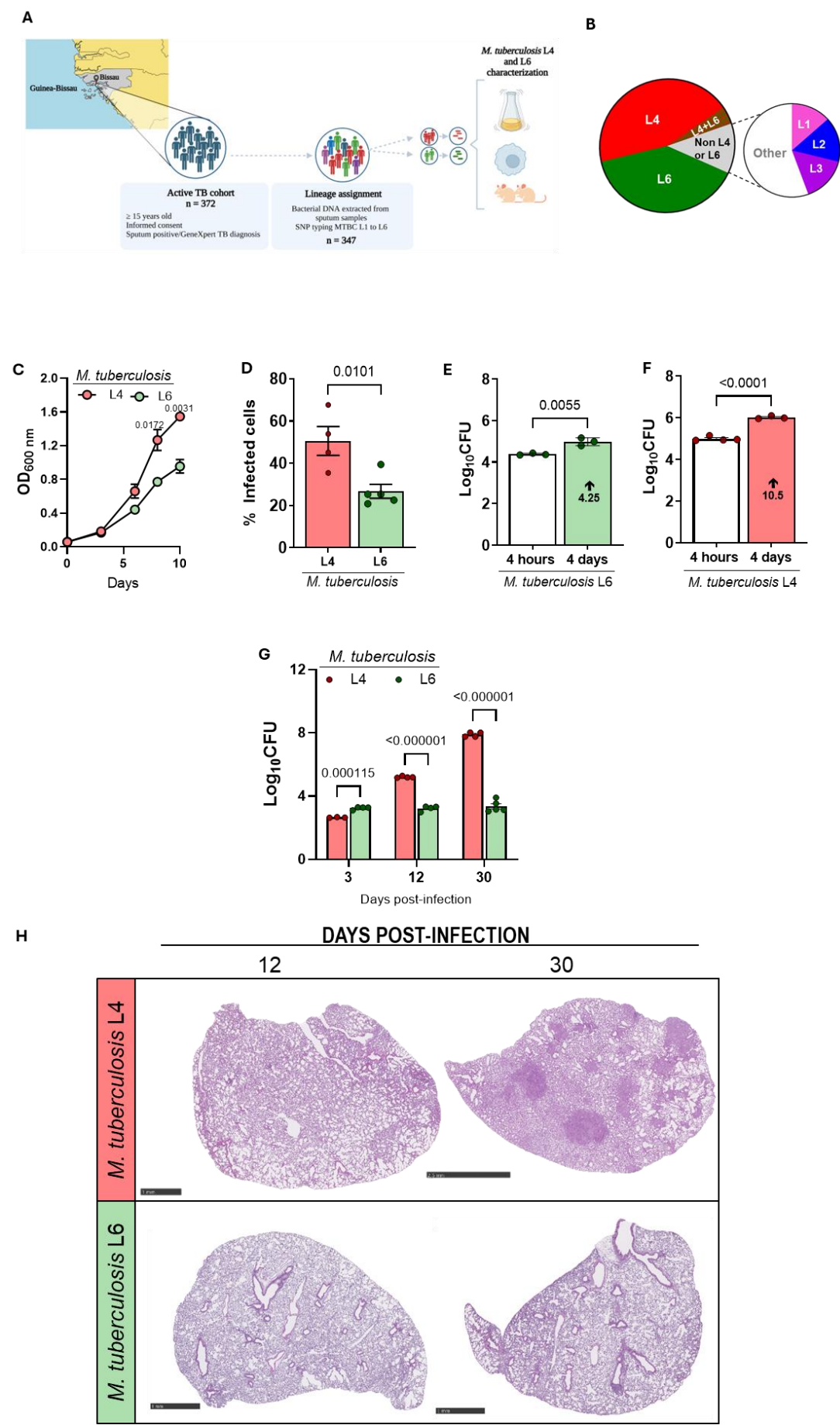


Figure 1. Characterization of *M. tuberculosis* L4 and L6 isolates: in vitro and in vivo analysis.

(A) Schematic representation of the experimental workflow used to characterize *M. tuberculosis* L4 and L6 isolates. The characterization included bacterial growth kinetics, intracellular replication, infection profile in macrophages, and in vivo infection outcomes. (B) Pie-charts showing the proportion of MTBC L1-6 in the Guinea-Bissau cohort. Lineage assignment was done by single nucleotide polymorphism typing in bacterial DNA extracted from the patient's sputum sample. "Other", refers to *M. tuberculosis* L7, L8 or L9, or non-human-adapted TB causing bacteria (C) Growth kinetics of *M. tuberculosis* L4 (pink) and L6 (green) in 7H9 medium. Bacterial growth was monitored by OD600 over time. (D) Infection rate of *M. tuberculosis* L4 and L6 BMDMs from C57BL/6 mice, assessed by flow cytometry using fluorescently labelled bacteria. Data show the percentage of infected cells for each isolate. (E, F) Intracellular replication of *M. tuberculosis* L6 and L4 in BMDMs, respectively, determined by CFU enumeration at 4 hours and 4 days post-infection. (G) Bacterial burden in lungs of C3HeB/FeJ mice infected with a high dose of *M. tuberculosis* L4 or L6. Lung CFUs were quantified at the indicated time-points post-infection. (H) Representative histological images of lung sections from C3HeB/FeJ mice infected with *M. tuberculosis* L4 or L6 at days 12 and 30 post-infection. H&E staining was used to evaluate tissue pathology. Scale bars are indicated in the figure. Each dot represents the mean of three wells (C), one well (D, E, F) or one mouse (G). Data are mean $\pm$ SEM. Statistical differences between the groups were identified by Mann-Whitney U tests: p-values $\leq$ 0.05 were considered significant. **Abbreviations:** CFU, colony forming unit; BMDM's, bone marrow derived macrophages; MTBC, *Mycobacterium tuberculosis* Complex; OD, Optical density; H&E, Hematoxylin & eosin; L, lineage.

The presence of *M. tuberculosis* L6 pathogens in a mixed infection reduces TNF- α levels by human macrophages

Taking the previous results into consideration, we questioned whether the immune response triggered by isolates of these two lineages independently or in the context of a mixed infection would vary. For that, we set up an in vitro infection system where different cells were infected with each *M. tuberculosis* isolate or with both together and different parameters were analysed (Fig.2).

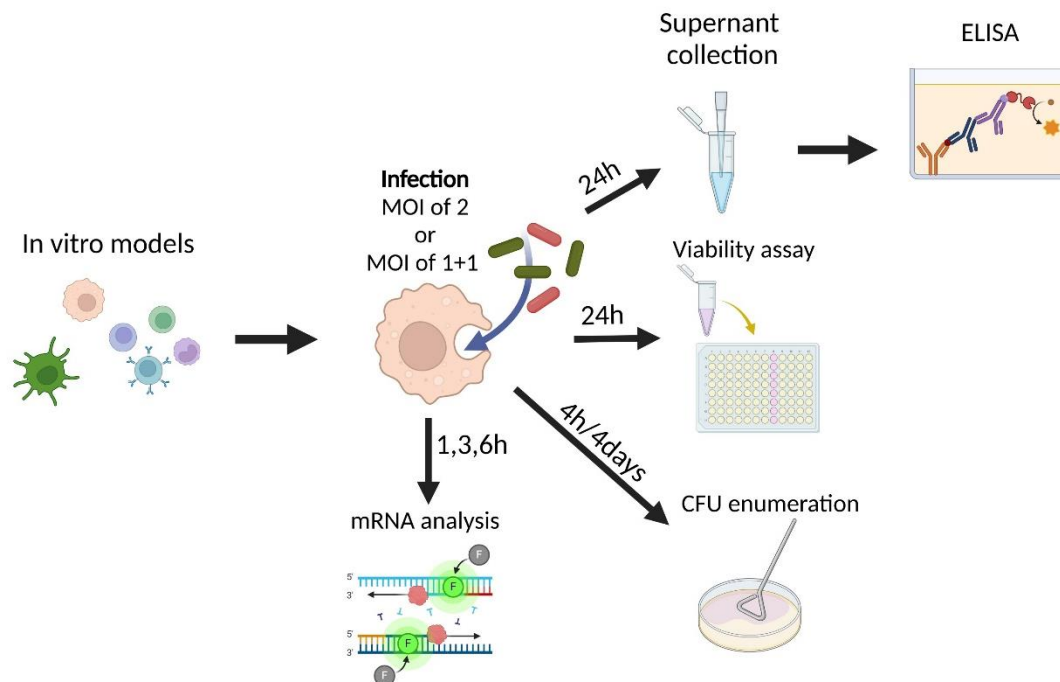


Figure 2. Schematic representation of single and mixed infections with *M. tuberculosis* isolates L4 and L6 in different in vitro models and their readouts.

We started by performing single or mixed infections in human phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells, and analysed the production of TNF- α as a proxy for cellular activation. We observed that the levels of TNF- α produced by infected THP-1 cells were significantly higher in response to *M. tuberculosis* L4 infections as compared to those induced by *M. tuberculosis* L6 infections (Fig.3A). Strikingly, we noticed that differentiated THP-1 cells infected with a mixed *M. tuberculosis* L4+L6 inoculum produced only minute TNF- α , similarly to what was observed in response to the single infection with the *M. tuberculosis* L6 strain (Fig.3A). Because this result was unexpected and surprising, we performed similar assays using peripheral blood mononuclear cells (PBMCs) isolated from 8-10 donors from the Guinea-Bissau cohort (Fig.3B). Again, in the mixed infection conditions, the detected TNF- α levels were significantly lower than those observed in *M. tuberculosis* L4 infections and even lower than those induced by *M. tuberculosis* L6

infections alone (Fig.3B). Next, we tested PBMCs from a different cohort in Porto, using IGRA+ donors (Fig.3C). Even with this variation in the donor population, the *M. tuberculosis* L6-mediated reduction in TNF- α production remained consistent, suggesting that host ancestry likely does not play a major role in the cytokine modulation observed in the presence of *M. tuberculosis* L6 infections. These observations suggest that the *M. tuberculosis* L6 isolate reduces the macrophage production of TNF- α in response to infections with *M. tuberculosis* L4, independently of the host cell genetic background.

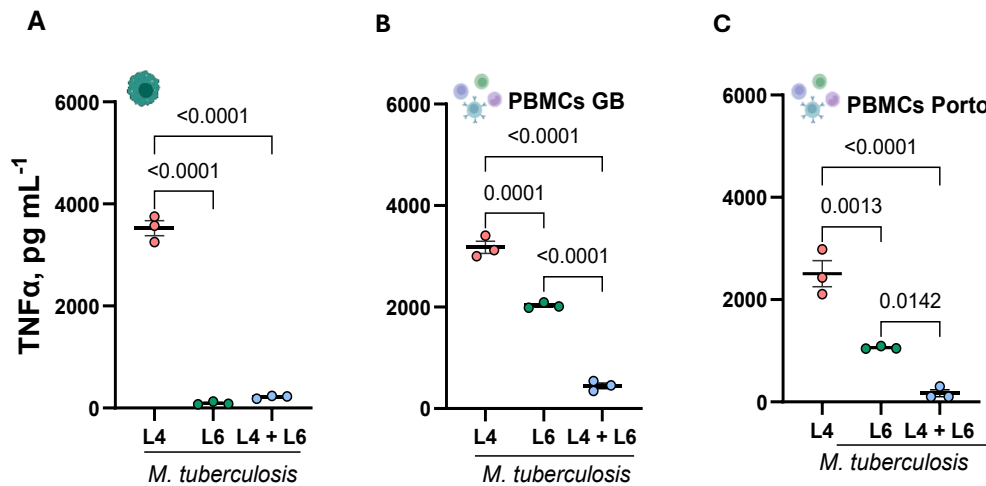


Figure 3. The presence of an L6 pathogen in L6/L4 mixed infections reduces TNF- α levels by human macrophages and PBMCs. (A) PMA-differentiated THP-1 cells after twenty hours of single or mixed infection with *M. tuberculosis* L4 and L6. (B) TNF- α production by PBMCs from the Guinea-Bissau cohort, or (C) PBMCs from the Porto cohort. The amount of secreted TNF- α was quantified in the culture supernatants by ELISA. Infections were performed at an MOI of 1 for single infections or 0.5+0.5 for mixed infections. Each dot represents the mean $\pm$ SEM of 8-10 donors in each PBMC's experiment (three independent experiments in total) and the medium of three independent wells for the THP-1 experiment. One-way ANOVA were used to determine statistical significance. p-values ≤ 0.05 were considered significant **Abbreviations:** PBMCs, peripheral blood mononuclear cells; TNF, tumor necrosis factor; ELISA, Enzyme-Linked Immunosorbent Assay.

Deciphering the immune response modulation by *M. tuberculosis* L6 across different cells

Our findings so far suggest that *M. tuberculosis* L6 interferes with the response of infected cells in terms of TNF- α production, even in the presence of a mixed infection with *M. tuberculosis* L4, across different human cells, regardless of their genetic background. To further investigate this possible modulation, we expanded our study to the mouse cells aiming to assess whether the cytokine modulation patterns induced by *M. tuberculosis* L6 were consistent across species. For that, we used BMDMs from two mouse strains: C3HeB/FeJ and C57BL/6. C3HeB/FeJ mice are known for their susceptibility to *M. tuberculosis* often displaying more severe disease progression and higher bacterial loads, making them a valuable model for studying acute and severe TB infections³². In contrast, C57BL/6 mice are more resistant to infection, showing more controlled bacterial growth and a more regulated immune response, which makes them a useful model for studying chronic TB and immune control mechanisms³⁵. BMDMs provide a physiologically relevant model, as they retain functional and phenotypic characteristics of tissue macrophages found in the lungs during infection^{36,37}. We assessed TNF- α production in single or mixed infections of C3HeB/FeJ and C57BL/6 BMDMs with *M. tuberculosis* L4 and *M. tuberculosis* L6 (Fig.4A, B). Consistent with the findings in human cells, mixed infections resulted in low TNF- α levels, comparable to those observed with *M. tuberculosis* L6 infection alone (Fig.4A, B). This pattern was similar across both mouse strains, reinforcing the idea of a specific immune evasion mechanisms employed by *M. tuberculosis* L6, independently on the host genetic background.

Having characterized the mixed infection profile using BMDMs from two different mouse models, we aimed to further investigate the interactions of *M. tuberculosis* L4 and L6 in other physiologically relevant cell types, specifically alveolar macrophages (AMs) and dendritic cells (DCs). AMs are crucial for initiating the immune response in the lungs, where *M. tuberculosis* typically establishes infection, while DCs play a key role in antigen presentation and the activation of T cells^{38,39}. By testing the mixed infections condition in these cells, we aimed to gain deeper insights into how the pathogenic mechanisms of *M. tuberculosis* may differ in the specialized lung environment compared to the systemic context provided by BMDMs. The results of *M. tuberculosis* L4 and L6 mixed infections in AMs (Fig.4C) and DCs (Fig.4D) are similar to those seen in BMDM. Given that all cell types tested exhibited comparable TNF- α responses, we proceeded with BMDMs for subsequent experiments, owing to their ease of isolation and practical advantages in the laboratory.

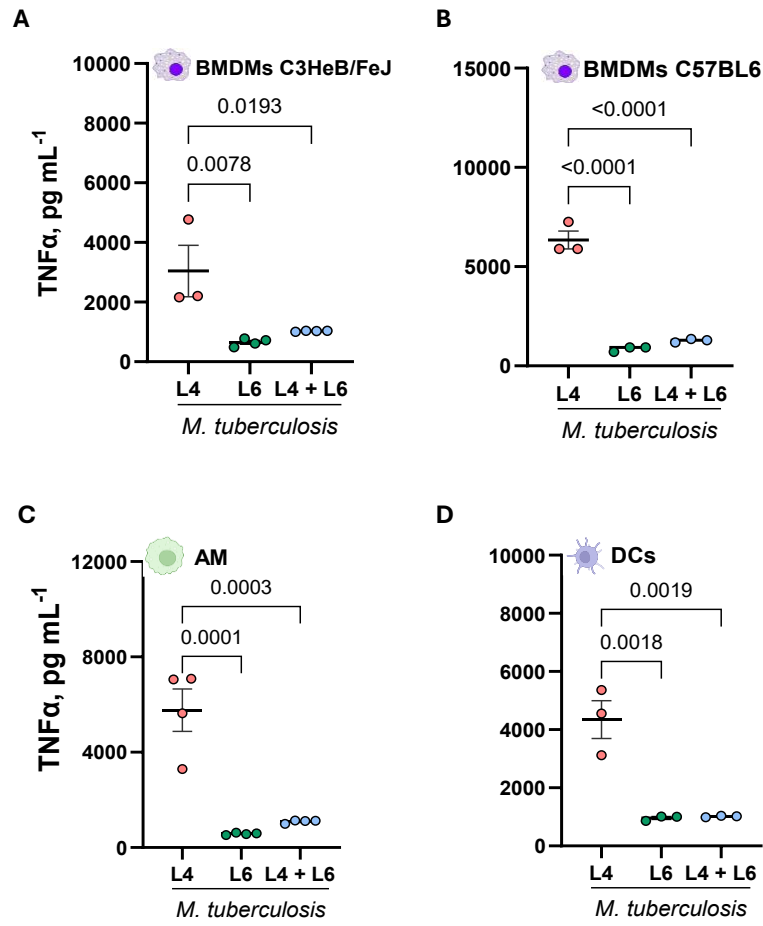


Figure 4. The presence of *M. tuberculosis* L6 in L6/L4 mixed infections reduces TNF- α levels across different cell types. Mouse BMDMs from C3HeB/FeJ (A) and C57BL/6 (B) strains, AMs(C), and DCs (D) were infected with *M. tuberculosis* L4, L6, or a combination of both isolates. Culture supernatants were collected twenty-four hours post-infection, and TNF- α concentrations were quantified by ELISA. Infections were performed at an MOI of 2 for single infections or 1+1 for mixed infections. Data are presented as mean $\pm$ SEM and is representative of two independent experiments. Each dot represents an independent well. Statistical significance was determined using one-way ANOVA, with $p \leq 0.05$ considered significant. **Abbreviations:** BMDMs, bone marrow derived macrophages; AMs, alveolar macrophages; DCs, dendritic cells; ELISA, Enzyme-Linked Immunosorbent Assay; TNF, tumor necrosis factor.

Prompted by these findings, we expanded our analysis to assess the effects of *M. tuberculosis* L6 in mixed infections on the levels of other key cytokines, particularly IL-10, IL-1 β , IL-1 α and IL-6. These cytokines were selected due to their critical roles in mediating inflammatory responses and regulating immune functions during *M. tuberculosis* infections⁴⁰. Remarkably, we found similar trends of lower levels for all these cytokines in the mixed infection condition (Fig.5A-E), further supporting that the host immune response to mixed infections is strongly dictated by *M. tuberculosis* L6.

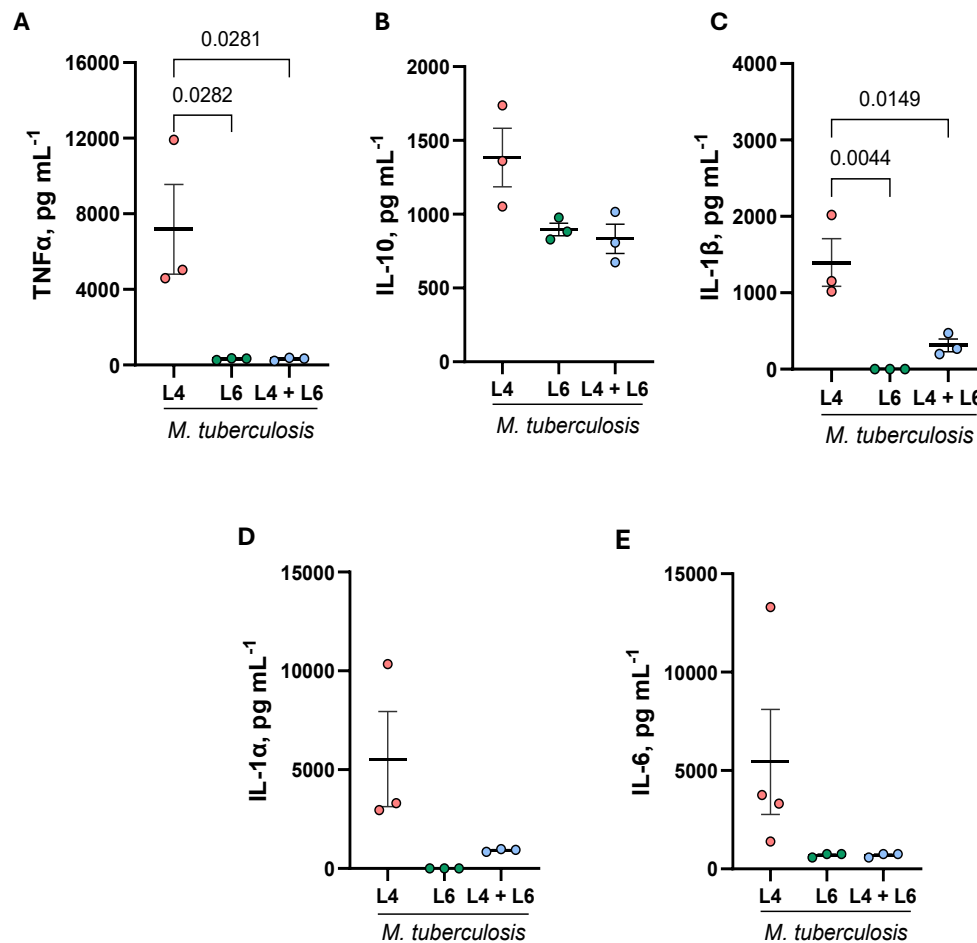


Figure 5. The presence of an L6 pathogen in L6/L4 mixed infections reduces the production of multiple cytokines by infected mouse macrophages. (A-E) Mouse BMDMs (C57BL/6) were infected with *M. tuberculosis* L4, L6, or a combination of both isolates. Culture supernatants were collected twenty-four hours post-infection, and the concentrations of the indicated cytokines were measured using LEGENDplex. Infections were performed at an MOI of 2 for single infections or 1+1 for mixed infections. Data are presented as mean $\pm$ SEM. Each dot represents an independent well. One-way ANOVA were used to determine statistical significance. p-values ≤ 0.05 were considered significant. **Abbreviations:** IL, interleukin; TNF, tumor necrosis factor; MOI, multiplicity of infection; BMDMs, bone marrow derived macrophages.

The macrophage modulation by *M. tuberculosis* L6 pathogen in mixed infections is independent of the co-infecting *M. tuberculosis* isolate

Thus far, our experiments have been focusing on mixed infections involving *M. tuberculosis* L4 and L6 clinical isolates from Guinea-Bissau, where both strains naturally coexist. To determine whether the observed effects are specific to this *M. tuberculosis* L4 isolate or reflect a broader interaction pattern of *M. tuberculosis* L6 with host cells, we expanded our analysis to include mixed infections with additional *M. tuberculosis* clinical isolates. These included *M. tuberculosis* L4 isolates from a distinct cohort in Porto, where *M. tuberculosis* L6 is highly uncommon, the reference laboratory strain H37Rv (L4), and the hypervirulent *M. tuberculosis* isolate HN878 (L2). The effects driven by *M. tuberculosis* L6 were consistently reproduced, regardless of the other co-infecting *M. tuberculosis* isolate (Fig.6A-B). This suggests that the suppression of TNF- α levels in the supernatant seen in mixed infections with *M. tuberculosis* L6 is not limited to a specific *M. tuberculosis* isolate, but rather represents a broader mechanism associated with *M. tuberculosis* L6 infections, which prevails during mixed infections.

Given these consistent findings across mixed infection scenarios with different *M. tuberculosis* strains, we questioned whether this immune modulatory effect of *M. tuberculosis* L6 would also be observed when cells were stimulated with single TLR agonists, such as lipopolysaccharide (LPS). To test this, we infected macrophages with *M. tuberculosis* L6 while simultaneously stimulating them with two different concentrations of LPS, a potent activator of the innate immune response³⁷. The results resembled what we have seen so far, a decrease in the TNF- α levels in the supernatant of LPS-stimulated BMDM when in the presence of *M. tuberculosis* L6 (Fig.6C).

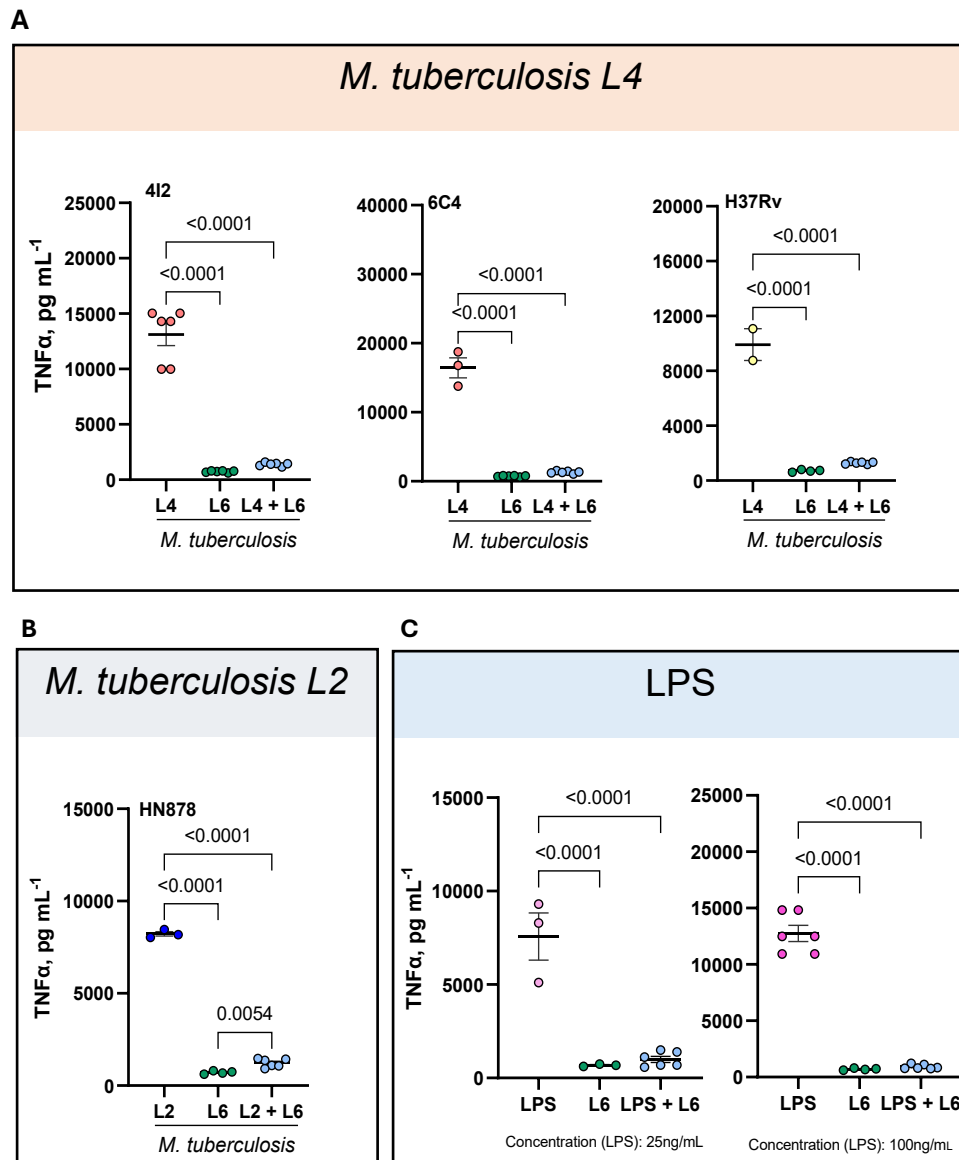


Figure 6. The macrophage modulation by L6 pathogen in mixed infections is independent of the other stimulus. Mouse BMDMs (C57BL/6) were infected with three different *M. tuberculosis* L4 strains (two clinical isolates from a cohort in Porto: *M. tuberculosis* 4I2 and 6C4, and the reference strain H37Rv (orange panel) (A) or a *M. tuberculosis* L2 isolate, HN878 (blue panel) (B) or stimulated with lipopolysaccharide (LPS) at a final concentration of 25 ng/mL or 100 ng/mL (pink panel), in a single or mixed infection with *M. tuberculosis* L6. Culture supernatants were collected twenty-four hours post-infection, and TNF- α concentrations were quantified by ELISA. Infections were performed at an MOI of 2 for single infections or 1+1 for mixed infections. Data are presented as mean $\pm$ SEM. Each dot represents an independent well. One-way ANOVA were used to determine statistical significance. p-values ≤ 0.05 were considered significant. **Abbreviations:** TNF, tumor necrosis factor; LPS, lipopolysaccharide; L, lineage, ELISA, Enzyme-Linked Immunosorbent Assay.

***M. tuberculosis* L6 modulates macrophage responses without impacting cell viability**

Our findings so far demonstrated that *M. tuberculosis* L6 infections decrease the levels of several pro-inflammatory cytokines secreted by BMDM in the context of mixed infections. To start investigating the underlying mechanisms, we assessed whether *M. tuberculosis* L6 would directly affect host cell viability as part of its immune evasion strategy. Since macrophage survival is critical for an effective immune response, we aimed to determine whether *M. tuberculosis* L6 infections were linked to a reduction in cell viability, potentially compromising immune function. The cell viability data measured 24h post-infection using ORANGU viability dye, show that *M. tuberculosis* L6 did not decrease the viability of macrophages as compared to those infected with *M. tuberculosis* L4 (Fig.7). In the case of mixed infections, the macrophage viability remained high, approaching the levels observed in *M. tuberculosis* L6 single infections. These findings suggest that *M. tuberculosis* L6 does not reduce the macrophage viability to modulate its response in a mixed infection condition. This increased viability in *M. tuberculosis* L6 single and mixed infections contrasts sharply with the low cell viability seen in *M. tuberculosis* L4 single infections, reinforcing the distinct behaviour of *M. tuberculosis* L6 in modulating host cell responses.

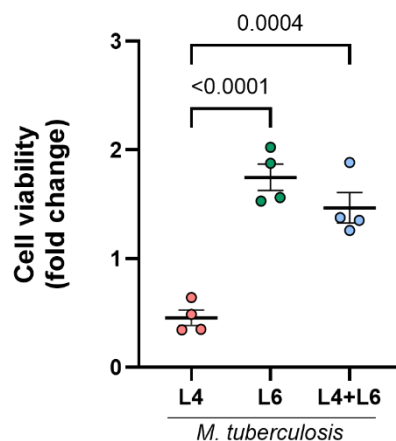


Figure 7. *M. tuberculosis* L6 infections does not compromise the BMDM viability. Mouse BMDMs (C57BL/6) were infected with *M. tuberculosis* L4, L6, or a combination of both isolates. Infections were performed at an MOI of 2 for single infections or 1+1 for mixed infections. Cell viability was measured 24 hours post-infection using the ORANGU cell counting solution colorimetric assay. Data are represented as log₂ (fold change relative to non-infected macrophages). Each dot represents an independent well. One-way ANOVA were used to determine statistical significance. p-values ≤ 0.05 were considered significant. **Abbreviations:** L, lineage; ELISA, Enzyme-Linked Immunosorbent Assay.

The modulation of the TNF- α levels by *M. tuberculosis* L6 is dependent on bacterial viability

To determine if the modulation of the immune response by *M. tuberculosis* L6 required live bacteria, we inactivated *M. tuberculosis* L6 with rifampicin overnight and evaluated the TNF- α levels in the supernatant of coinfecting cells. By comparing TNF- α levels in infections with live *versus* inactivated *M. tuberculosis* L6, we aimed to understand whether the reduced levels of TNF- α observed in the previous results requires active bacterial processes or if non-viable bacterial components alone are sufficient to trigger this effect. As compared to BMDM infected with live pathogens, those stimulated with rifampicin-treated *M. tuberculosis* L4 and L6 secreted significantly less TNF- α . However, whereas live *M. tuberculosis* L6 reduced the amount of TNF- α secreted in mixed infections of BMDM, as seen in the previous sections, inactivated bacteria did not (Fig.8). Thus, the modulation of TNF- α secretion by BMDM in mixed infections is driven by viable bacteria.

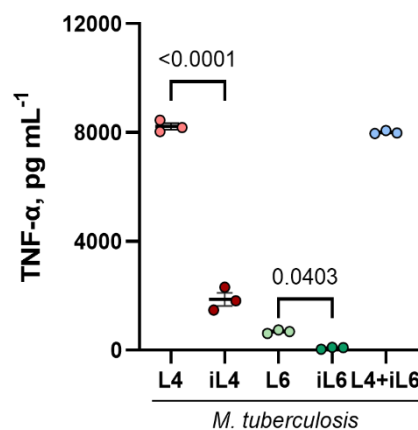


Figure 8: The presence of an inactivated L6 pathogen in a L6/L4 infection does not affect TNF- α secretion by the L4-infected macrophage. *M. tuberculosis* L6 was inactivated overnight with rifampicin (50 μ M) prior to infection. Mouse BMDMs (C57BL/6) were infected with *M. tuberculosis* L4, inactivated L6, or a combination of both isolates. Infections were performed at an MOI of 2 for single infections or 1+1 for mixed infections. Culture supernatants were collected twenty-four hours post-infection, and TNF- α concentrations were quantified by ELISA. Data are mean $\pm$ SEM. Each dot represents an independent well. One-way ANOVA were used to determine statistical significance. p-values ≤ 0.05 were considered significant. **Abbreviations:** L, lineage; TNF, tumor necrosis factor; I, inactivated; ELISA, Enzyme-Linked Immunosorbent Assay.

Transcriptional analysis of cytokine genes in mixed infections

In light of the promising cytokine profiles observed in the supernatant, we proceeded to assess the transcriptional expression of key cytokine-encoding genes implicated in the immune response to *M. tuberculosis*. For that, we infected BMDM's with the three conditions under study and evaluated the mRNA levels of several cytokines by real-time PCR, 3 hours post-infection. The transcript levels of the cytokine-encoding genes analysed in single versus mixed infections appeared to be comparable at the 3-hour post-infection time point (Fig.9A-D). However, some cytokines, such as *il6* and *il1 β* , showed only a modest induction. Importantly, the non-infected(NI) controls displayed negligible cytokine expression, confirming that the basal levels of transcription in the absence of infection were minimal. Because we only analysed one time point post-infection and the kinetics of mRNA expression in the different infections may impact the overall protein output, we next focused on TNF- α for a detailed analysis of its transcriptional profile over time. Importantly, as shown in Fig.9E, we observed that there were no significant alterations in the expression levels of this cytokine across the chosen time points in all three experimental conditions. The temporal analysis of TNF- α confirmed stable transcript levels across all conditions and time points evaluated.

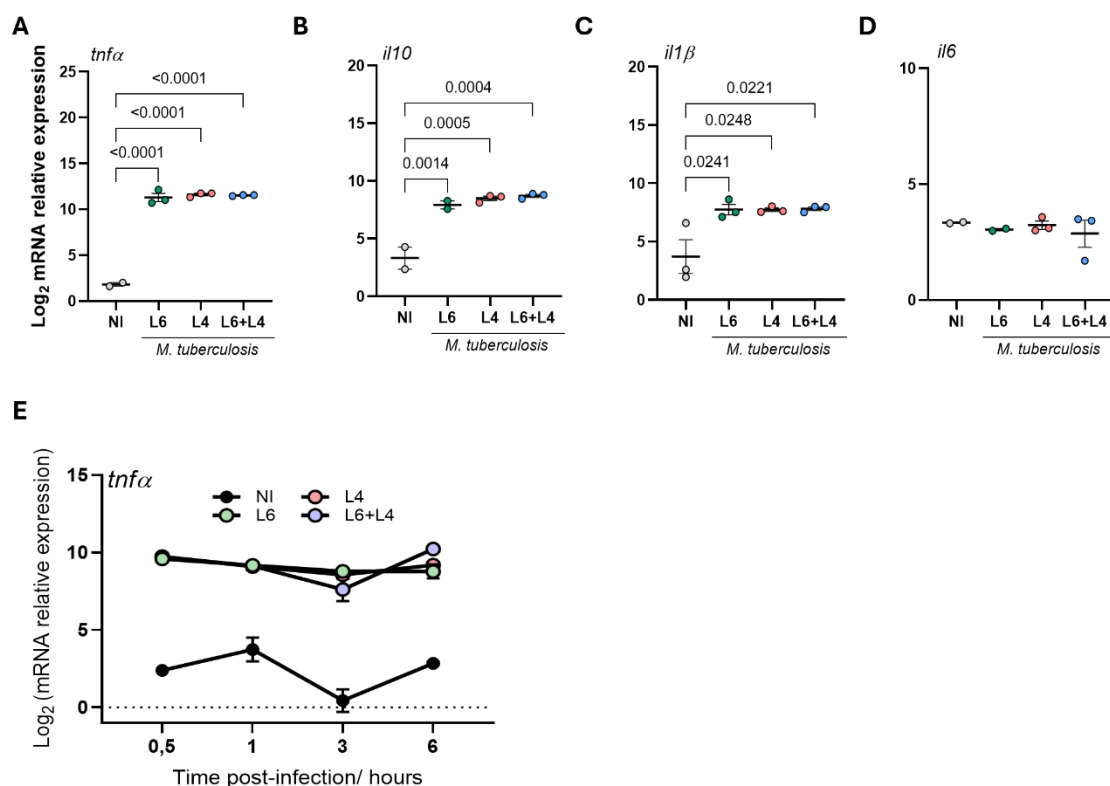


Figure 9. Transcriptional profiles of *M. tuberculosis* L4 or L6 clinical isolates in single and mixed infections are similar. Mouse BMDMs from C57BL/6 were infected for 3 hours (A-D) with *M. tuberculosis* L4 or L6 clinical isolates, at a multiplicity of infection (MOI) of 2, or 1+1 in the case of mixed infections. The expression levels of the indicated genes were measured by qPCR. TNF- α mRNA expression levels were measured by qPCR at 30 minutes, 1 hour, 3 hours, and 6 hours post-infection (E). Data are presented for samples where the gene expression was detectable. Data are shown as mean $\pm$ SEM. Each dot represents an independent well. One-way ANOVA tests were used to identify statistical differences between groups. p-values ≤ 0.05 were considered significant. **Abbreviations:** L, lineage; il, interleukin; tnf, tumor necrosis factor.

Discussion

The control of TB relies on understanding the interaction between the bacteria and the host, including mixed infections. This study combines in vitro experiments to characterize the underlying immune mechanisms and their impact on disease progression. Although the first mixed infection case was described in 1975, only recently have its implications for diagnosis, treatment, and TB control been recognized^{14,41}. A deeper understanding of these interactions is essential for developing new strategies to combat the disease. Our study highlights the complex interaction between *M. tuberculosis* L6 and L4 during mixed infections, focusing on the immune modulation observed in different conditions. Previous studies have reported attenuated immune responses in *M. tuberculosis* L6 infections, both in vivo and in vitro^{24-26,42}. The cohort in Guinea-Bissau is crucial for studying the dynamics of mixed infections, as both *M. tuberculosis* L4 and L6 naturally coexist in this region. Our findings indicate that the prevalence of the two lineages are similar in this area, and furthermore, both lineages can be found in the same individual. Interestingly, recent data from another study conducted in the same geographical region also demonstrated the existence of mixed infections with these two lineages⁴³. These results underscored the relevance of this population in understanding the interplay between these strains. *M. tuberculosis* L4 and L6 exhibit distinct genetic characteristics²⁴, and although they typically lead to different disease progression patterns, they often result in similar disease outcomes¹⁴.

The presence of a mixed infection could result in more severe disease and more significant alterations in the immune system, considering the different characteristics of the two bacteria involved. However, our results suggest that, despite the well-documented inflammatory potential of *M. tuberculosis* L4, the presence of the *M. tuberculosis* L6 strain leads to a marked suppression of a key pro-inflammatory cytokine: TNF- α . This observation was consistent across several experimental systems, including human PBMCs, THP-1 macrophages, as well as BMDMs from both TB-susceptible and resistant strains, and was further corroborated in alveolar macrophages and dendritic cells. The immune suppression seen for TNF- α was also verified for other cytokines as IL-1 β and IL-6 suggesting that *M. tuberculosis* L6 may manipulate the innate immune response in a broader way to create a less inflammatory environment, potentially allowing both *M. tuberculosis* L4 and L6 to persist longer within the host during the mixed infection. The lower cytokine production may also enable *M. tuberculosis* L6 to avoid detection by the host immune system, leading to a delayed disease progression.

Moreover, this reduction in TNF- α was not limited to mixed infections with L4: *M. tuberculosis* L6 also suppressed TNF- α levels in macrophages co-infected with other *M. tuberculosis* strains as H37Rv, HN878 or clinical isolates from different cohorts. In addition, infection of BMDM's with *M. tuberculosis* L6 concurrently with LPS stimulation also resulted in a reduction in TNF- α levels, indicating that the modulation by *M. tuberculosis* L6 transcends pathogen-specific pathways.

Importantly, our findings regarding cell viability suggest additional insights into the immune evasion strategy by *M. tuberculosis* L6. The increased macrophage viability observed during *M. tuberculosis* L6 and mixed infections, compared to *M. tuberculosis* L4 alone, suggests that the modulation of TNF- α secretion by the infected macrophage is not a consequence of decreased cell viability. Furthermore, this increased cell viability during *M. tuberculosis* L6 infections may contribute to the pathogen's ability to persist in a latent state or avoid immune-mediated cell death, a key aspect of *M. tuberculosis* pathogenesis⁴⁴⁻⁴⁶. These results indicate that *M. tuberculosis* L6's immune modulation may extend beyond cytokine suppression, potentially creating host environment that favours bacterial survival and long-term persistence, yet in the absence of a strong immune response. Furthermore, our findings also highlight that the modulation of TNF- α secretion requires live bacteria. It is appealing to conclude that live *M. tuberculosis* L6 bacteria actively interfere with host immune signalling pathway to dampen the pro-inflammatory response, rather than relying on passive recognition of bacterial components. These results suggest that *M. tuberculosis* L6 may influence the immune response at an early stage. The mRNA data show no significant differences in expression between the three experimental conditions, and the kinetics of TNF- α mRNA expression was also similar across all conditions, excluding a delayed response to *M. tuberculosis* L6 or mixed infections. These findings lead to the compelling conclusion that the observed differences in cytokine levels at the protein stage are likely regulated at a post-transcriptional level, rather than resulting from transcriptional suppression. Elucidating the mechanisms governing this post-transcriptional regulation is critical, as it may provide novel insights into how *M. tuberculosis* L6 modulates the immune response and affects cytokine production during mixed infections.

Interestingly, our findings align with previous observations in other pathogens that apply similar immune evasion strategies. For instance, SARS-CoV-2 manipulates cytokine production at the post-transcriptional level through non-structural proteins such as NSP1 and NSP3, which inhibit mRNA translation and remove immune modifiers like ISG15 from sensors, effectively dampening immune responses⁴⁷. Similarly, post-transcriptional regulatory mechanisms involving RNA-binding proteins such as Regnase-1 and miRNAs have been shown to modulate inflammasome activity in *Listeria monocytogenes* and

Helicobacter pylori infections, illustrating the critical function of such pathways in immune evasion^{48,49}. Moreover, pathogens such as *Staphylococcus aureus* and *Escherichia coli* leverage 3'UTR-mediated regulation of Nod-like receptor pathways to evade innate immune detection and manipulate cytokine responses, demonstrating a convergent evolutionary strategy among diverse microbes⁵⁰. Thus, it is possible that the modulation of innate immune responses by *M. tuberculosis* L6 rely on similar mechanisms. The parallels between these mechanisms and *M. tuberculosis* L6's ability to modulate host immunity suggest a broader applicability of targeting post-transcriptional regulatory networks in antimicrobial strategies. Furthermore, *M. tuberculosis* L6's reduced intracellular growth and persistence reflect survival strategies observed in *Candida albicans* and *Helicobacter pylori*, where slow replication and precise cytokine modulation enable long-term host colonization⁴⁸. This association emphasizes the importance of integrating our understanding of immunomodulatory mechanisms into therapeutic approaches for managing chronic and mixed infections. Collectively, these findings underscore the value of a comparative approach to unravelling immune evasion tactics employed by different members of the MTBC.

Material and Methods

Ethics statement for Human sample collection

The study protocol for blood and sputum collection was approved by the *Comité Nacional de Ética na Saúde* (CNES), Guinea-Bissau, reference number 003-CNES/INASA/2018. The PBMC samples study was reviewed and approved by the Portuguese *Comissão de Ética para a Saúde da ARS Norte* (#T792). For both studies, all participants signed an informed consent. To ensure confidentiality, data were anonymized by the assignment of a random identification number to each case. The study was conducted according to the principles expressed in the Declaration of Helsinki and the Oviedo Convention.

DNA extraction and MTBC L1-*M. tuberculosis* L6 assessment

Bacterial DNA was extracted from sputum samples using GenoLyse (Bruker, Cat. #51610) according to the manufacturer's guidelines and used for MTBC L1-*M. tuberculosis* L6 genotyping by a custom TaqMan® real-time PCR assay (Sup. Table 1; Applied Biosystems) based on the use of SNPs as stable genetic markers⁵¹. Results were analysed using the Bio-Rad CFX Manager™ 3.1 and the MTBC lineages assigned using the Allele Discrimination tool.

Peripheral blood mononuclear cells: isolation, culture and infection

Human peripheral blood mononuclear cells (PBMCs) were separated using Histopaque 1077 (Sigma-Aldrich, Cat. #10771) following the protocol for mononuclear cell separation of SepMate-50 tubes (StemCells, Cat. #85450). Purified PBMCs were frozen upon separation. Before infection, cells were thawed, cultured in RPMI (Gibco, Cat. #61870036) supplemented with 10% FBS (Gibco, Cat. #10500064), 1% HEPES (Gibco, Cat. #15630056) and 1% sodium pyruvate (Sigma-Aldrich, Cat. #58636), and rested overnight. PBMCs were infected with the selected *M. tuberculosis* isolates at total multiplicity of infection (MOI) of 1. Culture supernatants were collected 24 hours after infection/stimulation for protein quantification.

THP1 cells: differentiation, culture and infection

THP1 cells were grown according to ATCC instructions, differentiated with PMA (Sigma-Aldrich, Cat.#16561-29-8) for 24h, followed by a 48h rest before infection and then plated

in 24 well-plates at a density of 1×10^6 cell mL⁻¹ and infected with the *M. tuberculosis* isolates at a total MOI of 1. Culture supernatants were collected 24 hours after infection/stimulation for protein quantification.

Animal housing and infection

C57BL/6 and C3HeB/FeJ mice were bred and housed at the i3s animal facility; Males and females of 8 to 12 weeks old were used to generate BMDM's, alveolar macrophages and dendritic cells for posterior infection. Animals were maintained under specific-pathogen free conditions in the animal facility at i3s, in controlled temperature (20-24°C), humidity (45-65%), and a light cycle of 12h (light/dark). Water and food were provided *ad libitum*. Animal experiments followed the 2010/63/EU Directive and were approved by the i3S Animal Ethics Committee and the Portuguese National Authority for Animal Health (DGAV; #018413/2021-11-24).

Mice were infected with *M. tuberculosis* (isolates L4 and L6) through the aerosol route using an inhalation exposure system (Glas-Col)^{52,53}. To determine the dose of infection, the bacterial load in the lungs of 3 mice was quantified 3 days post-infection. Infected mice were weighed every week, or every other day when showing signs of disease. Mice were euthanized at the indicated time points or when reaching humane endpoints (weight loss over 20% or poor responsiveness to physical stimulation).

Lung processing

Lungs were aseptically excised and processed as previously described^{52,53}. Briefly, lungs were digested with Collagenase D (Roche, Cat. #11088882001) followed by physical disruption and filtration through 70 µm cell strainers (Falcon, Cat. #352350.0). Cell suspensions were used for colony forming units (CFU) determination, flow cytometry and mRNA analysis.

Histological analysis

Whole lungs were perfused in situ with PBS, the right upper lobe was excised, fixed in 10% buffered formalin and embedded in paraffin. Serial 3-µm thick sections were performed and used for hematoxylin and eosin (H&E) staining.

Bone marrow derived macrophages: differentiation, culture and infection

Mouse BMDM's were generated from C57BL/6 bone marrow suspension cells as previously described⁵⁴. On day 7 of culture, the macrophages were recovered, plated in 24 well-plates at a density of 1×10^6 cell mL^{-1} and infected with the selected *M. tuberculosis* isolates at a total multiplicity of infection (MOI) of 1 or 2 according to the aim of the experiment. For specific experiments, bacteria were pretreated with rifampicin (50 μM) overnight prior to infection. The bacteria were then pelleted by centrifugation and the concentration adjusted before infection. For conditions where the cells were stimulated with LPS (25 or 100 ng/mL), LPS was added to the cells and incubated in the same conditions as the infected wells. Culture supernatants were collected 24 hours after infection/stimulation for protein quantification. For CFU enumeration, extracellular bacteria were washed away with PBS after 4 h and bacterial growth was quantified at 4 hours (baseline) and 4 days post-infection. For mRNA extraction, TripleXtractor (GRiSP, Cat. #GB23.0100) was added to the cells 3h post-infection or in the kinetics experiments at 30 min, 1,3 and 6 hours post-infection, according to the manufacturer's instructions.

Mouse alveolar macrophages: isolation, culture and infection

AMs were collected directly from the bronchoalveolar lavage fluid (BAL) of mice. Briefly, the trachea of euthanized was exposed and 1mL of PBS (GIBCO) + 2% FBS using a 20G catheter. The PBS was flushed into the lung of the mice and then aspirated to recover the fluid. The procedure was repeated three times. Cells were then plated in complete (c)RPMI (RPMI (Gibco, Cat. #61870036) supplemented with 10% FBS (Gibco, Cat. #10500064), 1% sodium pyruvate (Sigma-Aldrich, Cat. #S8636), 1% HEPES (Gibco, Cat. #15630106)), containing Penicillin-Streptomycin (100 U mL^{-1} ; Invitrogen). Cells were plated at a density of 0.5×10^6 cell mL^{-1} in 96 well-plates and allowed to rest overnight prior to infection.

Dendritic cells: differentiation, culture and infection

DC's were generated from bone marrow cells of femurs and tibia of 6- to 9-weeks old C57BL/6 mice as previously described⁵⁵. Briefly, the cells were cultured in cRPMI supplemented with and 50ng/ml GM-CSF (R&D, Minneapolis, MN). Cells were maintained with fresh medium containing 50ng/ml GM-CSF every 2 days and used on day 7. DCs (>65% purity) were plated in 96-well plates at a density of 0.5×10^6 cell mL^{-1} with cRPMI medium and allowed to an overnight rest prior to infection.

Cytokine quantification

Cytokines levels in the cell supernatants of in vitro infected cultures were quantified by ELISA (Invitrogen, Cat.# 88-7324-88), following the manufacturer's instructions. Additionally, cytokine and chemokine levels were also measured using the LEGENDplex™ Mouse Inflammation Panel kit (IL-1 α , IL-1 β , IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN- β , IFN- γ , TNF- α , and GM-CSF; Biolegend, Cat.#740446_Mouse Inflammatory Panel) following manufacturer's specifications. The beads were acquired in an BD Accuri C6 (BD Biosciences), and the data analysed using the LEGENDplex™ Software version 8 (BioLegend).

mRNA analysis by real-time PCR

Total RNA from infected cells was extracted with TripleXtractor (GRiSP, Cat. #GB23.0100), according to the manufacturer's instructions and quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific). Complementary deoxyribonucleic acid (cDNA) was synthesized using the ProtoScript First-Strand cDNA Synthesis kit (ThermoScientific, Cat. #E6300L). Real-time PCR assays were performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad). Target gene mRNA expression was quantified by real-time PCR using iTaq Universal SYBR Green Supermix (Biorad, Cat. #1725121) and normalized to ubiquitin and Hmbs mRNA levels. The oligonucleotide sequences used are listed in Supp. Table 1. The specificity of amplification was confirmed by melting curve analysis.

Cell viability quantification

Twenty-four hours post-infection, Orangu™ (Cell Guidance Systems, Cat.# OR01-1000) was added to the cells and incubated for 2h at 37 °C. Absorbance was read at 450 nm.

CFU determination

Cells or lung cell suspensions were lysed with 0,1% of saponin, serial dilutions prepared and plated in Middlebrook 7H11 agar (BD Biosciences, Cat. #212203.0) supplemented with OADC and PANTA (BD BioSciences, Cat. # 245114). CFU were determined after 21-28 days of incubation at 37°C.

Bacteria growth and quantification

The *M. tuberculosis* L4 and *M. tuberculosis* L6 clinical isolates used in the study were recovered from the sputum of TB patients from Bissau and grown as before^{53,56}. These isolates and *M. tuberculosis* HN878 were expanded in Middlebrook 7H9 (BD Biosciences, Cat. #90003-876) liquid medium supplemented with 10% OADC and 0.2% glycerol (Sigma-Aldrich, Cat. #G5516-500M) and when in mid-log phase bacterial suspensions were aliquoted in cryovials, frozen, and stored at -80°C. Bacterial quantification was performed by thawing six vials of each stock and plating several serial dilutions in 7H11 agar medium supplemented with 10% OADC and 0.5% glycerol. The plates were incubated 21 to 28 days at 37 °C before colony count.

Fluorescent reporter bacteria strains

To generate *M. tuberculosis* reporter strains, parental *M. tuberculosis* L4 and L6 clinical isolates were transformed with the plasmid pBP10 (kindly provided by David Russel), as previously described⁵⁷. The *M. tuberculosis* L4 strain was transformed with pBP10 carrying a hygromycin resistance gene and expressing mCherry, whereas the *M. tuberculosis* L6 strain was transformed with a plasmid containing a kanamycin resistance gene and expressing GFP. Following transformation, colonies were selected on 7H11 agar plates supplemented with 50 µg/ml hygromycin B (Alfagene, Cat. #10687010.0) or kanamycin (Alfagene, Cat. #15160054.0), respectively. HygR/mCherry+ and KanR/GFP+ colonies were subsequently cultured to log phase in Middlebrook 7H9 broth supplemented with 10% OADC, 0.2% glycerol, and the corresponding antibiotic. Bacterial suspensions were aliquoted into cryovials and stored at -80°C until further use. Several clones were tested for mCherry or GFP intensity by flow cytometry and in functional assays in macrophages.

Flow cytometry

For surface staining, BMDM's suspensions were blocked with anti-mouse CD16/32 antibody (clone 93; BioLegend, Cat.#101320) at the concentration of 1:200 in PBS 1X at 4 °C for 10 minutes. Cells were then fixed for 20 min in 4% paraformaldehyde-PBS. Dead cells were excluded using ZombieAqua (Biolegend, Cat. #423102). Data were collected on an LSR Fortessa and LSR II (BD Biosciences). Analysis were performed using FlowJo software v10.5.3. Cells were acquired on a BD Fortessa II and data was analysed using FlowJo software (v10.1.r7).

Author contributions

Conceptualization: MLS, JC, BC, MS

Formal analysis: MLS, MC, MS

Funding Acquisition: MLS, MS

Investigation: MLS, JC, MaS MC,

Project administration: MLS, BC, MS

Resources: MV, BC

Supervision: PNSR, MS

Visualisation: MLS, JC, MS, MC

Writing of the draft: MLS, MS

Writing-review and editing: all authors

Competing interests

The authors declare no competing interests.

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Supplementary Table 1. List of primer-probes and oligonucleotides used in this study.

	Lineage	Primer - probe
<i>M. tuberculosis</i>	L1	Rv3597682CT
	L2	Rv3304966GA
	L3	Rv4266647AG
	L4	Rv2154724AC
	L5	Rv1377185GC
	L6	Rv378404GA
	Gene	Oligonucleotide Forward (5'-3') Reverse (5'-3')
Mouse	<i>Ifnb</i>	GCA CTG GGT GGA ATG AGA CT AGT GGA GAG CAG TTG AGG ACA
	<i>Il6</i>	ACACATGTTCTCTGGGAAATCGT AAGTGCATCATCGTTGTTCATACA
	<i>Il10</i>	ATTTGAATTCCCTGGGTGAGAAG CACAGGGGAGAAATCGATGACA
	<i>tnf</i>	GCCACCACGTCTTCTGTCT TGAGGGTCTGGGCCATAGAAC
	<i>Il1b</i>	ACC TTC CAG GAT GAG GAC ATG A AAC GTC ACA CAC CAG CAG GTT A
	<i>Il12p40</i>	CAA ATT ACT CCG GAC GGT TC AGA GAC GCC ATT CCA CAT GTC
	<i>ccl2</i>	CCT GGT GCA CAA CAT CGC C AAT CAA ACA GAG GCC GCA TGC
	<i>Hmbs</i>	GAAACTCTGCTTCGCTGCATT TGCCCATCTTTCATCACTGTATG
	<i>Ubiquitin</i>	TGGCTATTAATTATTCGGTCTGCAT GCAAGTGGCTAGAGTGCAGAGTAA

**CHAPTER V - THE BLOOD TRANSCRIPTOME OF TUBERCULOSIS
PATIENTS REVEALS TIMELY ACTIVATION OF THE LXR PATHWAY AS A
PROMISING HOST-DIRECTED THERAPY**

The blood transcriptome of tuberculosis patients reveals timely activation of the LXR pathway as a promising host-directed therapy

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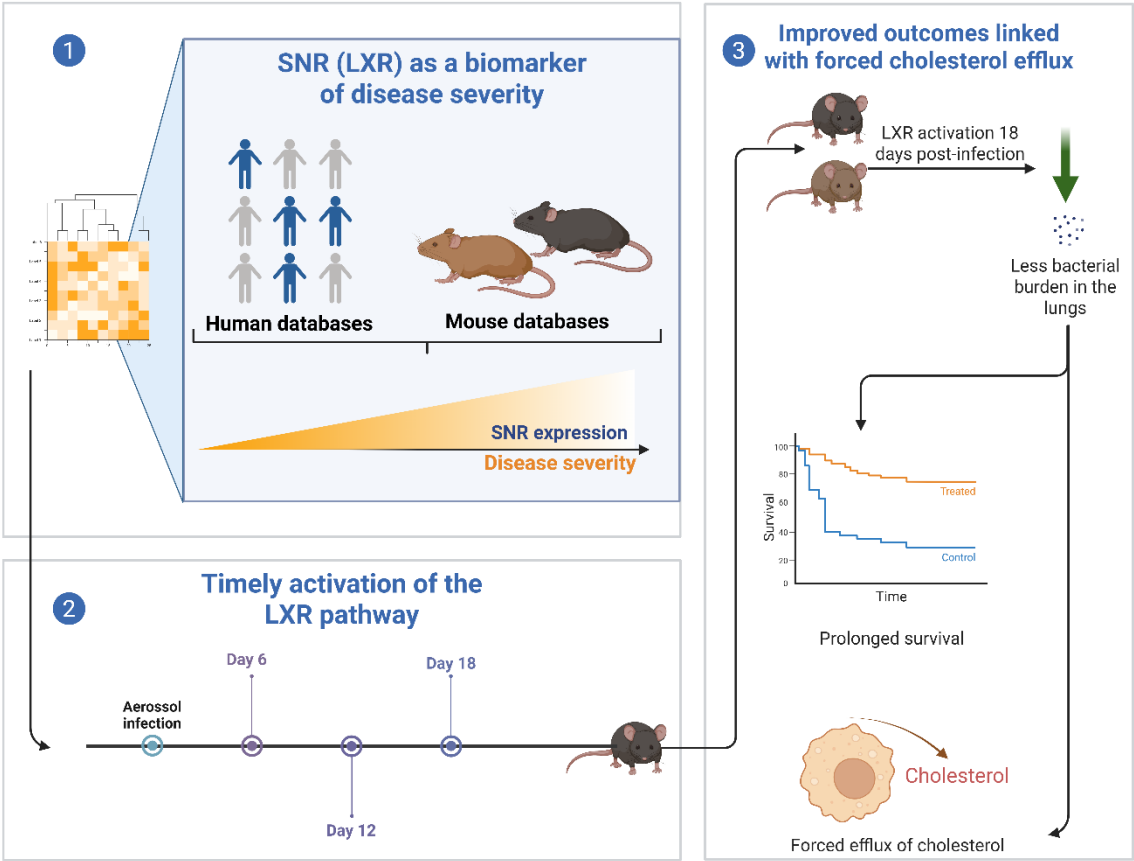
* These authors contributed equally to this work. I contributed to figures 2D, 3, 4 and Supplementary figures 1,2, and 3.

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Graphical abstract



Abstract

Tuberculosis (TB) remains a global health crisis, with host immune responses critically influencing disease outcomes. Here, we identify the "Signalling by Nuclear Receptors" (SNR) pathway as a transcriptional signature in whole blood that correlates with TB severity across diverse patient cohorts. Integrating clinical data from 372 TB patients in Guinea-Bissau with transcriptomic analyses, we demonstrate that SNR activation, particularly the liver-X-receptor (LXR) subpathway, correlates with disease progression. Using mouse models mirroring human TB signatures, we show that *Mycobacterium tuberculosis* infection induces time-dependent LXR pathway activation in both resistant (C57BL/6) and susceptible (C3HeB/FeJ) mice. Pharmacological LXR activation at later infection stages significantly reduced pulmonary bacterial burdens in resistant mice and extended survival in susceptible mice. Mechanistically, LXR agonism enhanced macrophage-mediated bacterial control and cholesterol efflux, depriving *M. tuberculosis* of this critical carbon source during chronic infection. Our findings position the LXR activation as a biomarker of TB severity and a host-directed therapy (HDT) candidate, emphasizing the importance of temporal precision in therapeutic intervention. By bridging patient-derived transcriptomics with mouse models of infection, this study offers a novel strategy to augment TB management through targeted modulation of host metabolic-immune crosstalk.

Keywords: tuberculosis, host-directed therapy, TB signatures, Liver-X-receptor.

Introduction

Tuberculosis (TB) remains a leading cause of death worldwide. In 2023, an estimated 10.4 million people fell ill with TB and 1.25 million died of this disease¹. The outcomes of infection by *Mycobacterium tuberculosis* vary from elimination, to latency to active disease of different severities^{2,3}. Because a central player in dictating these different outcomes is the immune response mounted against the pathogen, developing immunotherapies that harness the potential of the immune system to control *M. tuberculosis* has attracted a lot of interest. To this end, understanding the protective versus detrimental immune pathways triggered upon infection by *M. tuberculosis* is of fundamental importance. Recent advances have been made possible through the analysis of whole blood transcriptome of TB patients, which has identified immune signatures associated with TB disease, progression to infection, or response to treatment⁴⁻⁸. The neutrophil-driven type I interferon (IFN)-dependent signature has been widely identified in the blood of TB patients⁹. Importantly, this signature is recapitulated in the whole blood of C3HeB/FeJ mice infected with *M. tuberculosis* HN878 via the aerosol route. Previous studies showed that type I IFN blockade or neutrophil depletion in infected C3HeB/FeJ mice resulted in disease improvement¹⁰. Thus, the potential of exploring pathways discovered in human disease in tractable mouse models is high.

TB progression in the lung is accompanied by a series of tissue metabolic adaptations. In the mouse model, infection of alveolar macrophages with *M. tuberculosis* is characterized by a strong ox-phos signature, whereas infection of interstitial monocytes associates with a glycolytic shift¹¹. A realignment of the host lipid metabolism also occurs in the granuloma, with a high abundance of cholesterol, cholesteryl esters, triacylglycerols and lactosylceramide identified in the human caseum¹². Cholesterol is a substrate for *M. tuberculosis* during intracellular growth and the cholesterol catabolic pathway of *M. tuberculosis* is implicated in TB pathogenesis, mostly during the chronic phase of infection¹³. Indeed, disruption of *M. tuberculosis* cholesterol catabolism compromises its in vivo virulence and small chemical compounds capable of inhibiting cholesterol utilization by *M. tuberculosis* have been identified as promising therapeutic tools¹⁴. On the host side, nuclear receptors, a superfamily of transcriptional regulators that includes liver-X-receptors (LXR), are major orchestrators of the macrophage responses to *M. tuberculosis*¹⁵. Previous studies implicate the LXR pathway in TB pathogenesis. Single nucleotide polymorphisms in LXR genes associated with genetic susceptibility to TB in the Chinese Han population¹⁶. In the mouse model of *M. tuberculosis* infection, LXR deficiency associated with poorer outcomes of disease, whilst their activation improved bacterial control¹⁷. However, this

study used C57BL/6 mice intra-tracheally infected with the laboratory reference strain H37Rv, a model that presents some limitations with respect to the study of the immune response and progression of infection. Activation of the LXR in cultures of *M. tuberculosis*-infected THP1 cells resulted in a decrease in lipid body formation, and the activation of LXR by IL-36 improved bacterial control¹⁸.

Here, we show that the activation of the 'signalling by nuclear receptors' (SNR) pathway in the whole blood of TB patients positively correlates with TB severity across different TB cohorts. We then show that the expression of the SNR component LXR increases with time in the lungs of both the more resistant C57BL/6 and the susceptible C3HeB-FeJ mice upon infection with *M. tuberculosis* HN878 strain. Chemical modulation of the LXR in these mouse models revealed a time-dependent beneficial effect, with a marked decrease of lung bacterial burdens and improved survival of the susceptible mice. We further associate the beneficial effect of the LXR activation with improved macrophage control and cholesterol efflux in vitro and in vivo. In all, by combining whole blood transcriptomics of TB patients and experimental mouse data, we reveal a candidate molecular marker of TB severity and demonstrate the potential of fine tuning LXR activation to improve TB outcomes.

Results and Discussion

The signalling by nuclear receptors pathway is activated during TB and correlates with disease severity

A cohort of 372 active TB patients (over 15 years old; 29.6% females; with GeneXpert and sputum positive results; at presentation) was recruited in Bissau, and whole-blood RNAseq performed in samples collected at diagnosis from HIV negative TB patients, and from a control group of healthy donors. The activation of 23 transcriptional modules previously described in the whole blood of TB patients from London and South Africa¹⁹ was tested in the new cohort. The London dataset (referred to as Berry - London) comprises healthy donors, individuals with latent TB and those with active TB, whereas the South Africa dataset (referred to as Berry - South Africa) does not include healthy controls²⁰. The modular transcription pattern of the Bissau TB cohort was similar to the external ones, with no statistically significant differences detected (Fig.1A). Of special note are the overabundance of granulocyte and interferon modules, and the underabundance of T and B cell ones (Fig.1A) characteristic of TB signatures. These data are in line with the robustness of the TB whole blood transcriptional signature widely validated in different cohorts^{5,7,20-25}. We next performed gene set enrichment analysis (GSEA) in the differentially expressed genes (DEGs) from the Bissau TB cohort. Among the top 10 altered pathways were several TLR-related pathways, neutrophil degranulation and the SNR pathway (Fig.1B). Because the expression of several nuclear receptors has been reported in *in vitro* and *in vivo* *M. tuberculosis* infections and their potential as HDT acknowledged¹⁵, we decided to further investigate the activation of the SNR pathway in the whole blood of TB patients. The expression of the DEGs within the SNR pathway was notoriously higher in TB patients as compared with healthy controls, not only in the Bissau cohort, but also across the London and South Africa TB cohorts (Fig.1C). A certain degree of heterogeneity in the expression of the SNR genes was detected at the individual level (Fig.1C). Because the extent of the whole blood TB signature had been associated with the extent of radiological disease, we questioned whether the heterogeneous expression of the SNR pathway might be related to TB disease severity. To answer this question, a Pearson correlation was calculated between the SNR eigengenes and the TB severity classification based on TBscoreII values²⁶ (Fig.1D). We obtained a Pearson correlation value of 0.72 and corresponding p-value of 0.006 considering the 15 samples with available clinical data. Similarly, in the Berry London dataset, increased expression of the SNR pathway accompanied disease severity even though a different classification (chest x-ray) was used to stratify patients in this cohort (Fig.1E). Collectively, our data reveal the expression of the

SNR pathway in TB patients and points to its relation with the stage of disease, thus positioning the SNR pathway as a candidate biomarker of TB severity.

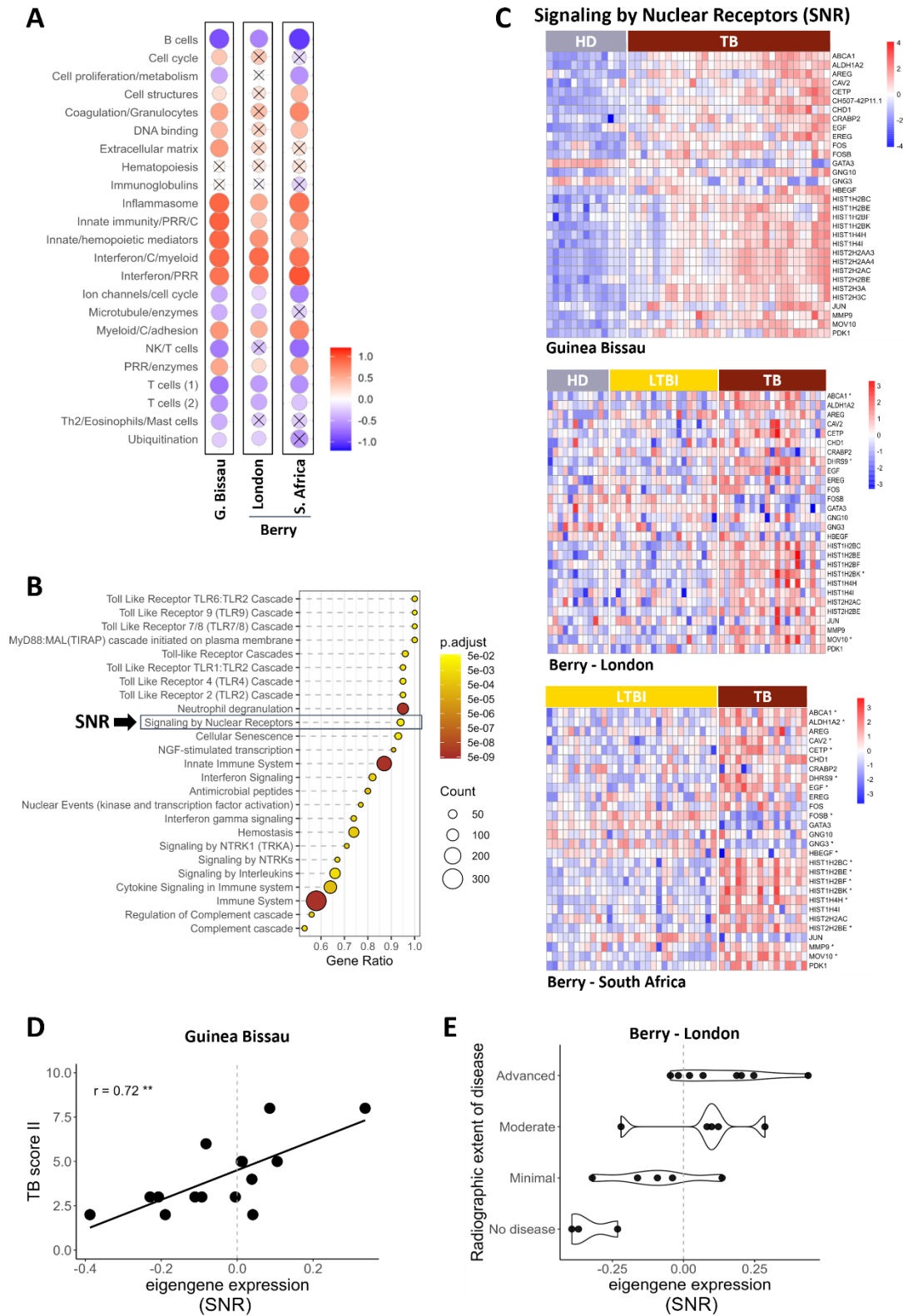


Figure 1. The signalling by nuclear receptors pathway associates with disease severity. (A) Dotplot with the activation level of the 23 previously described transcription modules for the public datasets Berry London and Berry South Africa, and for the new Guinea-Bissau cohort. Log2 fold enrichment scores for modules detected as altered with statistical significance (FDR-adjusted $p < 0.05$) between TB patients and controls are presented. Red and blue indicate over or under-abundant modules compared to the controls, respectively, with colour intensity and size representing the degree of enrichment. (B) Dot plots showing the top 10 altered pathways from gene set enrichment analyses for the Guinea-Bissau cohort. (C) the relative expression of the DEGs from the SNR pathway for the for healthy donors (HD) and TB patients from the Guinea-Bissau TB cohort (top heatmap), for the HD, latent TB infection (LTBI) and active TB (aTB) from the Berry-London cohort (middle heatmap) and the LTBI and aTB groups from the Berry-South Africa cohort (bottom heatmap). Heatmaps (C) present a normalized gene expression by gene (Z-score by row). (D) Pearson correlation analysis between TBscoreII and the eigengene expression of the SNR pathway with $** p \leq 0.01$ (E) Violin plot showing the distribution of the eigengene expression of the SNR across the radiographic extent of disease classifying the aTB individuals from the Berry-London cohort. **Abbreviations:** SNR, signalling by nuclear receptors; LTBI, latent TB infection; HD, healthy donors.

Increased expression of the liver-X-receptor pathway in mouse upon *M. tuberculosis* infection

To further investigate the link between the SNR activation and TB, we decided to focus on the LXR pathway, which is part of the SNR super-family and plays a role in the regulation of the immune response and protection to several intracellular bacteria²⁷⁻²⁹, including *M. tuberculosis*^{16-18,30}. We started by analysing the activation of the LXR pathway in the Guinea-Bissau and the Berry TB cohorts. Overall, the expression of the LXR genes was higher in TB patients than in healthy donors, although among patients this expression was heterogeneous (Fig.2A). This pattern of expression of genes of the LXR pathway paralleled that of genes of the SNR pathway (Fig.1C).

We then analysed the activation of the LXR pathway in relatively TB-resistant (C57BL/6) or TB-susceptible (C3HeB/FeJ) mice infected via aerosol with the hypervirulent *M. tuberculosis* isolate HN878, which were shown to recapitulate the human TB blood signature⁹. Using available RNAseq datasets generated at the peak of disease, we found that the expression of the genes encoding components of the LXR pathway was altered both in the blood (Fig.2B) and the lung (Fig.2C) of infected mice as compared with non-infected ones. Moreover, the observed alterations were overall most pronounced for C3HeB/FeJ mice (Fig.2B and C), which recapitulate more severe forms of TB disease. To obtain the kinetics of expression of the different LXR genes as the infection progressed, we measured their mRNA transcripts in the lungs of C57BL/6 or C3HeB/FeJ at days 6, 12 and 25 post-infection. These time-points match the progression of infection in terms of weight loss and bacterial burdens (Sup. Fig.1A and B). We found that, with exception of *ApoE*, the other tested genes were upregulated over-time upon infection (Fig.2D), accompanying disease progression. Furthermore, the mRNA expression profiles of the tested genes were comparable between the two mouse models.

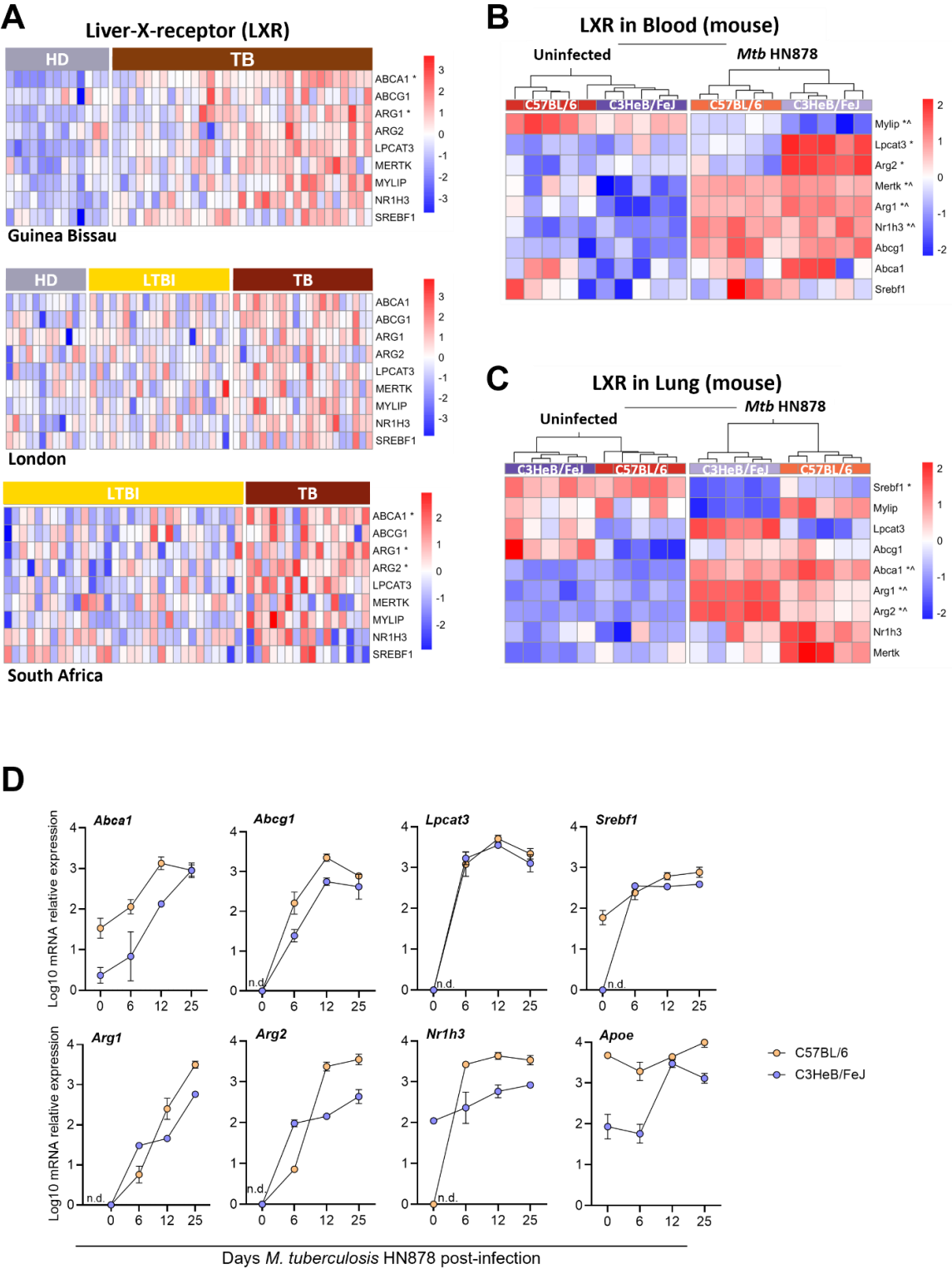


Figure 2. The LXR pathway is activated during TB. (A) Heatmaps showing the relative expression of the indicated genes of the LXR pathway across the indicated groups and cohorts (Guinea-Bissau, Berry London and Berry South Africa). Normalized gene expression by gene (Z-score by row) is presented. * indicates DEGs. (B,C) Heatmaps showing the relative expression of selected genes from the LXR pathway in the blood (B) and lung (C) of uninfected or aerosol-infected C3HeB/FeJ or C57BL/6 mice. Data are from infections with *M. tuberculosis* HN878, from a publicly available RNAseq dataset. Normalized gene expression by gene (Z-score by row) is presented and DEGs for C3HeB/FeJ or C57BL/6 are identified with an (*) or (^), respectively. (D) Transcriptional analysis of the indicated genes of the LXR pathway by qPCR in the lungs of C57BL/6 animals infected with *M. tuberculosis* isolate HN878 (500-1000 CFU delivered to the lung) or C3HeB/FeJ animals infected with *M. tuberculosis* isolate HN878 (100-200 CFU delivered to the lung) at different time-points post-infection. **Abbreviations:** LXR, Liver-X-receptor; HD, healthy donors; LTBI, Latent TB infection; Mtb, *M. tuberculosis*.

Timely modulation of the LXR pathway improves the outcome of *M. tuberculosis* infection in C57BL/6 mice

Our data expand on a previous study showing the progressive expression of a smaller set of LXR responsive genes in the bronchoalveolar lavage cells of mice infected intra-tracheally with *M. tuberculosis* H37Rv strain. In that study, LXR $\alpha\beta$ double deficient mice were more susceptible to *M. tuberculosis* infection, showing higher CFUs and lung histopathology at 5 weeks post-infection. Moreover, mice treated with an LXR agonist prior or post-infection, also demonstrated improved lung bacterial burdens, possibly due to increased CD4⁺ T cell responses¹⁷. Motivated by these promising results, we questioned whether pharmacologic activation of LXR in mouse models known to better reproduce key features of TB⁹ would also improve the infection outcomes. We started by infecting C57BL/6 mice with *M. tuberculosis* HN878 and administering the LXR agonist T0901317 at different time points: from 7 days prior to infection, to day 6, 12 or 18 post-infection. These time points were chosen based on the progression of infection informed by weight loss and lung bacterial burden (Sup.Fig.1A and B), and considering the dynamics of LXR genes expression in the lungs (Fig.2D). Whereas no alterations to the weight of vehicle or treated mice were observed for the prophylactic administration of the LXR agonist or for administration on days 6 or 12 (Fig.3A), mice that received the treatment on day 18 did not lose as much weight as the vehicle group (Fig.3A), suggesting a time dependent beneficial effect of the administered regimen. On day 25 post-infection, at which point the control (vehicle) group reached the peak of disease, we analysed lung bacterial burdens for all groups. Prophylactic administration of the LXR agonist resulted in a minimal decrease of lung CFUs (Fig.3A), a finding that contrasts with previous results, possibly due to the difference in the route of administration (aerosol vs intra-tracheal) and the strain of *M. tuberculosis* used (HN878 vs H37Rv). Early activation of the LXR pathway (day 6 post-infection) did not impact lung bacterial burdens, whereas activation from day 12 post-infection resulted in a modest decrease in lung bacterial burdens (Fig.3A). Activation of the LXR pathway at 18 days post-infection led to the most pronounced decrease in bacterial numbers in the lungs (Fig.3A). Indeed, the increase in the lung bacterial burden from day 18 to the end-point of the experiment (day 25) was much higher in vehicle than in LXR-treated mice (respectively, 47.9 vs 5.58 increase between the start of the administration and the end-point; Fig.3B). Surprisingly, despite this improved control of bacterial growth, the % of lesioned lung area was not altered in LXR-treated mice (Fig.3C). A more detailed lung histopathological analysis of the mice subjected to this regimen was indicative of a small reduction ($p = 0.0635$) of the lesion inflammatory score for the LXR-treated animals (Fig.3D), which is composed of four different parameters (Sup. Fig.1C)³¹. These data

suggest reinforcing the LXR pathway as a potential HDT for TB, supporting a link between this pathway and improved bacterial control, likely independent of tissue damage restriction. Furthermore, our data show that it is at later time points post-infection that the LXR potentiation works better.

LXR activation enhances the macrophage bacterial control and the extracellular levels of cholesterol

To gain further insights on the immune response accompanying the activation of the LXR at day 18 post-infection, we analysed the immune cell populations present in the lungs of vehicle or LXR-treated mice (Sup. Fig.1D). Two major immune populations with opposing effects in TB are neutrophils, which are largely detrimental, and CD4⁺ T cells, which are protective. We found that LXR treatment did not significantly impacted the number of lung neutrophils, but did increase that of CD4⁺ T cells (Fig.3E). A significant increase in the numbers of CD11c⁺ cells, CD8⁺ T cells, and B cells, and no alterations in monocytes or alveolar macrophages were seen in the lungs of infected LXR-treated mice (Sup. Fig.1E). The generalised increase in immune cells detected in the lungs of C57BL/6 mice, may on one hand contribute to the bacterial control and on the other explain the fact that the lung infiltrated area remains similar to that of control mice. Despite the increase in the numbers of CD4⁺ T cells, we did not observe an increase of IFN- γ , IL-17 or TNF- α in the lungs of LXR-treated animals, as measured by multiplexed immunoassay (Fig.3F). These results suggest that in contrast to what was reported in the intra-tracheal model of *M. tuberculosis* (H37Rv) infection¹⁷, the beneficial effect of LXR activation in *M. tuberculosis* HN878-aerosol infected C57BL/6 mice is likely independent of improvement of CD4⁺ T cell responses.

The used LXR agonist (T0901317) is described to promote cholesterol efflux from host cells. In line with this, increased levels of cholesterol were found in the supernatants of lungs of *M. tuberculosis* HN878 infected C57BL/6 mice treated with the LXR agonist from day 18 to day 25 post-infection (Fig.3G). Because it is at later stages post-infection that *M. tuberculosis* relies on cholesterol as a carbon source in vivo³¹⁻³⁷, we hypothesise that the LXR agonist might be acting on macrophages to deprive *M. tuberculosis* of this carbon source. To test this hypothesis, we infected mouse bone marrow-derived macrophages (BMDM) with *M. tuberculosis* HN878 and allowed the infection to progress for 4 days in the absence (vehicle) or presence of the LXR activator. Activation of the LXR pathway decreased intracellular bacterial growth as compared to vehicle controls (Fig.3H) and simultaneously increased the extracellular cholesterol levels (Fig.3I). This was not due to differential phagocytosis of the bacteria, as the presence of the LXR activator did not impact the number of intracellular bacteria after the first 4 h of infection (Sup. Fig.2A). It was also not due to a direct effect of the LXR agonist on the pathogen, as its presence in axenic media did not impact the growth of *M. tuberculosis* even in the presence of cholesterol (Sup. Fig.2B). Moreover, the protective effect of the LXR activator in BMDM was not exclusive to infection with *M. tuberculosis* HN878, as similar results were obtained upon infection of

C57BL/6 BMDM with a clinical isolate from lineage 4 (Sup. Fig.2C), nor to BMDM, as similar findings were observed in THP1 cells (Sup. Fig.2D). Invariably, increased bacterial control was accompanied by increased extracellular cholesterol amounts (Sup. Fig.2C-D).

To show that in vivo this effect is time-dependent, we administered the LXR agonist to C57BL/6 mice infected with *M. tuberculosis* HN878 between day 6 and 12 post-infection and monitored the evolution of lung CFUs and the variation in extracellular cholesterol. Although the vehicle group showed significantly higher lung bacterial burden than the treated mice at the end of treatment (day 12; Sup. Fig.2E), the increase in CFUs between day 6 and day 12 was much more similar than that observed when the LXR activator was administered later post-infection (Fig.3B). Moreover, this difference was not sustained in time as by day 25 post-infection no differences in the lung CFUs were observed between the two groups (Sup. Fig.2E). Also, at this time point, no differences in the levels of extracellular cholesterol were detected (Sup. Fig.2F).

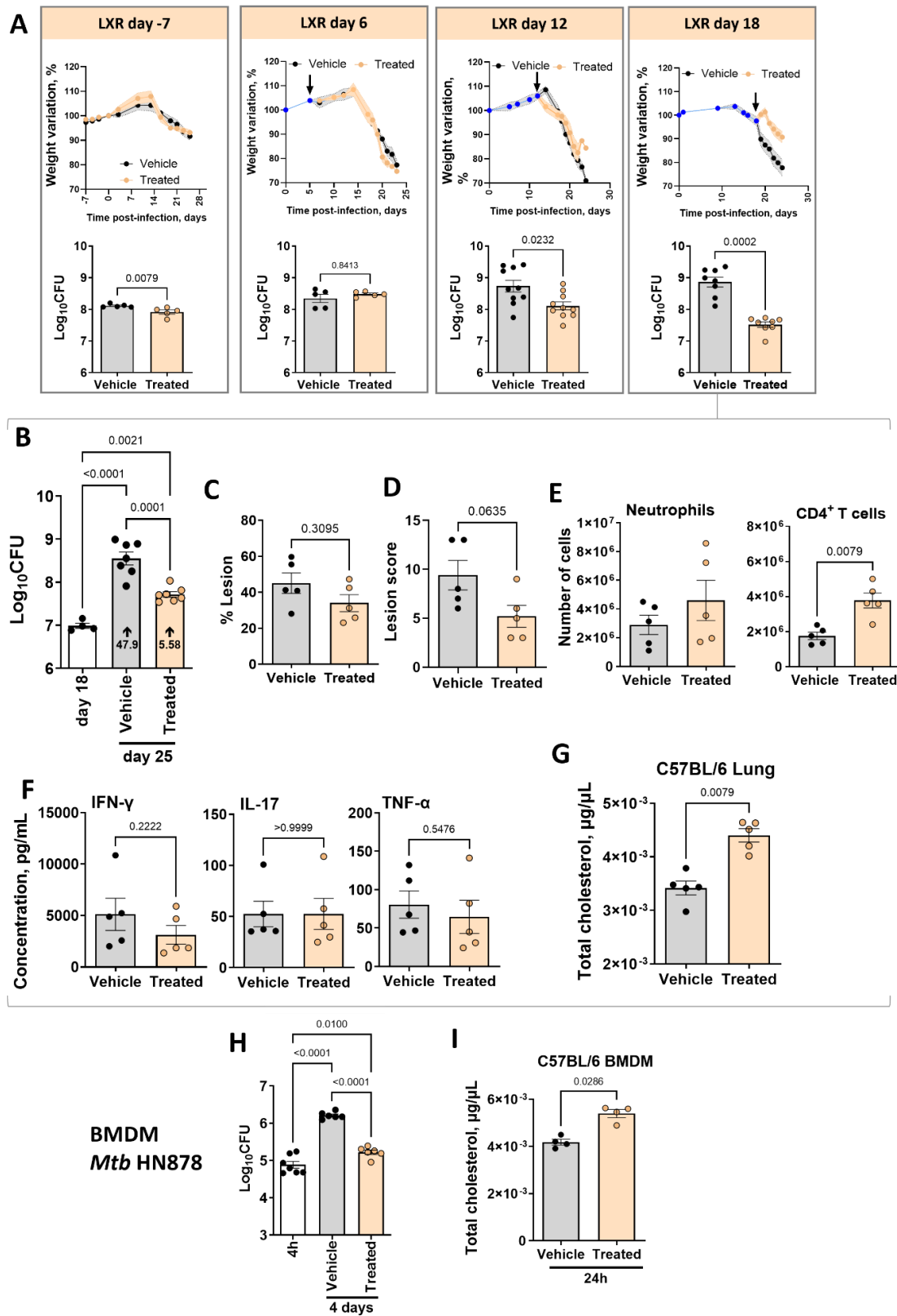


Figure 3. Timely liver-X-receptor activation limits the growth of *M. tuberculosis* in the lungs of TB-resistant mice. (A) C57BL/6 mice were aerosol-infected with *M. tuberculosis* isolate HN878 (500-1000 CFU delivered to the lung) and treated with the LXR activator T0901317 from day -7, 6, 12 or 18 post-infection. Shown is the body weight variation for control (vehicle; grey) or treated (orange) mice, at the indicated time points post-infection. Each dot represents the mean of five animals, with SEM shaded. The arrow represents the start of vehicle or T0901317 administration. For each time point is represented the lung bacteria burden assessed on day 25 post-infection (A). (B) Variation in the lung bacterial burden from the day 18 to day 25 post-infection in treated (orange) and non-treated animals (grey). The fold increase in lung bacterial burden from day 18 to day 25 is represented in the respective bars. (C) % of lesioned area and (D) lesion score were determined for control (vehicle; grey bars) or treated (orange bars) mice. (E) Total cell counts of the indicated lung immune cell populations in control and LXR-treated animals. (F) Total concentrations of the indicated cytokines in lung supernatants measured by Legendplex of LXR-treated and control mice at day 25 post-infection. (G) Total cholesterol content determined on day 25 post-infection in lung supernatants of C57BL/6. (A-G) Data are mean $\pm$ SEM. Each dot represents an individual mouse. Mann-Whitney U tests were used to identify statistical differences between the two groups. p-values ≤ 0.05 were considered significant. (H) Bacterial loads in BMDMs infected with *M. tuberculosis* HN878 from C57BL/6 mice, measured 4 hours post-infection and 4 days in the absence (vehicle) or presence (treated) of the LXR activator (10 μ M). (I) Total cholesterol content determined 24 hours post-infection in BMDMs supernatant with or without the LXR activator. **Abbreviations:** LXR, Liver-X-receptor; CFU, colony forming unit; IFN, interferon; BMDM, bone marrow derived macrophages; Mtb, *M. tuberculosis*; IL, interleukin; TNF, tumor necrosis factor.

Impact of LXR activation in *M. tuberculosis* infections of TB-susceptible mice

Having demonstrated a protective effect of late LXR activation in the relatively TB-resistant C57BL/6 mice, we tested the same intervention in TB-susceptible ones. Treatment of C3HeB/FeJ mice with the LXR activator 18 days post-aerosol infection with *M. tuberculosis* HN878 led to a decrease in lung bacterial burdens (Fig.4A). In contrast with C57BL/6 mice, the only altered immune cell populations in LXR-treated infected C3HeB/FeJ were neutrophils and B cells, where the frequencies were increased (Sup. Fig.3A). Given the specificity of the LXR-induced alterations in lung immune cell composition, it is tempting to speculate that neutrophils may exacerbate tissue damage, while B cells contribute to a protective response.

As before, no significant differences were found in IFN- γ , IL-17 or TNF- α in the lungs of LXR-treated mice versus non-treated (Sup. Fig.3B). Similarly to the results obtained in TB-resistant mice, the activation of the LXR pathway also resulted in a significant increase in the extracellular cholesterol levels (Fig.4B). In both mouse models, and despite their divergent immune response to *M. tuberculosis*⁹, the main effect of LXR activation was the reduction of lung bacterial loads. Because LXR activation is known to exert combined metabolic and anti-inflammatory actions that result in the modulation of the immune response³⁸, several mechanisms may underlie our observations.

Given the promising results evidenced by the CFU decrease in the lungs of these mice, we reasoned that prolonged activation of the LXR pathway might further impact the progression of infection and enhance the survival of animals challenged with a hypervirulent strain. To test that, the administration of the LXR activator was prolonged up to day 48 post-infection. Whereas the vehicle-treated animals all succumbed before day 40 post-infection, those receiving the LXR activator survived longer, with five animals still performing well at the end-point (Fig.4C). Treated mice continued to gain weight throughout the experiment, in clear contrast to the vehicle-treated group, which uniformly reached the humane endpoint (Sup. Fig.3C). Of note, despite some variation in the lung CFU (Fig.4D) at the time of death, LXR treated animals showed overall lower bacterial burdens as compared with control ones. Therefore, LXR administration also improves the outcome of infection in TB-susceptible mice, which similarly to TB-resistant mice was likely due to enhanced bacterial control and dissociated from lung pathology.

C3HeB/FeJ; Mtb HN878 with LXR activated from day 18

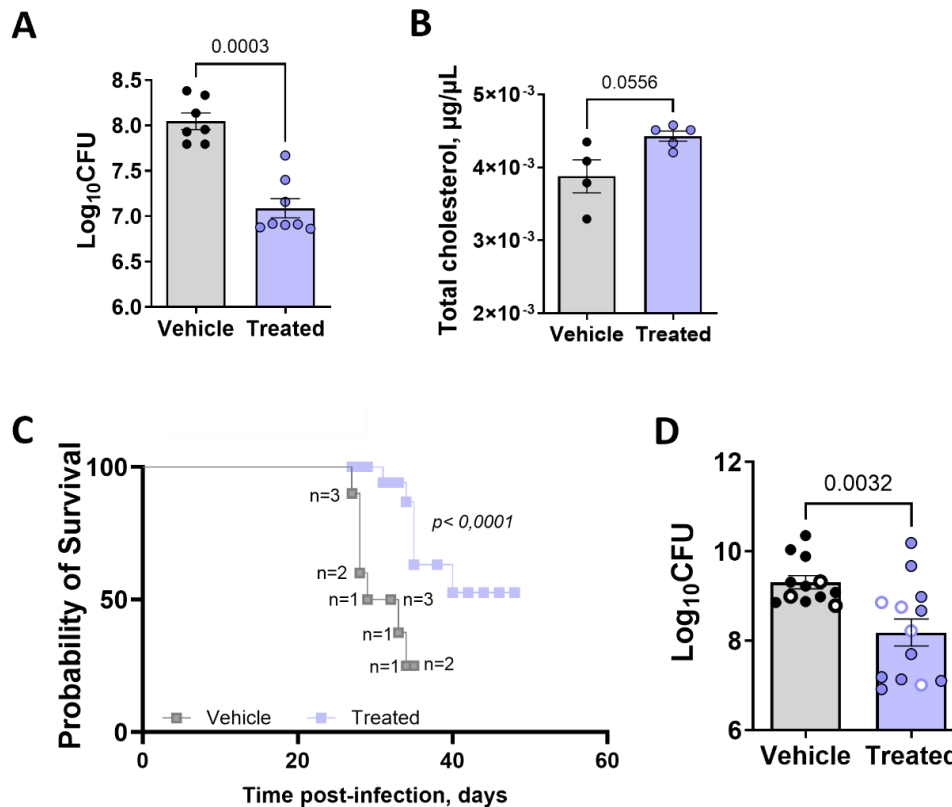


Figure 4. LXR activation reduces lung bacterial burdens and prolongs survival of infected TB-susceptible mice. C3HeB/FeJ mice were aerosol-infected with *M. tuberculosis* isolate HN878 (average of 203 CFU delivered to the lung) and treated with the LXR activator T0901317 from day 18 post-infection. On day 25 post-infection, the lung bacterial burdens (A) and the total cholesterol in the lung supernatant (B) were determined for control and treated mice. (C) Survival curve (humane endpoints defined based on over 20% weight loss or severely compromised respiratory function) for C3HeB/FeJ mice infected with *M. tuberculosis* as above and treated with the LXR agonist for 48 days. Vehicle controls are also shown. (D) Lung bacterial burdens for each animal enrolling the survival curve at the respective end-point. Three animals in the vehicle group are not included due to premature death. (A-D) Vehicle and treated groups are represented by grey or blue bars, respectively. Data are mean ± SEM. Each dot represents an individual mouse. Mann-Whitney U tests were used to identify statistical differences between the two groups. p-values ≤ 0.05 were considered significant. (C) A Log-rank (Mantel-Cox) test was used to compare the survival curves; the p-value is indicated. **Abbreviations:** CFU, colony forming unit; Mtb, *M. tuberculosis*; LXR, Liver-X-receptor.

Conclusions

With the present study, by leveraging a unique cohort of TB patients exhibiting high strain diversity and a wide range of disease severities, we were able to associate the differential activation of the SNR pathway with TB disease progression. This integrative analysis of global whole-blood transcriptomes not only established the SNR pathway as a promising biomarker of disease severity but also paved the way for uncovering a novel host-directed therapeutic strategy. The increased control of *M. tuberculosis* seems to be correlated to the forced efflux of cholesterol promoted by the used LXR agonist used (T0901317), possibly by depriving the pathogen of a key energy source and counteracting the cholesterol importing systems evolved by the bacilli^{32,33}. The beneficial effect of the LXR activation specifically at later stages of infection supports the link to cholesterol efflux, as it is during the chronic phase of infection that *M. tuberculosis* mostly relies on cholesterol for survival^{33-36,39}.

Other cholesterol targeting strategies were tested for their potential to control experimental *M. tuberculosis* infection. In line with our study, administration of all trans-retinoic acid (ATRA), which induces cholesterol efflux in macrophages³⁸, to 129S2 mice during *M. tuberculosis* infection reduced lung bacterial burdens, with little impact in tissue pathology⁴⁰. Cholesterol-lowering drugs (statins) or pharmacologic inhibition of the pathogen cholesterol pathway by activating the *M. tuberculosis* protein Rv1625c, a stimulator of cAMP synthesis reduced the bacterial burdens in infected mice, although not to the extent we report here^{41,42}. Combined targeting of both host and pathogen cholesterol metabolism may further improve the infection outcome. Also likely is that the use of LXR agonists as adjuvants to TB antibiotherapy will further limit TB progression and severity.

Collectively, by incorporating the genetic diversity of *M. tuberculosis* and combining patient stratification with global transcriptome analyses and different mouse models, our findings position the fine-tuned modulation of LXRs as a promising HDT to TB, also pinpointing the SNR as a candidate molecular pathway reflecting TB disease progression.

Study limitations: Increasing the number of TB participants with complete clinical data will strengthen the potential correlation of SNR expression and disease stage, thus helping in defining the SNR pathway as a possible biomarker of disease progression. Our pre-clinical testing of LXR potentiation used the chemical agonist T0901317, which may have adverse effects due to the LXR α -mediated activation of a lipogenic program⁴³. Testing improved LXR agonists, capable of promoting selective LXR functions⁴⁴, in experimental models of TB is a promising and exciting avenue of research.

Methods

Ethics statement

The study protocol for blood and sputum collection was approved by the *Comité Nacional de Ética na Saúde* (CNES), Guinea-Bissau, reference number 003-CNES/INASA/2018. The PBMC samples study was reviewed and approved by the Portuguese *Comissão de Ética para a Saúde da ARS Norte* (#T792). For both studies, all participants signed an informed consent. To ensure confidentiality, data were anonymized by the assignment of a random identification number to each case. The study was conducted according to the principles expressed in the Declaration of Helsinki and the Oviedo Convention.

Animal experiments followed the 2010/63/EU Directive and were approved by the i3S Animal Ethics Committee and the Portuguese National Authority for Animal Health (DGAV; #018413/2021-11-24).

Study cohort and sample collection

The study was based in the West African country Guinea-Bissau which has a TB incidence of 361 cases/100.000 habitants. We have included new TB cases in the Demographic Surveillance Site study area of Bissau since 2003 and found a stable high incidence of TB⁴⁵. A total of 372 individuals (over 15 years old; 29.6% females) with active TB were recruited at presentation, between 2019 and 2021 from different areas in the region of Bissau, both within and outside the Bandim Health Project (BHP) study area (<https://www.bandim.org/about-bandim-health-project/background/>). Collected sputum was used to confirm the disease diagnosis and to perform *M. tuberculosis* lineage determination by SNP genotyping. For some participants, HIV testing (Determine-HIV Early Detect set, Abbott; followed by First Response HIV 1-2.O Card test (Version 2.0), Premier Medical Corporation Private Limited) was performed (n = 130/372) and clinical parameters were recorded to obtain a score of disease (n = 51/372). Disease severity was scored employing the Bandim TBscoreII, which examines clinical severity using symptoms and clinical examination²⁶ and is based on three self-reported symptoms (cough, dyspnoea, chest pain), together with three signs identified at examination (anaemia, body mass index (BMI) < 18 kg/m², and mid-upper arm circumference (MUAC) < 220 mm). Each of the 6 clinical variables contributes 1 point to the score, while BMI and MUAC contribute an extra point if <16 kg/m² and < 200 mm, respectively, being 8 the maximum TBscoreII. A volume of approximately 9 mL of blood was collected into Tempus™ Blood RNA Tubes (Applied Biosystems, Cat. #4342792) followed by vigorous mixing and immediate storage at -80°C. Samples were shipped from Bissau to i3s (Porto) in dry ice and stored at -80°C until further use. Thirteen healthy donors were included in the blood collection.

RNA extraction and cDNA library preparation for RNAseq

Whole-blood samples were selected for RNAseq analysis based on the following criteria: (1) HIV negative; (2) within the BHP study area and with available clinical data; and (3) sex match. Total RNA was isolated using the Tempus™ Spin RNA Isolation Kit (Invitrogen, Cat. #4380204) and globin RNA was depleted using the GLOBINclear™-Human Kit (Invitrogen, Cat. #AM1980), both according to the manufacturer's instructions. The yield, quality, and integrity of the globin-depleted RNA were assessed by assigning an RNA Quality Score (RQS) to each sample, and 46 samples (33 from individuals with active TB and 13 from healthy individuals) with RQS above 6 were further selected for sequencing. cDNA libraries were prepared using the Nexterra kit (Illumina, Cat. #FC-131-1024) and sequenced on an Illumina NextSeq sequencer at a targeted 35 million single-end reads with 75 base pairs (bp) per sample. Library preparation and high-throughput sequencing were performed at the EMBL Gene Core facility (Heidelberg, Germany).

RNAseq data analyses

The quality of raw sequencing data and the presence of adaptors were initially assessed using FastQC. Low-quality reads with less than 36 nucleotides long, adapters, leading bases with quality below 3, and trailing bases when the average quality per base was below 15 for regions of 4 bases were removed using Trimmomatic v0.39⁴⁶. HISAT2 v2.2.1 was then used to align the remaining reads to the *Homo sapiens* genome Ensembl GRCh38 (release 84) followed by the usage of StringTie v2.1.6 to obtain gene-level counts^{47,48}. The obtained counting table was imported into R v4.1 for further analysis. Raw counts were processed using edgeR v3.36.0 and limma v3.50.0 packages^{47,48}. Initial processing of raw counts was performed with edgeR and genes with counts per million (CPM) lower than 10 in at least 12 samples were discarded from further analysis. Differential expression analysis on the remaining genes was performed using generalized linear models from the limma package. The effect of the sex covariate was taken into account by adding it as a variable to the model. Genes were deemed significant if log2 fold change was ≥ 1 or ≤ -1 and the false discovery rate (FDR) p-value ≤ 0.05 after Benjamini-Hochberg correction for multiple testing. Pathway over-representation analysis (ORA) and gene set enrichment analysis (GSEA) were performed using the ReactomePA package⁴⁹. Activation of the previously described blood TB transcriptional modules⁸ or the identified relevant pathways for each sample was computed using the QuSAGE package⁵⁰. Cell type composition was deconvoluted using the CYBERSORTx software⁵¹ and differences for each cell type between the four groups were tested by performing a Kruskal-Wallis test followed by a

Wilcoxon post-hoc test for the cell types that presented significant results. Plots were obtained using ggplot2, pheatmap, ggcorrplot, and ReactomePA packages⁵⁰. Whole-blood RNAseq count matrices from the Berry London and Berry South-Africa cohorts⁸ were downloaded from GEO (accession codes GSE107992 and GSE107991, respectively), imported into R, and analysed using the aforementioned pipeline.

Mouse whole-blood and lung RNAseq data from⁹ were downloaded from SRA (GEO accession code GSE140945) and processed using the same pipeline. The reference genome used for alignment was the *Mus musculus* genome Ensembl GRCm38 (release 84).

Bacteria growth and quantification

The *M. tuberculosis* L4 clinical isolate used in the study were recovered from the sputum of TB patients from Bissau and grown as before^{52,53}. This isolate and *M. tuberculosis* HN878 were expanded in Middlebrook 7H9 (BD Biosciences, Cat. #90003-876) liquid medium supplemented with 10% OADC and 0.2% glycerol (Sigma-Aldrich, Cat. #G5516-500M) and when in mid-log phase bacterial suspensions were aliquoted in cryovials, frozen, and stored at -80°C. Bacterial quantification was performed by thawing six vials of each stock and plating several serial dilutions in 7H11 agar medium supplemented with 10% OADC and 0.5% glycerol. The plates were incubated 21 to 28 days at 37 °C before colony count.

Cytokine determination by multiplexed immunoassay

Cytokine and chemokine levels in the lung supernatants were measured using the LEGENDplex™ Mouse Inflammation Panel kit (IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α, and GM-CSF; Biolegend, Cat. #740446_Mouse Inflammatory Panel) following manufacturer's specifications. The beads were acquired in an BD Accuri C6 (BD Biosciences), and the data analysed using the LEGENDplex™ Software version 8 (BioLegend).

In vitro cultures and infections

Mouse BMDMs were generated from C57BL/6 or C3HeB/FeJ bone as previously described^{54,55}. On day 7 of culture, the macrophages were recovered, plated in 24 well-plates at a density of 1x10⁶ cells ml⁻¹ and infected with the selected *M. tuberculosis* isolates at a multiplicity of infection (MOI) of 2. THP1 cells were grown according to ATCC instructions, differentiated with PMA (Sigma-Aldrich, Cat. #16561-29-8) for 24 h, followed by a 48 h rest before infection and then plated in 24 well-plates at a density of 1x10⁶ cells ml⁻¹ and infected with the *M. tuberculosis* isolates at a MOI of 1. Bacterial burdens were

determined at 4 h and 4 days post-infection by CFU enumeration; the cell viability was analysed using the Orangu kit (Cell Guidance systems, Cat. #OR01-1000), according to the manufacturer's instructions; and the culture supernatants were collected 24h post-infection for cholesterol analysis. Uninfected cells were used as controls.

Cholesterol quantification

Cholesterol content in the supernatants was measured using the Cholesterol Quantification kit (Sigma-Aldrich, MAK043-1KT) following the manufacturer's instructions.

Growth of *M. tuberculosis* in axenic media

M. tuberculosis HN878 was grown in 7H9 supplemented with 10% OADC and 0.05% Tween-80 (Sigma, Cat. #P1754) until mid-exponential phase (OD600 of 0.6-0.9). At this stage, a single bacterial suspension was obtained and bacteria were plated in 96-well plates at a final OD of 0.01 in 7H9 medium alone, or with cholesterol and in the presence or absence of the LXR activator T0901317 at a final concentration of 10 μ M. Cholesterol (Sigma, Cat. #C3045) was dissolved in a solution of tyloxapol and ethanol (Sigma, Cat. #1.00983) (1:1) and added to the medium at a concentration of 200 μ M. Cultures were replenished with cholesterol at day 7. Each condition was tested in triplicate. Growth was monitored by measuring the optical density (OD600) over the time course of the experiment.

Animal housing, infection, LXR agonist administration and monitoring

C57BL/6 and C3HeB/FeJ mice were bred and housed at the i3s animal facility. Males and females of 8 to 12 weeks old were used for infections, and maintained under contention conditions in the animal biosafety level 3 facility at i3s, in controlled temperature (20-24°C), humidity (45-65%), and a light cycle of 12h (light/dark). Water and food were provided *ad libitum*. Mice were infected with *M. tuberculosis* (isolate HN878) through the aerosol route using an inhalation exposure system (Glas-Col)^{54,55}. To determine the dose of infection, the bacterial load in the lungs of 3 mice was quantified 3 days post-infection. Doses of infection are indicated in the Figure legends. Infected mice were weighed every week, or every other day when showing signs of disease. Mice were euthanized at the indicated time points or when reaching humane endpoints (weight loss over 20% or poor responsiveness to physical stimulation). For the LXR modulation, mice were treated with 50 μ g of LXR agonist T0901317 (Abcam, Cat. #ab142808) or with the vehicle (0,1% DMSO/PBS) administered intraperitoneally every other day, starting on day -7, 6,12 or 18 until the end of the experiment.

Lung processing

Lungs were aseptically excised and processed as previously described^{9,55}. Briefly, lungs were digested with Collagenase D (Roche, Cat. #11088882001) followed by physical disruption and filtration through 70 µM cell strainers (Falcon, Cat. #352350.0). Cell suspensions were used for colony forming units (CFU) determination, flow cytometry and mRNA analysis.

RNA extraction, qPCR, and lung targeted RNAseq

Total RNA was extracted from infected mouse lungs using TRIzol reagent (GRI SP, Cat. #GB23.0100) according to the manufacturer's instructions. For qPCR analysis, cDNA was synthesized with the SuperScript First-Strand Synthesis System for RT-PCR (ThermoScientific, Cat. #E6300L). Target gene mRNA expression was quantified by real-time PCR, using Taqman Primer Probes (Biorad, Cat. #1725121) and intron spanning oligonucleotides specific for the target genes. Gene expression was normalized to Hprt1/Hmbs (Taqman). Primer probes sequences are listed in Sup. Table 2.

CFU determination

Lung cell suspensions were lysed with saponin, serial dilutions prepared and plated in Middlebrook 7H11 agar (BD Biosciences, Cat. #212203.0) supplemented with OADC and PANTA (BD BioSciences, Cat. # 245114). CFU were determined after 21-28 days of incubation at 37°C.

Flow cytometry

Mouse lung cell suspensions were stained for surface antigens for 30 min at 4° C and fixed for 20 min in 4% paraformaldehyde-PBS after erythrocyte lysis. Dead cells were excluded using ZombieAqua (Biolegend, Cat. #423102). Cells were acquired on a BD Fortessa II and data was analysed using *FlowJo* software (v10.1.r7). All antibodies used are listed in Sup. Table 1. Gating strategies are shown in Sup. Fig.1D

Histological analysis

Whole lungs were perfused *in situ* with PBS, the right upper lobe was excised, fixed in 10% buffered formalin and embedded in paraffin. Serial 3-µm thick sections were performed and used for hematoxylin and eosin (H&E) staining. Morphometric analysis of the lung pathology was performed as before^{54,55}, with the software Interactive Learning and Segmentation Toolkit (Ilastik version 1.3.3). The probability maps of the whole lung and the

lesions were analyzed in CellProfiler Analyst (version 3.1.5). Lesion % was defined by the area occupied by lesion in each lung and lesion score by scoring histopathological features (peribronchiolitis, perivascularitis, alveolitis and necrosis/cell debris) as previously reported³¹.

Statistical analysis

Data were analysed using GraphPad Prism software v8.1.0 or R v4.1. Statistical significant differences between two groups were determined using non-parametric two-tailed Mann-Whitney U tests. Kruskal-Wallis test was used to determine differences between more than two groups, followed by Dunn's post-hoc test when the initial test was statistically significant. Pearson correlation analysis was performed between TBscoreII and SNR activation to determine the existence of a significant correlation between the two variables. A Log-rank (Mantel-Cox) test was used to analyse the survival curve. Differences were considered significant for $p \leq 0.05$ and represented as follows: * $p \leq 0.05$; ** $p \leq 0.01$; and *** $p \leq 0.001$.

Data availability

The sequencing data generated for the current study are available on the NCBI GEO repository under accession code GSE248425 (token: uzuloswiblkhvyr). All other data are available from the corresponding author upon reasonable request.

Code availability

The code for data processing and all downstream analysis will be available at the Github repository https://github.com/RaquelMaceiras/WBtranscriptome_L4_L6 before publication.

Author contributions

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Competing interests

The authors declare no competing interests.

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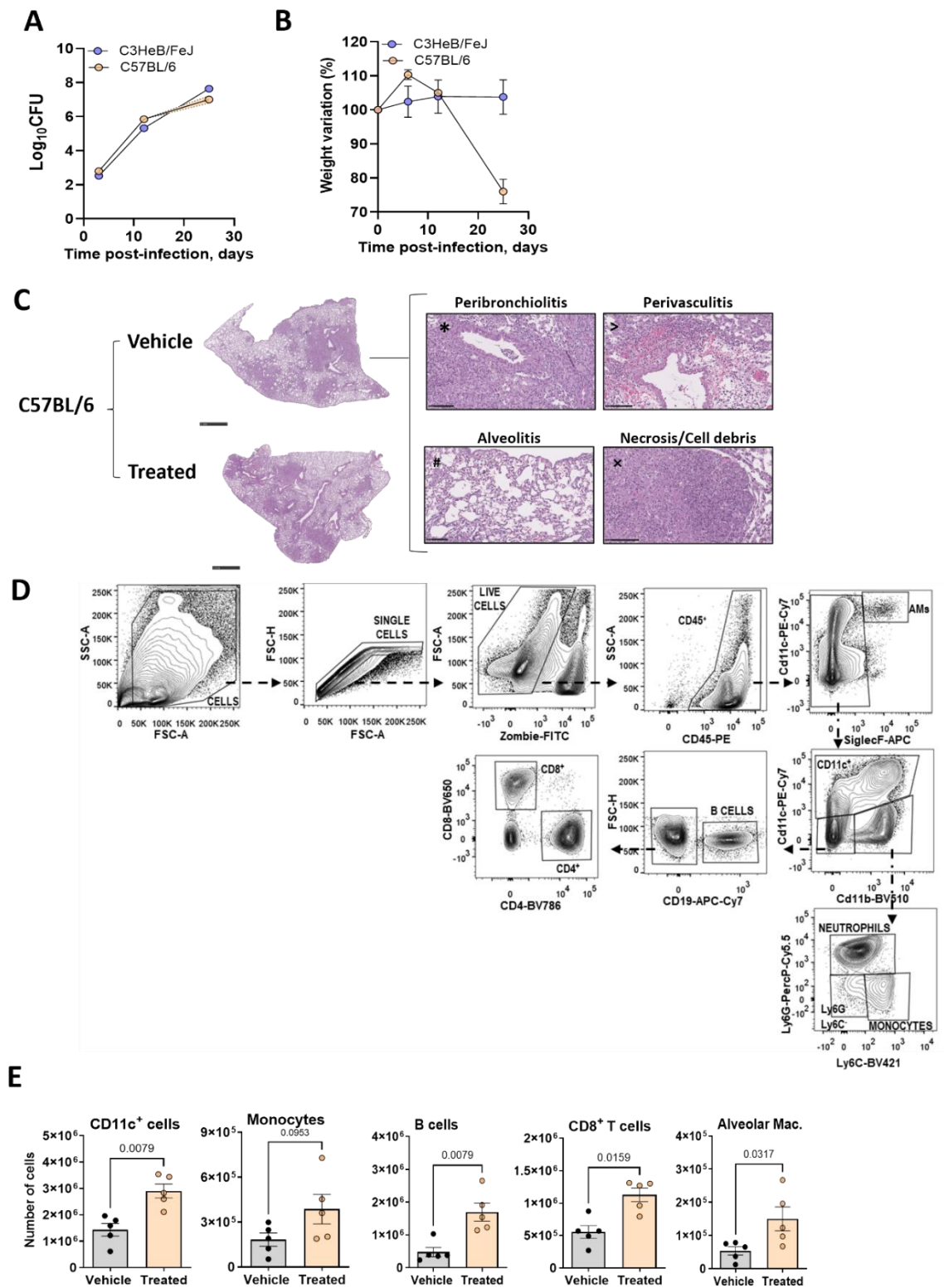
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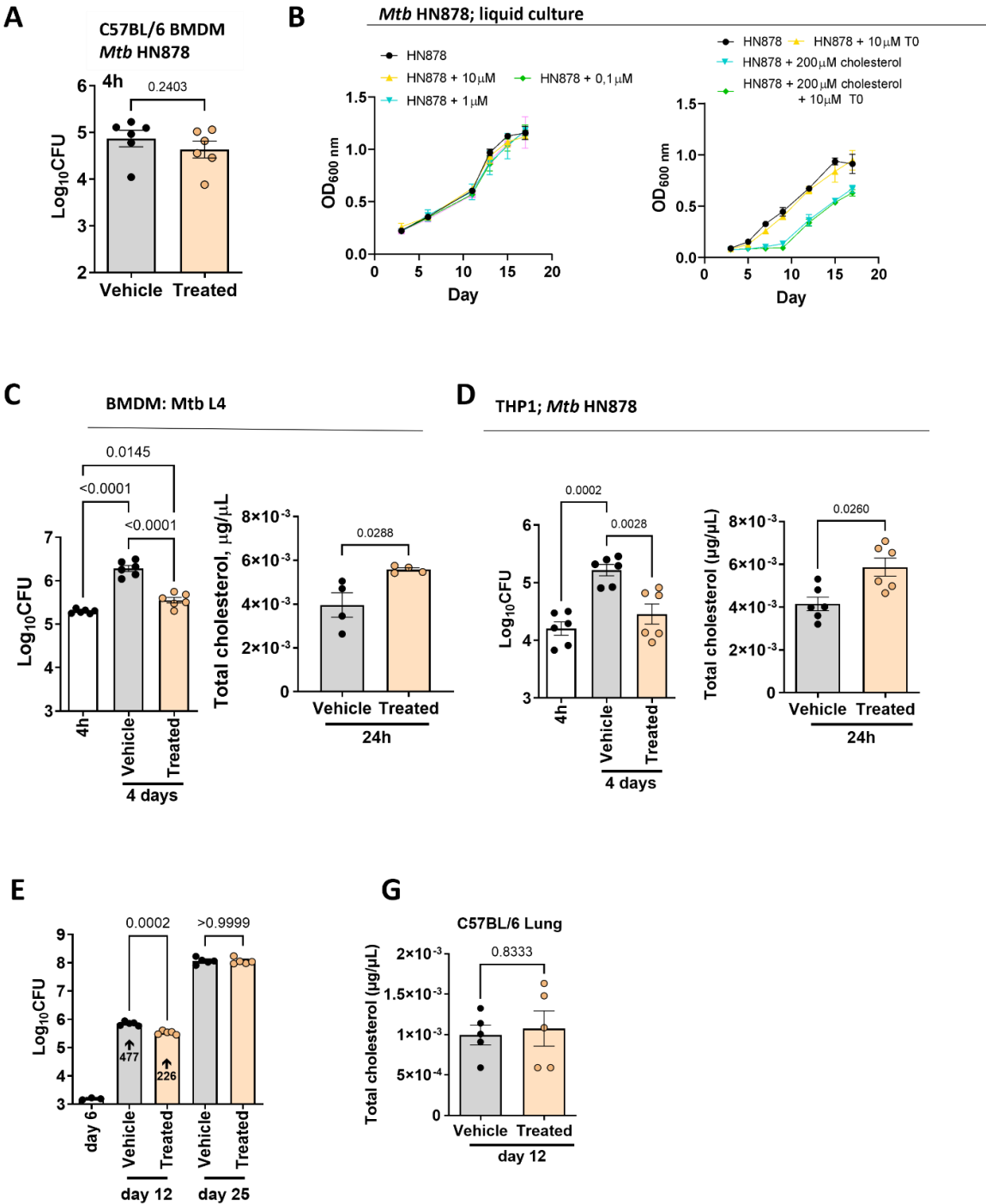
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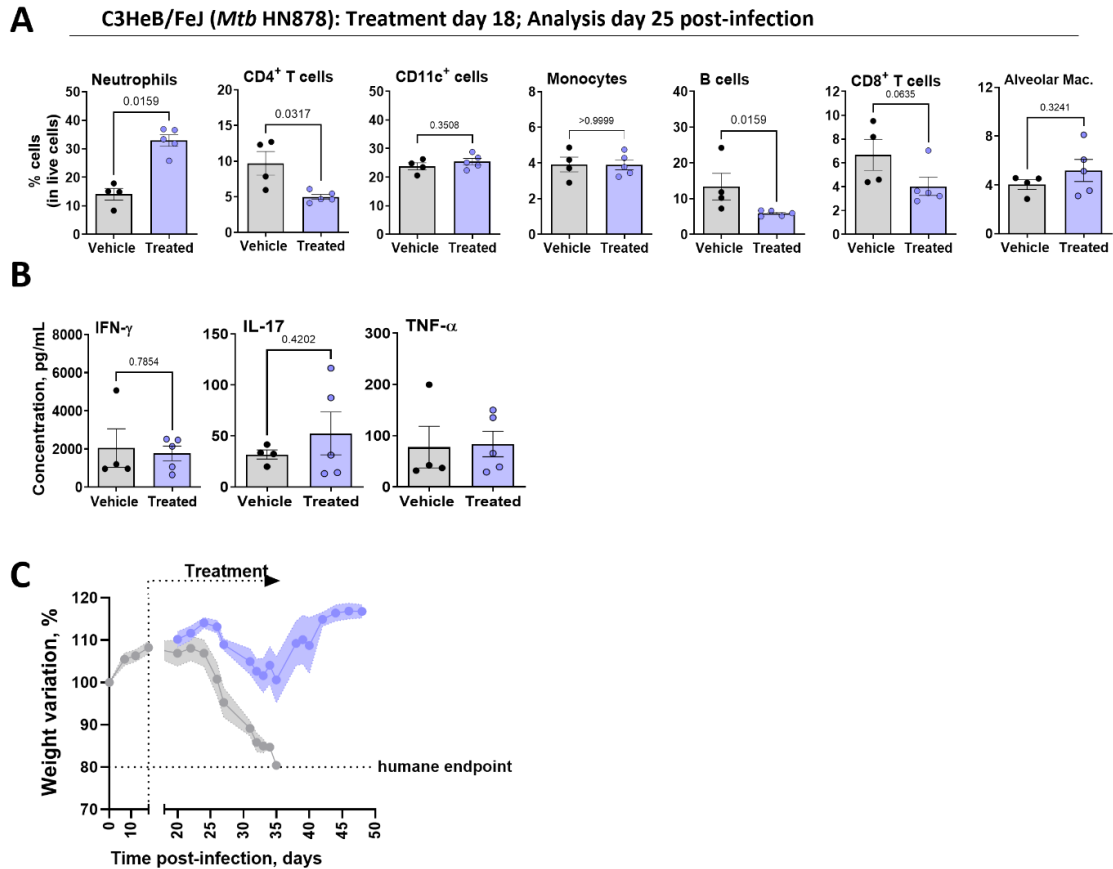
Supplementary materials



Supplementary Figure 1. Lung bacterial loads (A) and body weight curves (B) of C57BL/6 and C3HeB/FeJ mice upon aerosol infection with *M. tuberculosis* HN878. Bacterial loads were determined on days 6, 12 and 25 post-infection. Each dot represents the Mean and the SEM for 3-5 animals per time point in one experiment. (C) Images representing the vehicle and treated animals and the histologic parameters (peribronchiolitis, perivascularitis, alveolitis and necrosis/cell debris) used to obtain the combined lesion score presented in Fig.3D. Scale bars 100 μ m. (D) Gating strategy used to analyse the lung immune cell populations in this study. (E) Cell numbers of distinct lung immune cell populations: CD11c⁺ cells, monocytes, B cells, CD8⁺ T cells, and alveolar macrophages. Vehicle and treated groups are represented by grey or orange bars, respectively. Each dot represents one mouse. Data are mean $\pm$ SEM. Statistical differences between the groups were identified by Mann-Whitney U tests: p-values $\leq$ 0.05 were considered significant. **Abbreviations:** CFU, colony forming unit.



Supplementary Figure 2. (A) Bacterial loads in BMDMs from C57BL/6 mice, measured 4 hours post-infection in the absence (vehicle) or presence (treated) of the LXR activator (10 μ M). (B) Growth curves obtained for *M. tuberculosis* HN878 in axenic medium alone or with increasing concentrations of the LXR activator (left panel) or in media supplemented with 200 μ M cholesterol in the absence or presence of the LXR activator (10 μ M; right panel). (C) Bacterial loads and total cholesterol content in BMDMs infected with *M. tuberculosis* L4 from C57BL/6 mice, measured 4 hours post-infection and 4 days (bacterial loads) or 24 hours (total cholesterol), in the absence (vehicle) or presence (treated) of the LXR activator (10 μ M). (D) Bacterial loads and total cholesterol content determined in Thp1 cells infected with *M. tuberculosis* HN878 measured 4 hours post-infection and 4 days (bacterial loads) or 24 hours (total cholesterol) with or without the LXR activator. (E) Lung bacterial loads of animals treated between days 6 and 12 post-infection, with bacterial loads assessed on days 6 (prior to LXR activation), 12 (after LXR activation) and 25 (experimental endpoint) post-infection. (G) Total cholesterol determined on day 12 post-infection in the lungs of C57BL/6 infected-mice treated with LXR activator between days 6 and 12 post-infection. **Abbreviations:** Mtb, *M. tuberculosis*; BMDM, bone marrow derived macrophage; CFU, colony forming unit;



Supplementary figure 3. TB-susceptible (C3HeB/FeJ) mice were aerosol-infected with *M. tuberculosis* HN878 (average of 203 CFU delivered to the lung). On day 18 post-infection the LXR activator or the vehicle control were administered. The experiment was terminated on day 25 post-infection. (A) Cell percentages of distinct lung immune cell populations: neutrophils, CD4⁺ T, CD11c⁺ cells, monocytes, B cells, CD8⁺ T and alveolar macrophages. (B) Total concentrations of the indicated cytokines in lung supernatants measured by Legendplex of LXR-treated and control mice at day 25 post-infection. (C) Body weight variation for control (vehicle; grey) or treated (blue) mice, at the indicated time points post-infection, in the survival experiment. Each dot represents one mouse. Data are mean $\pm$ SEM. Statistical differences between the groups were identified by Mann-Whitney U tests: p-values ≤ 0.05 were considered significant. **Abbreviations:** Mtb, *M. tuberculosis*; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

Supplementary Table 1. List of flow cytometry antibodies used in this study.

Target	Clone	Company (Catalog #)	RRID
CD4	GK1.5	BioLegend (100453)	AB_2565843
CD8	53-6.7	BioLegend (100741)	AB_2563056
CD11b	M1/70	BioLegend (101245)	AB_2561390
CD11c	N418	BioLegend (117318)	AB_493568
CD19	6D5	BioLegend (115530)	AB_830707
CD45	30-F11	BioLegend (103106)	AB_312971
Ly6C	HK1.4	BioLegend (128014)	AB_1732079
Ly6G	1A8	BioLegend (127616)	AB_1877271
SiglecF	S17007L	BioLegend (155508)	AB_2750237

Supplementary Table 2. List of Taqman probes used in this study.

Mouse	Gene	Primer - probe
	<i>Arg1</i>	Mm00475988_m1
	<i>Arg2</i>	Mm00477592_m1
	<i>Abca1</i>	Mm00442646_m1
	<i>Abcg1</i>	Mm00437390_m1
	<i>Nr1h3</i>	Mm00443451_m1
	<i>ApoE</i>	Mm01307193_g1
	<i>Hprt1</i>	Mm03024075_m1
	<i>Lpcat3</i>	Mm00520147_m1
	<i>Srebf1</i>	Mm00550338_m1
	<i>Hmbs</i>	Mm01143545_m1

CHAPTER VI - GENERAL DISCUSSION

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TB remains a daunting global health challenge¹, and despite notable advances in diagnostic and therapeutic modalities^{2,3}, the complex interplay between host immunity and *M. tuberculosis* continues to delay disease control. In this thesis, we addressed critical questions regarding the immunopathogenesis of TB by investigating lineage-specific immune evasion mechanisms, the interplay of cytokine responses in mixed infections, and the potential of host-directed therapeutic targets. By combining immunoinformatic analyses, clinical cohort studies, and transcriptomic profiling, we aimed to elucidate how molecular adaptations of *M. tuberculosis* to its host contribute to disease progression, and how these insights might inform innovative treatment strategies.

Our immunoinformatic investigations revealed that non-synonymous substitutions in functionally validated antigens⁴ of *M. tuberculosis* L6 substantially reduced the predicted binding affinity of peptide fragments to prevalent HLA Class I and II molecules highly prevalent in West African populations. This observation supports the hypothesis that *M. tuberculosis* L6 may have evolved mechanisms to evade antigen presentation and subsequent T cell recognition, which likely contributes to its restricted geographic distribution and attenuated virulence in comparison to the globally distributed *M. tuberculosis* L4. Nevertheless, these results are based on immunoinformatic predictions and require further experimental validation. Future studies should prioritise the functional testing of antigen variants in host immune cells, stratified by HLA type and infecting *M. tuberculosis* lineage. Such efforts will not only confirm the immunogenic potential of L6-specific epitopes predicted in our immunoinformatic analysis, but also inform the development of lineage-adapted vaccines and host susceptibility markers tailored to affected populations. This integrative approach will be key to translating computational predictions into actionable insights for TB control strategies. These results highlight the importance of genetic adaptation in shaping host-pathogen interactions and offer a possible molecular explanation for the differential geographical restriction and disease progression observed between these lineages.

Complementing these findings, our analysis of a novel TB cohort from Guinea-Bissau provided clinical evidence of similar prevalence in infections caused by *M. tuberculosis* L4 or L6, as previously reported^{5,6}, along with the occurrence of mixed infections⁷. Importantly, by characterizing clinical isolates from the cohort, we observed that single infections with *M. tuberculosis* L4 induced a robust cytokine response in myeloid cells, whereas infections with *M. tuberculosis* L6 evoked a markedly diminished pro-inflammatory profile. This lower response in *M. tuberculosis* L6 echoes the *in vivo* phenotype described previously⁸, in which

M. tuberculosis L6 clinical isolates establish a slow-progressing infection in mice with minimal leukocyte influx, reduced transcription of key inflammatory mediators, and attenuated lung pathology, despite persistent bacterial loads. In contrast, other work with *M. tuberculosis* L4 clinical isolates, showed distinct outcomes⁹. Clinical isolate 6C4, which was associated with severe disease, restricted phagosome rupture in host macrophages, limiting downstream activation of inflammasome, cGAS and RIG-I pathways, ultimately suppressing IL-1 β and IFN-I responses⁹. These signatures were consistent with increased disease severity⁹. Taking into account these two studies on different isolates, these data suggest that a low cytokine signature may arise via two distinct strategies: true attenuation in *M. tuberculosis* L6 versus active immune subversion in certain *M. tuberculosis* L4 strains.

Strikingly, during mixed infections of macrophages with the two isolates, viable *M. tuberculosis* L6 suppressed the strong cytokine production induced by *M. tuberculosis* L4. This dynamic interaction suggests that *M. tuberculosis* L6 may not only evade immune detection through molecular modifications but may also actively modulate the host immune environment during mixed infections. Such an inter-lineage interplay is consistent with previous reports on lineage-specific immune responses and may have profound implications for disease progression and transmission dynamics¹⁰⁻¹². Future studies should aim to dissect the molecular basis of this immunomodulation, with particular attention to whether the suppression operates via post-transcriptional mechanisms, such as mRNA stability, translation efficiency, or modulation of cytokine secretion. Identifying the specific bacterial or host pathways affected by *M. tuberculosis* L6 in this context will be critical. Moreover, validating these findings in in vivo models and clinical settings with confirmed mixed infections will clarify the physiological relevance and potential impact on TB diagnosis and treatment strategies.

Expanding the scope of our research to identify novel TB biomarkers, whole-blood transcriptomic profiling of TB patients from Guinea-Bissau identified the SNR pathway as a critical modulator of disease severity. This finding was corroborated by analyses of other published cohorts¹³, underscoring the relevance of this pathway across regions with diverse lineage distributions. Building upon previous studies that have implicated LXR in modulating and potentially improving disease outcomes¹⁴⁻¹⁹, we performed functional validation experiments in mouse models, demonstrating that pharmacological activation of the LXR, a key component of the SNR pathway, significantly reduced bacterial loads and enhanced cholesterol efflux in late-stage infection without inducing deleterious immune remodelling. This discovery positions LXR activation as a promising host-directed therapeutic strategy, offering an innovative adjunct to conventional bactericidal treatments. Such host-directed approaches are increasingly recognized for their potential to circumvent the challenges

posed by multidrug-resistant TB²⁰, as they target the host metabolic pathways exploited by the pathogen rather than the bacteria directly^{21,22}.

Our integrative analysis reveals that lineage-specific genomic adaptations in *M. tuberculosis*, particularly within L6, are associated with predicted reductions in antigen presentation to prevalent HLA-I and HLA-II molecules in West African populations. These data, combined with *M. tuberculosis* L6 ability to suppress pro-inflammatory cytokine responses, suggest that *M. tuberculosis* L6 has developed immune-modulatory mechanisms that may contribute to its attenuated virulence and geographical restriction. Moreover, we identify the LXR signalling pathway as a host-regulated determinant of TB severity, with timely therapeutic LXR activation, limiting bacterial burden and immunopathology in vivo. These findings support a model in which host-pathogen co-evolution and metabolic immune regulation converge to shape TB pathogenesis, and highlight the potential of lineage-informed, host-directed strategies for TB control. This work, therefore, not only addresses key research objectives regarding the importance of pathogen diversity but also opens new directions for future investigations.

Future directions should focus on exploring the long-term effects of modulating the SNR (LXR) pathway in diverse patient populations and to examine the impact of mixed infections on TB transmission and treatment outcomes in endemic regions. Positioning our findings within the broader context of contemporary TB research, including recent WHO estimates and emerging literature on host-directed therapies, our work contributes significantly to the evolving paradigm of TB pathogenesis and management, laying the groundwork for novel, more personalized approaches to fight this ancient disease.

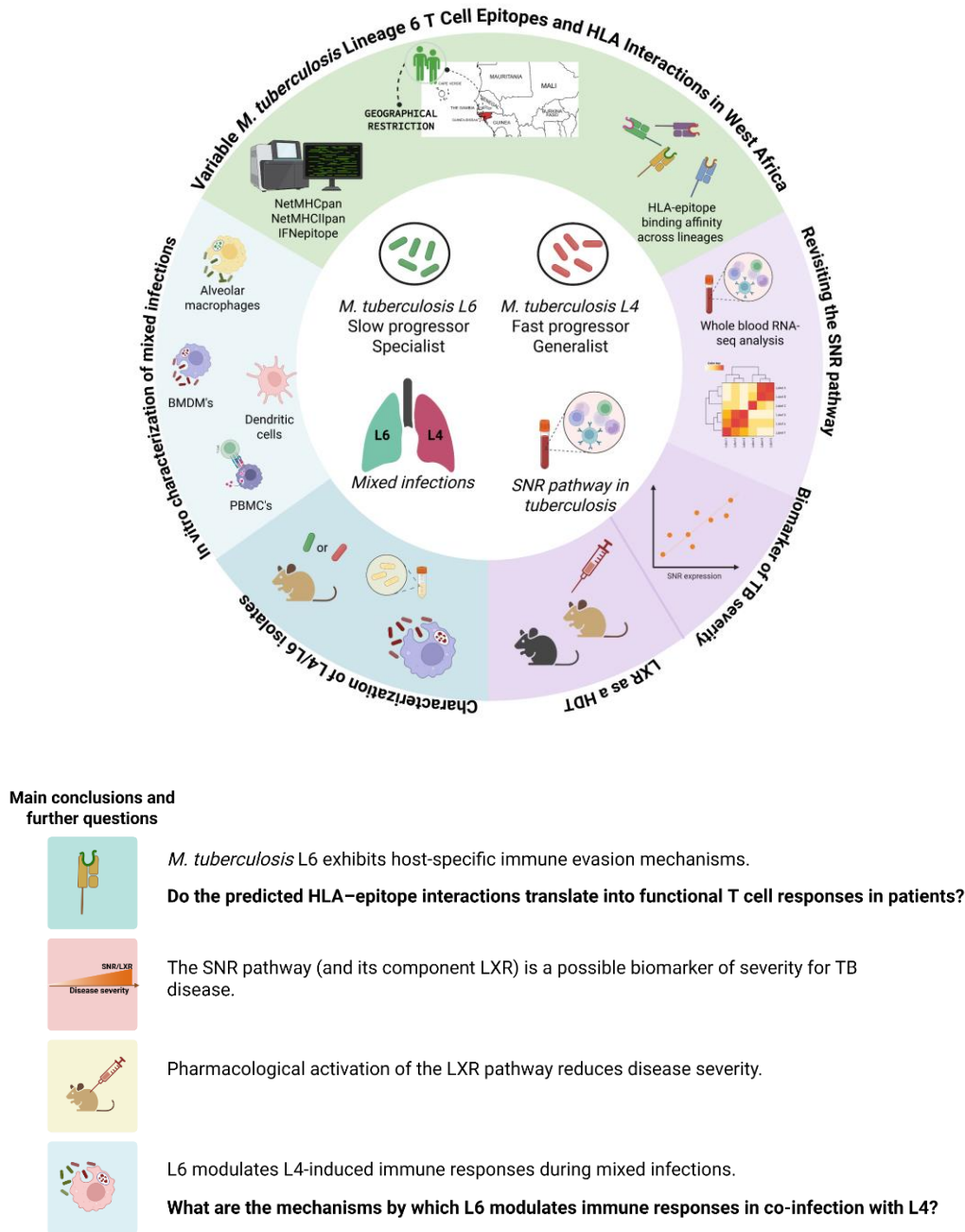


Figure 1. PhD project main findings. Research framework, including the integrated projects, methodologies applied, main findings, and remaining open questions. This figure summarizes how distinct yet complementary approaches were employed to address host-pathogen interactions in *Mycobacterium tuberculosis*, highlighting key immunological and microbiological insights gained, while outlining unresolved challenges and directions for future investigation.

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