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Exploring *Mycobacterium tuberculosis* immune evasion strategies to improve host immunity and tuberculosis outcome: the IL-1 axis

Diogo Fonseca Silvério

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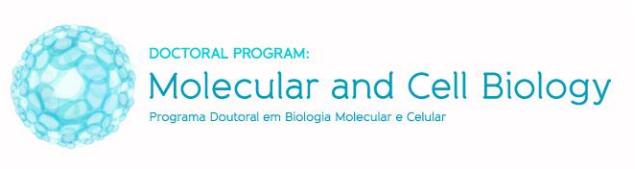

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter I – General introduction

Chapter III – The IL-1 receptor signaling is essential for the initiation of the immune response by alveolar macrophages in *Mycobacterium tuberculosis* infections

Chapter IV – Infection with hypervirulent *Mycobacterium tuberculosis* triggers emergency myelopoiesis but not trained immunity

LIST OF TABLES

Chapter III – The IL-1 receptor signaling is essential for the initiation of the immune response by alveolar macrophages in *Mycobacterium tuberculosis* infections

Chapter IV – Infection with hypervirulent *Mycobacterium tuberculosis* triggers emergency myelopoiesis but not trained immunity

LIST OF ABBREVIATIONS

AECs	Alveolar epithelial cells
AIM2	Absent in melanoma 2
AMs	Alveolar macrophages
APCs	Antigen presenting cells
ASC	Apoptosis-associated speck-like protein containing a CARD
B6	C57BL/6J
BAL	Broncho-alveolar lavage
BCG	Bacille Calmette-Guerin
BM	Bone marrow
CASP	Caspase
C3H	C3H/HeJ
CCL2	Chemokine C-C motif ligand 2
CD	Cluster of differentiation
CFUs	Colony forming units
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors
CXCL2	Cytokines chemokine C-X-C ligand 12
DCs	Dendritic cells
DEGs	Differentially expressed genes
DR-TB	Drug-resistant tuberculosis
EM	Emergency myelopoiesis
C-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDT	Host-directed therapy
H&E	Hematoxylin and Eosin
HIV	Human immunodeficiency virus
HSPCs	Hematopoietic stem and progenitor cells
HSCs	Hematopoietic stem cells
IFN	Interferon
IGRA	Interferon-γ release assay
IL	Interleukin
IL-1R$^{-/-}$	IL-1R deficient
IL-1RA	Interleukin-1 receptor antagonist
IMs	Interstitial macrophages

Lin	Lineage
LK	$Lin^{-}cKit^{+}$
LPS	Lipopolysaccharides
LSK	$Lin^{-}Sca\text{-}1^{+}cKit^{+}$
LTB	Latent tuberculosis
LT-HSCs	Long term hematopoietic stem cells
M-CSF	Macrophage colony-stimulating factor
mIL-1R$^{-/-}$	Myeloid IL-1R deficient
MPPs	Multi potent progenitors
MTBC	*Mycobacterium tuberculosis* complex
MyD88	Myeloid differentiation primary response 88
NLRP3	Nucleotide-binding oligomerization domain-like receptors family pyrin domain containing 3
NRF2	Nuclear factor erythroid 2-related factor 2
NO	Nitric oxide
PBMCs	Peripheral blood mononuclear cells
PGE2	Prostaglandin E2
PRR	Pattern recognition receptors
R	Receptor
ROS	Reactive oxygen species
SCF	Stem cell factor
ST-HSCs	Short term hematopoietic stem cells
TB	Tuberculosis
Th	T helper
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TST	Tuberculin skin test
WHO	World Health Organization
WT	Wild type

RESUMO

Palavras-chave: Tuberculose; Imunidade inata; Interleucina-1; Macrófagos alveolares; Mielopoiese

Tuberculose (TB) é uma doença grave causada por infeções com *Mycobacterium tuberculosis*. Apesar de vários anos de declínio moderado no número de casos e mortes por TB, em 2021 os números aumentaram pela primeira vez em décadas. Isto evidencia a necessidade de continuar a investigação sobre a TB na procura de estratégias preventivas e tratamentos inovadores. A resposta imune desempenha um papel central na TB, e é necessário desvendar as interações imunes entre a bactéria, o hospedeiro e os fatores externos que podem influenciar a resposta imune à *M. tuberculosis*, com o objetivo de desenvolver metodologias inovadoras para um diagnóstico e tratamento rápidos e precisos da TB. As interações imunes iniciais do hospedeiro com o patógeno são muito importante para o estabelecimento da resposta imune à TB. Nesta tese, exploramos como os eventos iniciais da infeção podem moldar a resposta imune inata contra a TB, tendo em consideração como a modulação da resposta imune pode ser influenciada por diferentes isolados clínicos de *M. tuberculosis*.

Anteriormente, o nosso grupo estratificou uma coleção de isolados clínicos de *M. tuberculosis* em pacientes cuja gravidade clínica variava entre casos leves e graves de TB. A gravidade da doença foi associada a uma diminuição na indução da interleucina (IL)-1β em macrófagos e a uma evasão dos sistemas de vigilância citosólica *in vitro*. Nesta tese, usando o isolado clínico 4I2, um indutor de grandes quantidades de IL-1β, proveniente de um caso leve de TB, e o isolado clínico 6C4, um indutor de pequenas quantidades de IL-1β, proveniente de um caso grave de TB, demonstramos que o isolado 4I2 induziu consistentemente maiores quantidades de IL-1β em diferentes células mieloides infectadas *in vitro*, bem como no modelo de infeção por aerossol em murganho. Dada a modulação diferencial de IL-1β pelos isolados 4I2 e 6C4, o nosso próximo objetivo foi desvendar o impacto da falta de sinalização do recetor de IL-1 (IL-1R) no curso da infeção, realizando infeções com estes isolados clínicos em murganhos deficientes em IL-1R. A carga bacteriana nos pulmões aumentou em ambas as infeções numa fase posterior da doença, mas foi particularmente proeminente nas infeções por 4I2, acompanhada por uma diminuição no número de macrófagos alveolares (AMs). Os AMs são os primeiros intervenientes na infeção por *M. tuberculosis*, e a sinalização do IL-1R é tida como fundamental na migração dos AMs dos alvéolos para os pulmões. Assim, examinamos o

perfil transcricional dos AMs de murganhos deficientes em IL-1R infetados por 4I2, onde observámos uma diminuição drástica na ativação em comparação com AMs de murganhos controlo infetados por 4I2. Estes resultados apontam para um papel crucial da IL-1 na reprogramação transcricional dos AMs infetados por 4I2. Adicionalmente, procurámos compreender o papel da sinalização do IL-1R especificamente no compartimento mieloide, relacionado com a resposta imune inicial. Verificámos que a falta de sinalização mieloide do IL-1R não afetou as infeções com o isolado 6C4, mas resultou num agravamento da doença em infeções com o isolado 4I2 desde os momentos iniciais da infeção, comprovando que a modulação das respostas de IL-1 por estes isolados clínicos tem impacto no resultado da doença, onde a sinalização mieloide do IL-1R é protetora nas infeções com o isolado 4I2.

Outro aspeto importante da resposta imune inata abordado nesta tese foi a capacidade da *M. tuberculosis* de induzir mielopoiese de emergência (EM) no hospedeiro e como isso afetaria a produção de células imunes inatas. Para além disso, a EM está também relacionada com a imunidade treinada, a capacidade das células imunes inatas de ter uma resposta imune aprimorada a um segundo estímulo após um estímulo inicial. A *M. tuberculosis* pode infiltrar na medula óssea (BM) e há evidências de alterações hematológicas em pacientes com TB. No entanto, estudos com a estirpe de referência da *M. tuberculosis*, H37Rv, mostraram que a EM e a imunidade treinada parecem limitadas no modelo de murganho após infeção. Nesta tese, demonstramos que a infeção de murganhos com o isolado híper-virulento da *M. tuberculosis*, HN878, levaram a um influxo de células CD45+ nos pulmões, particularmente da linhagem mieloide. O aumento de células mieloides também foi observado no sangue e na BM, sugerindo que a hematopoiese possa estar alterada. As bactérias também foram detetadas na BM. Estes resultados indicam que a hematopoiese na BM é desviada para EM nas infeções pelo isolado HN878. Quando examinámos as células progenitoras da BM, observamos uma diminuição das células estaminais hematopoéticas de longo prazo (LT-HSCs) e um aumento das células progenitoras multipotentes (MPPs). A percentagem de células progenitoras de granulócitos/macrófagos (GMP) também aumentou, com uma diminuição subsequente das células progenitoras mieloides comuns (CMP) e das células progenitoras de megacariócitos/eritrócitos (MEP). Estes dados corroboram a hipótese de que a EM pode estar a ocorrer no modelo de murganho testado. No entanto, a EM induzida não se refletiu em imunidade treinada, como observado em infeções *in vitro* em macrófagos e monócitos derivados da MO de murganhos infetados ou não infetados, onde o controlo da *M. tuberculosis* foi semelhante. Assim, apesar do isolado HN878 disseminar até à BM e induzir a EM, aumentando a produção de células mieloides, estas células mieloides não apresentam uma resposta imune aprimorada a um estímulo secundário.

Combinados, os resultados desta tese demonstraram a importância da diversidade da *M. tuberculosis* em manipular diferencialmente a resistência do hospedeiro e o seu potencial como ferramenta para melhor compreender a resposta imune inicial. Isto também evidencia que a dinâmica entre o hospedeiro e a *M. tuberculosis* pode afetar os eventos imunes iniciais e a resposta imune inata, e consequentemente pode moldar a resposta imune subsequente e levar às inúmeras manifestações clínicas que se observam na TB.

ABSTRACT

Keywords: Tuberculosis; Innate immunity; Interleukin-1; Alveolar macrophages; Myelopoiesis

Tuberculosis (TB) is a severe disease caused by infections with *Mycobacterium tuberculosis*. Despite several years of slow, but steady, decline in the number of new cases and deaths by TB, in 2021 these numbers have increased for the first time in decades. This evidences the need to continue TB research towards novel preventive and therapeutic options. The immune response is a central piece in TB and so there is also a need to unravel the immune interactions between the bacteria, the host and the extrinsic factors that may influence the immune response to *M. tuberculosis*, so that novel methodologies can be developed to allow for a fast and accurate diagnosis and treatment of TB. The early host immune interactions with the pathogen are gaining importance for the establishment of the immune response to TB. In this thesis, we have addressed how the initial events of infection may shape the innate immune response against TB, also taking into account how the modulation of the immune response may be shaped by different *M. tuberculosis* clinical isolates.

Previously, our group stratified a collection of *M. tuberculosis* clinical isolates that caused mild to severe cases of TB in patients. Higher disease severity was associated with decreased induction of interleukin (IL)-1β in macrophages and evasion from cytosolic surveillance systems *in vitro*. Here, using clinical isolate 4I2, a mild TB/high IL-1β inducer, and clinical isolate 6C4, a severe TB/low IL-1β inducer, we have demonstrated that isolate 4I2 consistently induced higher amounts of IL-1β in different myeloid cells infected *in vitro*, as well as in the mouse model of aerosol infection. Given the differential modulation of IL-1β by isolates 4I2 and 6C4, we next aimed at understanding the impact of lack of IL-1 receptor (IL-1R) signaling in the course of infection, by performing infections with these isolates in mice lacking IL-1R. The lung bacterial load was increased in both infections at later stages of disease, but it was particularly prominent in 4I2 infections, which was accompanied by a decrease in the number of alveolar macrophages (AMs). AMs are the first responders to *M. tuberculosis* infection and IL-1R signaling has been shown crucial in the migration of AMs from the alveoli into the lung. Thus, we looked at the transcriptional profile of AMs from 4I2-infected IL-1R deficient mice, where we observed a drastic decrease in activation when compared to AMs from 4I2-infected wild-type mice. These results point at a crucial role for the IL-1 pathway in the transcriptional reprogramming of 4I2-infected

AMs. Furthermore, we aimed at understanding the role of IL-1R signaling specifically in the myeloid compartment, pertaining to the initial immune response. We verified that lack of IL-1R myeloid signaling did not impact infections with isolate 6C4, but resulted in an aggravated state of disease in infections with isolate 4I2 from early time-points of infection, evidencing that the modulation of IL-1 responses by these clinical isolates has an impact in the outcome of disease, where myeloid IL-1R signaling is protective in infections with isolate 4I2.

Another important aspect of innate immune response addressed in this thesis was the ability for *M. tuberculosis* to induce emergency myelopoiesis (EM) in the host and how that would affect the production of innate immune cells. Furthermore, EM is linked to trained immunity, the ability of innate immune cells to have an enhanced response to a second stimulus after an initial stimulus. *M. tuberculosis* can reach the bone marrow (BM) and there is evidence pointing at hematologic alterations in TB patients. However, studies with the laboratory reference *M. tuberculosis* strain H37Rv showed that EM and trained immunity appear limited in the mouse model upon infection. In this thesis, we showed that infections with *M. tuberculosis* hypervirulent isolate HN878 in the mouse model resulted in a lung influx of CD45+ cells, particularly of the myeloid lineage. This increase in myeloid cells was also observed in the blood and BM, suggesting a possibly altered hematopoietic output. Bacteria were also detected in the bone marrow. Altogether, these results pointed at the adaptation of BM hematopoiesis towards EM upon isolate HN878 infections. When looking at BM progenitor cells, we observed a decrease of long-term hematopoietic stem cells (LT-HSCs) and an increase in multipotent progenitors (MPPs) cells. The percentage of granulocyte/macrophage progenitor (GMP) cells also increased, with a subsequent decrease in common myeloid progenitor (CMP) and megakaryocyte/erythrocyte progenitor (MEP) cells. These data further supported that EM may be occurring in the mouse model tested. Nonetheless, EM did not reflect on trained immunity, as observed by *in vitro* infections of BM-derived macrophages and BM-derived monocytes from either infected or non-infected mice, which showed similar control of *M. tuberculosis*. Thus, while isolate HN878 reaches the BM and induces EM, increasing the output of myeloid cells, these resulting myeloid cells do not show an enhanced immune response to a secondary challenge.

Put together, results from this thesis demonstrated the importance of *M. tuberculosis* diversity to differentially impact host resistance, and its potential as a tool for better understanding the initial immune response. It also clearly evidences that the host-pathogen dynamics in TB may impact the initial immune events and the innate immune response, and consequently may shape the subsequent immune response and lead to the multitude of clinical manifestations that encompass TB.

CHAPTER I – GENERAL INTRODUCTION

1. Tuberculosis

1.1. Epidemiology, diagnosis and treatment

Tuberculosis (TB) is an ancient disease that has resulted in many fatalities across the globe throughout human history (1). This disease remains an immense burden for humankind. In 2021, there were an estimated 10.6 million new cases of TB, and 1.6 million deaths (2). Currently, TB remains a major killer, ranking at 13th place in leading causes of death, rising to 2nd if we only account for casualties by infectious agents (2). Despite a steady decrease in the number of cases and deaths of TB over recent years, this progress was halted due to the COVID-19 pandemic and its repercussions on decreased testing and access to healthcare (3). Indeed, these recent years showed us how progress can be hampered by unforeseeable events such as a pandemic. There is an increased need to continue to address the progression, transmission and treatment of TB, particularly in low-income areas and countries that are most affected by this continuing epidemic (2).

There is still an attempt to achieve the proposed targets and milestones of the World Health Organization (WHO)'s End TB strategy, which aim to reduce incidence and mortality by 90% and 95%, respectively, until 2035 (4). It is estimated that about one quarter of the world population is affected by latent TB (LTB) and that approximately 5-10% of individuals with LTB are at risk of developing active disease (2, 5). It is particularly important to identify hotspots of latently infected individuals and to develop methods to detect those at greater risk of transitioning into active TB, as they will continuously feed the TB epidemics (6). This is particularly important in areas where human immunodeficiency virus (HIV) incidence is high. Being infected with HIV drastically increases the risk of developing TB, which remains the main killer in HIV-infected individuals (2, 7). In 2021, there were above 700 000 new cases of HIV/TB co-infections that resulted in 187 000 deaths (2). Another great cause of concern is the increasing occurrence of drug-resistant TB (DR-TB) (8). The latest reports indicate that almost half a million people presented with DR-TB in 2021. While TB is curable, resistance to antibiotics dramatically decreases the efficacy of treatment, dropping the success rate of treatment to only 60% (2).

The most widely used method for diagnosing TB is by collecting sputum from suspected cases and performing an acid-fast bacilli staining (9). This is a cheap and quick method to identify mycobacterial species, being particularly important in lower-income areas (10). As this method lacks in being able to distinguish between different *Mycobacterium*, positive sputum samples are often cultured for further analysis of the presence of *Mycobacterium tuberculosis* (9). *Mycobacterium* may be cultured in either liquid or solid media. The latter

is preferable to detect the possible presence of different bacteria in the sample, where growth rate and colony morphology is helpful not only to distinguish *Mycobacterium* from other bacteria, but also within the genus (9). Nevertheless, it is still difficult to distinguish *M. tuberculosis* from other bacteria belonging to the *M. tuberculosis* complex (MTBC) solely from classical microbiological techniques (9). The introduction of molecular techniques allowed for a fast and precise confirmation of *M. tuberculosis* presence in samples, whether taken directly from patient's sputum or in cultured specimens (11). Additionally, the screening for *M. tuberculosis* genetic material allows the identification of antibiotic resistance mutations and treatment may be personalized accordingly (9, 11). Due to this, the WHO is effectively recommending the employment of fast molecular techniques, allowing for an initial screening of TB and simultaneously for antibiotic resistance to first line antibiotics, in order to accelerate TB diagnosis and improve treatment (12). Often paired with bacteriological or molecular methods, radiological methods such as chest -X-ray or positron emission tomography-computed tomography scans are used to further confirm the TB diagnosis and to check for the progression of lesions caused by the disease (9).

Other frequently used methodologies to detect exposure to *M. tuberculosis* are immunological tests, such as the tuberculin skin test (TST) or the IFN (interferon)-γ release assay (IGRA) (13). Both methods consist of measuring the host immune response through the activation of memory T cells by *M. tuberculosis* antigens (14). TST measures immune responses against a cocktail of antigens delivered intradermally, assessing the migration of memory cells into the spot of injection (14). However, this cocktail of antigens is not very specific and may also activate memory cells that are specific to other *Mycobacterium*. On the contrary, IGRA measures the immune responses by quantifying the amount of IFN-γ released upon stimulating blood samples with *M. tuberculosis* specific antigens (14). Comparative studies have demonstrated that IGRA is a more suitable testing method, as it presents higher predictive performance and specificity than TST (15, 16). Nonetheless, both tests present important limitations. Both tests have a low predictive value in individuals with compromised immune responses, which is particularly worrying in the case of TB-HIV co-infections (17). Furthermore, as they rely on memory T cell responses, which can persist over years, a positive result does not necessarily mean that the bacteria are viable or that the infection is progressing, making it difficult to distinguish active from latent TB (18). Thus, it is crucial to further develop diagnostic tools so that we are able to pinpoint the stage of the TB spectrum someone might be in.

The current treatment of active TB patients consists in administering a cocktail of antibiotics during a 6-month regimen (19). Although effective, this is met with some difficulties, such as the long duration of treatment, with different pills, which ultimately impact patient compliance, which is not always optimal (19). Recently, a 4-month rifapentine-based

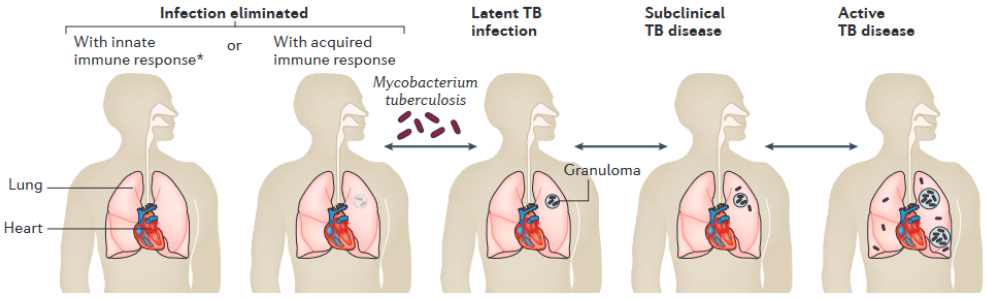
regimen containing moxifloxacin was found equivalent to the standard 6-month regimen, which is an important step to reduce the duration of treatment and consequently improve the outcome (20). The occurrence of DR-TB is also a major problem for the treatment of the disease, as *M. tuberculosis* may acquire resistance to most front-line drugs. This evidences the urge to find novel drugs with different mechanisms of action that allow a more efficient and shorter treatment (19). Thankfully, in 2019, a 6-month drug regimen containing bedaquiline, pretomanid and linezolid was developed for the treatment of highly DR-TB, greatly diminishing the 9-24-month regimen that usually took place (21). Ideally, drug treatments should develop into even more personalized therapies based on the host immunity, clinical symptoms and the characteristics of the infectious agent (19).

1.2. The dynamic spectrum of tuberculosis

TB presents mainly as a pulmonary disease, established upon inhalation of aerosol droplets containing *M. tuberculosis* (13). As for other respiratory infections, coughing is believed to be the main transmission cause of *M. tuberculosis* (22). However, *M. tuberculosis* is detectable in particles exhaled just by breathing, which may lead to an underestimation of the transmissibility risk of TB (23). After being infected, individuals would be classified as either presenting LTB (asymptomatic, but with evidence of infection confirmed by immunological tests) or active TB (disease presenting with symptoms) (24). However, the binarity of this concept has been challenged within recent years, as the variety of different outcomes of infection among individuals points at the development of TB within a dynamic spectrum of disease, from initial clearance to severe infection (Fig. 1) (24, 25). There are several different types of techniques (e.g. bacteriological, molecular, radiological or immunological) that can be useful in diagnosing with a certain degree of confidence the state of disease (26).

Upon infection, there are individuals who may be resistant to infection or who may be infected but are able to clear the bacteria due to their immune system. Others may be infected but show no symptoms or signs of progression, as they conceal the bacteria in a dormant form. These individuals are believed to being able to eventually develop into disease during their lifetime (25). In both clearance and concealing infection, there would be no clinical manifestations or positive results for bacteriological, radiological or molecular diagnostics. However, both cases could result in negative or positive TST/IGRA test results. If positive, both cases would be classified as LTB; however, the implications for further disease development and transmission risk are very different (24). 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 (24, 27).



TST	Negative	Positive	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Intermittently positive	Positive
Sputum smear	Negative	Negative	Negative	Usually negative	Positive or negative
Infectious	No	No	No	Sporadically	Yes
Symptoms	None	None	None	Mild or none	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy	Multidrug therapy

Figure 1. The dynamic spectrum of TB. After acquiring infection, individuals may eliminate the disease right away with their innate immune response or acquired immune response. Both would have no clinical manifestations, but in the latter case there could be positive results from TST or IGRA. If the pathogen is not eliminated, disease may present in a dynamic continuum from LTB to subclinical TB to active TB. LTB or subclinical TB patients would both be positive for TST or IGRA. Subclinical patients may not develop symptoms but may test positive for *M. tuberculosis* culture. Active TB patients will most of the times test positive for TST or IGRA, as well as for culture and sputum smears, and will develop mild to severe symptoms. From (13).

Recently, there has been an increasing demand for biomarkers that are able to assess the stage of TB disease a patient may be at. These can be either pathogen or host markers, that would allow to distinguish between a state of normal biological processes, to a state of immune response or exposure and, finally, in a pathogenic process (28). For instance, it was demonstrated that there is a specific IFN-inducible neutrophil-driven blood signature in human TB patients (29). When looking at the LTB patients, there were a few that showed a similar blood signature to that of active TB patients. This could indicate that these individuals with the same signature are at a higher risk or progressing to clinical manifestation (29, 30).

People who are infected with *M. tuberculosis* and end up developing active TB may start at a subclinical level, where they may be sputum-culture positive and TST/IGRA positive, but still present no overt clinical symptoms (25). Eventually, with progression of disease, bacterial detection becomes increasingly evident by multiple methods, including lung

lesions detectable by radiography. In the most severe cases of disease, symptoms may progress into coughing (possibly with haemoptysis), weight loss, fever, night sweats and eventually develop dyspnoea due to partial destruction of the lung which, if left untreated, may result in disease dissemination and eventually death (26).

1.3. *Mycobacterium tuberculosis* – the etiological agent of tuberculosis

Robert Koch was the first to isolate the main pathogen responsible for TB, *M. tuberculosis* (31). Other *Mycobacterium* very closely related to *M. tuberculosis* (99.21 to 99.92% identical on the whole genome analysis) can also cause TB, mainly in immuno-compromised hosts. These bacteria are all part of the MTBC (32). While the 9 *M. tuberculosis* lineages of the MTBC have adapted to human hosts, others like *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium canetti*, *Mycobacterium orygis*, *Mycobacterium caprae*, *Mycobacterium pinnipedii Mycobacterium suricattae* and *Mycobacterium mungi* typically infect mammalian species, being collectively known as MTBC animal lineages. Nonetheless, they are still clinically relevant in public health safety (33). A recent study based on phylogenetic analysis even suggests that all these bacteria should be considered as variants of *M. tuberculosis* rather than individual species (32).

Within the MTBC, human-adapted bacteria are further divided into phylogeographically structured lineages (L1-L9). L4 (Euro-American) is the most widely distributed, followed by L2 (East-Asian), while L5-L9 are geographically restricted to parts of Africa (34-36). Most importantly, it is now clear that the *M. tuberculosis* lineages associate with distinct disease presentation and immune responses. Both L4 and L2 are widely used in scientific research, likely due to their higher pathogenicity and transmission, with the latter being commonly considered a hypervirulent variant of *M. tuberculosis* (37). For instance, infections with the laboratory strains H37Rv (L4) and HN878 (L2) in resistant C57BL/6J (B6) and susceptible C3HeB/FeJ (C3H) mice demonstrated that there are differences in the course of infection as well as distinct modular transcriptional signatures in the blood, with HN878-infected C3H mice resembling that of active TB disease in humans (30, 38). But even within lineages there is evidence for differences in the immune response triggered which have been associated to different severities of clinical isolates (39). All this shows that there is a need to further dwell into the immune interactions between bacteria and host so that we can improve clinical outcomes and tackle the threat that TB still represents worldwide (40).

2. Host immunity to tuberculosis

As previously mentioned, TB is now classified within a spectrum of disease, mainly due to the several clinical outcomes that may arise upon *M. tuberculosis* infection (24). It is the interaction between the bacteria, the environment and the host immune response that will determine if the patient develops symptomatic disease (of different severities) or is able to fight off the infection (40, 41). The major events of the immune response to TB have been extensively studied (Fig. 2) (41-45) and will be discussed below.

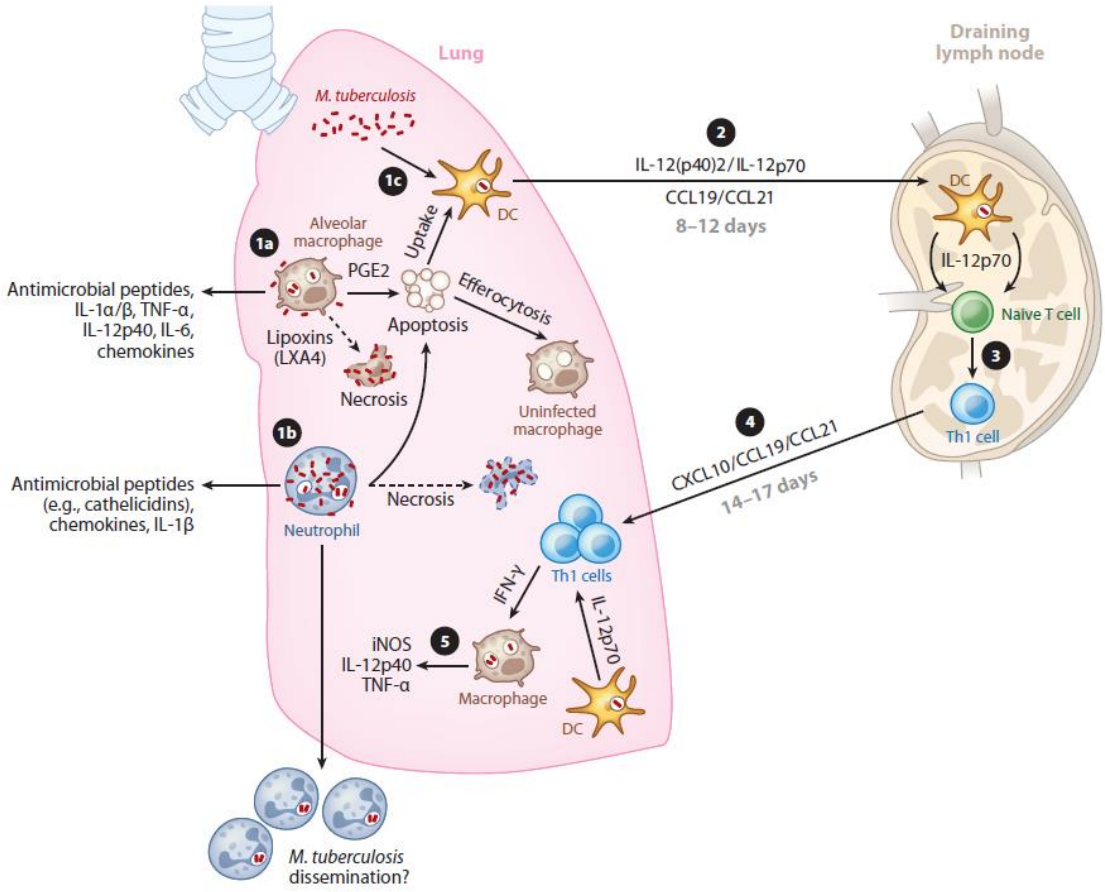


Figure 2. Immune response to TB. Alveolar macrophages **(1a)**, neutrophils **(1b)** and dendritic cells (DC) uptake *M. tuberculosis* and produce several factors, amongst them IL-1. Dendritic cells migrate to the lymph nodes **(2)**, where they activate naive T cells **(3)**, which once differentiated take a reverse route back to the lung **(4)** where they potentiate the immune response by macrophages. From (42).

2.1. Onset of the innate immune response in tuberculosis

The immune response to TB starts following the inhalation of bacteria, and its internalization by the resident macrophages in the lung – alveolar macrophages (AMs) (46). These will eventually migrate into the lung interstitium, where the bacteria will establish and the subsequent immune response will continue (46). In the context of this thesis, and due to their importance in the early immune response to TB, AMs and their role in TB immunopathology will be further discussed in the next section of this introduction. Also part of the alveolar space, but less studies than AMs are alveolar epithelial cells (AECs). There is evidence for an important role of AECs in the onset of infection, as the decrease of surfactant proteins produced by these cells leads to an uncontrolled growth of *M. tuberculosis* after infections of these cells *in vitro* (47). Indeed, the interactions between AMs and AECs are crucial for lung homeostasis (48) and their interaction in *M. tuberculosis* infections is likely fundamental for consequent disease development.

Following the migration of AMs into the lung interstitium, other cells of the innate immune system take part in the fight against *M. tuberculosis*, including eosinophils, neutrophils, macrophages and dendritic cells (DCs) (Fig. 2) (42, 49). Eosinophils were shown to be the first cell to be recruited into the airways to fight off TB infection (50). The exact role of eosinophils in TB is still not completely understood; however, mice lacking eosinophils have shown to be more susceptible to TB (51). There also appears to be an inverse correlation between eosinophil numbers and patient clinical status, with less amount being linked to more severe TB cases (49). Curiously, the other main granulocytes involved, neutrophils, show an opposite pattern of this correlation, with higher numbers usually associated with more severe clinical presentations (49).

Neutrophils are the main phagocyte infected with *M. tuberculosis* in the airways of TB patients with clinical manifestations of disease (52). In fact, they have been associated with a protective role in an early stage of infection in both human and mice (53, 54). However, increased neutrophilic responses clearly play a detrimental role in TB pathogenesis (49). In fact, rather than being apt at killing *M. tuberculosis*, neutrophils appear to be poor at phagocyting this bacterium and may instead act as a niche for bacterial growth (55, 56). It was also demonstrated that limiting neutrophilic infiltration could ameliorate lung inflammation during infection (57). Even in certain cases where bacterial loads are similar, increased neutrophilic influx leads to increased lung pathology with consequent higher mortality in infections with the mouse model (58). Thus, although having its importance at an early stage, in the long term it appears that neutrophils are mostly detrimental to the host during TB infection (49).

While AMs are responsible for migration of bacteria into the lung parenchyma, other subsets of macrophages are recruited during TB infections (59). Interstitial macrophages (IMs), along with AMs, form the majority of macrophage subsets present in the lung (60). Ims have been shown to be more restrictive to *M. tuberculosis* growth, while AMs seem to be more permissive (61). Other macrophages are also recruited from blood monocytes in a chemokine C-C motif ligand 2 (CCL2) dependent manner to help fight off infection (62).

2.1.1. Alveolar macrophages in tuberculosis

AMs are the largest macrophage population in the lung (63). They reside within the lumen of the alveolar space and are in direct contact with airborne pathogens that enter the lung (63, 64). They are surrounded by AECs (type I and type II), endothelial cells and fibroblasts that, through the production of cytokines, help maintain the homeostasis of the alveoli and the correct functioning of AMs (64). Unlike macrophages that are recruited to the lung derived from the aforementioned hematopoietic process, AMs originate from embryonic yolk-sac precursors and possess a self-renewal ability (60). In both mice and humans, granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by type II AECs is crucial for the development and functioning of AMs (60, 64). Some factors like ageing, tissue injury or infection may lead to a reduction or even depletion of AMs, which may be substituted by their monocyte-derived counterparts (60).

During TB infections, AMs seem to have an impaired control over bacteria replication and thus provide an ideal dissemination niche into the lung interstitium (46, 61). This impaired response was reported to be driven by nuclear factor erythroid 2-related factor 2 (NRF2), which promotes cell protective features but not microbicidal ones. Furthermore, infected AMs display a deficient inflammatory response upon infection when compared to their uninfected counterparts (65). AMs from TB patients showed a different transcriptional profile from AMs from healthy donors, with increased expression of pro-inflammatory mediators, as well as genes related to cell recruitment and tissue damage, which may be related to their poor anti-bacterial performance (66). Furthermore, it was shown that AMs, unlike IMs, were unable to adapt their metabolism into a glycolytic state important to promote bacterial elimination (61, 67).

In influenza infections, interleukin (IL)-1 signaling is directly linked to host protection by preventing depletion of AMs (68). In TB, the lack of IL-1 receptor (R) also leads to increased bacterial loads in AMs and other phagocytes. However, the lack of IL-1 signaling does not seem to influence the phagocytic capacities of AMs itself, but rather acting on multiple

pathways that promote cooperation of hematopoietic and non-hematopoietic cells to help clear infection (69). Furthermore, IL-1 appears to have a crucial role in the early stages of TB, as the migration of infected AMs from the alveoli into the lung are dependent of the host IL-1R signaling (46, 70). It has also been suggested that the heterogenicity of *M. tuberculosis* can manipulate this IL-1R dependent migration mechanism to promote transmissibility (70).

These studies demonstrate that the early events of TB infection are very important for the evolution of disease, and both AMs and the IL-1 signaling are a crucial aspect of the initial host defense. There is a great amount of previously published studies that shed light on the role of IL-1 in TB, which will be further reviewed later in section 2.3.1. of the introduction.

2.2. Establishment of the acquired immune response

These early immune events and the interaction of innate immune cells with the bacteria helps shaping the subsequent immune response. In order to establish an adaptive immune response, antigen presenting cells (APCs) need to migrate into the lymph nodes to initiate a T cell response (Fig 2.) (71). This is performed mainly by DCs, as well as blood monocytes, which carry *M. tuberculosis* antigens and prime naive T cells, which will migrate into the lung about 2 to 3 weeks after infection in the mouse model (42). *M. tuberculosis* manipulates APC responses and maturation (72), which could have different implications for the mounted adaptive immune response and consequent development of disease.

Both cluster of differentiation (CD)4+ and CD8+ T cells are primed by APCs to mount a response to *M. tuberculosis (42)*. Upon antigen presentation, naïve CD4+ T cells will differentiate into effector T helper (Th) responses. Th1 cells are responsible for the production of IFN-γ, which is a cytokine crucial for the immune response to TB (73). It has been shown that IFN-γ critical for the activation of bactericidal mechanisms by macrophages and by boosting their phagocytic capacity (74). The importance of CD4+ T cells for TB immunity has been demonstrated in animal models that lack these cells (75, 76). Moreover, the deficiency of CD4+ T cells observed in HIV patients is the reason for the increased susceptibility and mortality in TB-HIV coinfections (77). As for CD8+ T cells, their role in fighting TB seems less prevalent than for CD4+ T cells, although still non-redundant (78). However, CD8+ T cells are able to produce a number of immune mediators important to activate other cells, as well as possessing cytotoxic features that eliminate other infected cells (78).

B cells are far less researched in TB than T cells and so their role is still not well understood. However, they produce many immune factors important for the immune system and seem to modulate other immune cells responses to TB infection (79). The role of B cells appears to be phase-dependent, seeming more important for an acute phase of infection (80). There is also evidence for the importance of B cells in bacterial containment within the granuloma (81, 82).

The granuloma is considered a hallmark structure of TB. Long thought to be an important part of bacterial containment and elimination, it is now clear that it can be used to the bacteria's advantage to promote dissemination and transmission of *M. tuberculosis* (83). Granulomatous structures are characterized by having a caseous necrotic core filled with bacteria, surrounded by aggregates of infected macrophages and epithelioid macrophages (activated macrophages that resemble epithelial cells that can merge into each other to form aggregates). The outer core is usually formed by surrounding T and B cells (83). A deficient immune response can eventually lead to poor granuloma formation, with consequences for bacterial escape that eventually may exit the host trough aerosol droplets and perpetuate infection among individuals (43). While the granuloma has long been regarded as a crucial structure in host defense, this notion has been challenged in recent years (83). Granulomas are now regarded as important sites of bacterial replication, and may prevent immune mediators to reach the bacteria contained inside, shielding them against the host immune response (83). Thus, it is possible that *M. tuberculosis* may use the granuloma as an advantage to escape the host immune defenses and to ensure bacterial replication.

2.3. Cytokines in tuberculosis

Cytokines are small proteins produced by cells that act as communicators between them. Virtually any nucleated cell is able to produce and respond to other cytokines. Responsible for maintaining the homeostasis of the body, their influence in infection, particularly in the immune response to TB, is of great importance (45). Below, the importance of some of the main immune mediators responding to TB will be discussed.

Tumor necrosis factor (TNF)-α is a pro-inflammatory cytokine mainly produced by macrophages, but also by other immune and non-immune cells. The importance of this cytokine for TB control was made clear by the increased bacterial burden and mortality seen in mice lacking TNF-α (84). It has also been demonstrated that granulomas fail to correctly develop in the absence of TNF-α (85). This could be due to the subsequent reduced production of other cytokines that allow for the assembly of the granuloma. This can result

in poor bacterial containment that allows bacterial migration to other parts of the body (45). Importantly, TNF-α is required throughout the life of infected hosts, as neutralization of this cytokine for immunotherapy has been linked to TB reactivation (86).

Another set of important cytokines in TB are IFNs. While structurally related, type I IFN (IFN-α and IFN-β) and type II IFN (IFN-γ) have opposite roles in TB immune response. Type I IFN has been shown to be mostly detrimental to the host, possibly even being manipulated by the bacteria for its own benefit; on the other hand, type II IFN is essential to survive *M. tuberculosis* infection (45). Increased type I IFN features have been linked to the pathogenesis of the hypervirulent strain HN878, by reducing the amount of other important cytokines at play (45). Type I IFN was also shown to interfere with the anti-bacterial effect of IFN-γ (87), as well as being associated with a neutrophil blood signature in active TB patients (29).

As for IFN-γ, the importance of this cytokine is evident for TB control. Genetic deficiencies in humans in the IFN-γ pathway, know as mendelian susceptibility to mycobacterial diseases, are linked to increased susceptibility to TB and poorer control of disease outcome (88). Likewise, lack of IFN-γ in mice infected with *M. tuberculosis* results in very high susceptibility to infection (89). IFN-γ is mostly produced by CD4+ T cells, which instructs macrophages and other cells to activate phagocytic actions (45). Additionally, IFN-γ also limits the infiltration of neutrophils that can be detrimental to the host. Mice lacking IFN-γ signaling have shown to have increased neutrophilic accumulation that related to the increased immunopathology observed (57, 89).

There are several other immune mediators of great importance in the context of TB infection. As previously mentioned, IL-1 is one of these mediators, and its role on the immune response to TB, particularly in the early events, can be critical for the outcome of disease. Since in the context of my thesis I investigated new aspects on the role of IL-1 in TB, I provide next a more detailed introduction to this cytokine.

2.3.1. IL-1 and tuberculosis

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

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Advances on the Role and Applications of Interleukin-1 in Tuberculosis

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2.3.1.1. IL-1

IL-1 was described almost 80 years ago, by Menkin, as a factor mediating tissue injury due to inflammation (90). At the time, this factor was termed "leukocytic pyrogen". Thirty years later, this pyrogen protein was shown to induce the activation and proliferation of lymphocytes (91). Upon introduction of the term "interleukin" in 1979, the leukocytic pyrogen was coined "IL-1" (92). Two agonist molecules with IL-1 biological activity were later discovered and named IL-1α and IL-1β (93). The discovery of other related molecules (IL-18, IL-33, IL-36 and IL-38) led to the definition of the IL-1 super family (94). Members of this super-family exert their function by binding to one of the several members of the IL-1 family of receptors and co-receptors (95). Both IL-1α and IL-1β (collectively referred to as IL-1) exert their activity by binding the IL-1R1 (95). Interestingly, the antagonist molecule (IL-1RA) also binds to this receptor, preventing the binding of IL-1 molecules and consequently blocking their biological effects (95).

IL-1 plays many different roles, from mediating the immune response to infection (96), to the regulation of vascular permeability and angiogenesis (97). Deregulated IL-1

responses have been associated with the development and progression of cancer (98) and also with autoimmune diseases, such as rheumatoid arthritis (99). These findings eventually led to the development of Anakinra, a drug that mimics the IL-1RA, thus blocking IL-1 responses (100). Anakinra signals the potential of IL-1 modulation to treat disease, as it proved effective in the case of rheumatoid arthritis and other acute or chronic auto-inflammatory conditions (100). Nevertheless, the administration of Anakinra may result in increased risk of infections, including TB (101-103).

2.3.1.2. IL-1 plays (mostly) a protective role in tuberculosis

Several studies were performed in cohorts of TB patients with the aim of defining the contribution of IL-1 for TB protection/susceptibility. The genetic variants -511(T/C) and +3953(T/C) of the *IL1B* gene have been addressed in multiple studies, but varying associations with TB susceptibility have been described (104-111). As an example, the T allele in position -511, which is functionally related to lower IL-1β production by stimulated cells, has been associated with increased TB susceptibility in a Gambian population, whereas in the same study no association was found for polymorphisms in the +3953 position (105). However, in another study involving a Colombian population, the very opposite findings were reported, with the +3953 T allele being associated with low IL-1β expression and conferring TB protection (106). Furthermore, the high IL-1β-producing T allele in the promoter position -31 was shown to associate with increased risk of active TB and poor clinical outcomes, likely due to increased neutrophil infiltration (109). The observed discrepancies may reflect variable numbers of patients, as well as the genetic makeup of both the host and the pathogen populations in the study cohorts. Additionally, different populations are subjected to distinct infection pressure, which may influence transmission rates, dose of infection and outcomes of TB. These variations may in turn interfere with a protective or detrimental role for IL-1 in TB. Therefore, collectively, findings coming from genetic studies highlight the importance of a tight regulation of IL-1β during TB. This is further supported by observations coming from the clinical manipulation of IL-1β. As mentioned above, therapeutic blockade of IL-1 with Anakinra has been associated with risk of TB, supporting a protective role of IL-1 in TB (102, 103). However, increased levels of IL-1β and the ratio of IL-1β/IL-1RA were shown to associate with tissue necrosis and cavity formation in TB patients (112).

Studies performed in the mouse model of infection unequivocally illustrate the importance of IL-1 for the host defense against TB. *M. tuberculosis* infection of genetically

engineered mouse models with abrogated synthesis of IL-1α and IL-1β or of their target receptor IL-1R1 revealed high mortality, accompanied by increased bacterial burdens and extensive pathology in the lungs (69, 113-118). Furthermore, abrogation of the adaptor molecule myeloid differentiation primary response 88 (MyD88) resulted in high susceptibility to experimental infection and premature death (119-121), which did not result from defective TLR signaling, but instead from defective IL-1R signaling (116, 122). Although the protection afforded by a competent IL-1R in experimental *M. tuberculosis* infection is indisputable, whether the determinant protective role is played by IL-1α or IL-1β is still a matter of debate. Mice lacking IL-1β displayed acute mortality upon *M. tuberculosis* infection, similarly to IL-1R1 deficient mice, in support of a major role played by IL-1β (116). However, in other studies, genetic deficiency or neutralization of IL-1α, but not of IL-1β, conferred higher susceptibility to infection (113, 123). Moreover, while IL-1α or IL-1β double deficiency consistently recapitulated the phenotype of IL-1R1 deficient mice, the presence of either cytokine ensured bacterial burden and pathology control (124), thus suggesting a certain degree of redundancy. Although these various studies use similar mouse (B6) and *M. tuberculosis* (H37Rv strain) genetic backgrounds, there are some experimental differences that may account for the distinct results: the infection route (aerosol vs intra-nasal); the dose of bacteria in infection (low vs high); the time points chosen for the analyses; the inactivating mutations in the *Il1b* gene, and the use of genetic abrogation vs antibody neutralization. Furthermore, it is possible that imbalances in the animal microbiome may explain some current contradictions, giving the emerging evidence for a cross-talk between the microbiota and TB outcomes (125). Of note, the reasons underlying discrepant IL-1 impacts on TB in human and mouse studies are therefore parallel. Different doses of bacteria used in experimental infections may relate to differences in infection pressure in human populations; data collected in different time points *in vivo* may reflect differences across the spectrum of disease in human TB; and the influence of microbiome is likely to occur in mouse and humans alike, especially considering the diverse environments and life quality that different populations experience around the globe. Thus, although the interpretation of the IL-1 role in TB needs to be taken with care, the parallel study of human and mouse data, as well as the modulation of the discussed variables in experimental models are opening very challenging and promising avenues in TB research.

As discussed above, the protective role of IL-1 in TB seems to be a dynamic one. Both in non-human primates and in a susceptible mouse, the administration of Anakinra two weeks post- *M. tuberculosis* infection, as an adjunct to Linezolid treatment, proved beneficial in controlling of inflammation and lung damage (126). Thus, whereas initial IL-1 responses may be critical for protection, at a later stage they may become detrimental and contribute to tissue damage. It is tempting to speculate that this dynamic role, leading to

protective or detrimental effects of IL-1, results from changes in the cellular and molecular micro-environments. During early time points post-infection, IL-1 would mainly affect stromal and innate immune cells possibly activating protective mechanisms. Later on, the establishment of acquired immune responses induces a dramatic change in the lung micro-environment, with IL-1 potentially acting on different cell types and possibly controlling different steps of the immune response. The tuning of these different steps is likely set by the characteristics of the host, the infecting *M. tuberculosis*, as well as doses of infection. Importantly, a dynamic role of IL-1 has been also demonstrated in other infectious diseases, where excessive production leads to inflammatory damage, but too little of this cytokine is insufficient to trigger an immune response to fight off the pathogen (127, 128).

2.3.1.3. Cellular sources and cellular targets of IL-1 in tuberculosis

Innate immune cells are described as the main *in vivo* cellular source of IL-1β during experimental *M. tuberculosis* infection. Inflammatory monocytes, macrophages and DCs all upregulate their production of IL-1 upon *M. tuberculosis* uptake in the lung (129). Neutrophils are also recruited to the lung and uptake *M. tuberculosis*, but their contribution to IL-1 production seems to be much lower (129). Interestingly, lung residing myeloid-derived suppressive cells have also been described to release IL-1β during *M. tuberculosis* infection, despite maintaining their suppressive activity (130). More recently, AMs were also found to produce IL-1β *in vivo* upon infection with *M. tuberculosis* (46). These findings are paralleled *in vitro* as the secretion of IL-1α and β (and of other cytokines) were reported in a model of *M. tuberculosis*-infected AMs (131). The molecular mechanisms regulating the production of IL-1 by innate immune cells are discussed in more detail in the next section.

Myeloid cells are also well described as cellular targets of IL-1 during TB. The functional consequences of the IL-1R activation in myeloid cells are mainly studied *in vitro* and suggest that IL-1 triggers several microbicidal mechanisms, thus offering a potential explanation for the protective role of this cytokine during infection. Among the described microbicidal mechanisms are the induction of autophagy in the macrophage cell line Raw264.7 (132) and the potentiation of the production of TNF, through a caspase (CASP)-3 dependent mechanism, in both human- and murine- derived macrophages (133). Without the presence of a functional IL-1R, mouse macrophages are more easily infected by *M. tuberculosis*, hinting at a further role for this molecule in preventing bacterial dissemination (69). Furthermore, IL-1 appears to instruct the type of cell death upon phagocytosis of bacteria, by enhancing the production of prostaglandins, particularly prostaglandin 2 (PGE2) (115).

Low prostaglandin production is described to drive infected macrophages into a necrotic rather than apoptotic cell death, favoring the spreading of *M. tuberculosis* within the host (134). The crosstalk between IL-1 and the production of PGE2 is also evident in human macrophages and *in vivo*, as the blocking of IL-1 or IL-1R results in lower production of PGE2 in mice, culminating in poor control of *M. tuberculosis* (115).

A growing body of evidence highlights an important role for non-hematopoietic cells as *in vivo* targets of IL-1. The course of *M. tuberculosis* infection has been assessed in bone marrow (BM) chimeric mice lacking a competent IL-1R in the hematopoietic or non-hematopoietic compartments. In one study, deficiency of IL-1R in hematopoietic cells resulted in high susceptibility to infection, with mice succumbing to disease similarly to full IL-1R deficient mice (113), therefore suggesting that immune cells are the main targets of IL-1 during *M. tuberculosis* infection. However, another study reported that IL-1R signaling in hematopoietic or non-hematopoietic cells was sufficient to afford a degree of protection similar to that seen in WT (wild type) mice (69). This conclusion was further reinforced by the observation that CD45-restricted IL-1R deficient mice did not show increased susceptibility to *M. tuberculosis* infection (69). Since in these two studies (69, 113) the experimental setup was similar, with equivalent mouse and bacteria genetic backgrounds, as well as route and dose of administration, further studies are required to unlock the cellular targets of IL-1 during *M. tuberculosis* infections. For this, the study of the infection in hematopoietic vs non-hematopoietic cell-restricted IL-1R deficient mice and the detailed analysis of non-hematopoietic cells upon *in vivo* infection would be important.

More recently, the migration of *M. tuberculosis* infected AMs into the lung interstitium was shown to be dependent on IL-1R signaling triggering in lung epithelial cells (46). Also, in an *in vitro* co-culture system of macrophages and small airway epithelial cells, macrophages produced IL-1β in response to *M. tuberculosis* infection, but this IL-1β acted on the epithelial cells, inducing their production of the antimicrobial peptide DEFB4/HBD2 which was effective in controlling *M. tuberculosis* replication in these cells (135). The fact that IL-1 increases the expression of adhesion molecules on endothelial cells (136, 137), in this way promoting the migration of immune cells from the vasculature to the tissue, may also uncover a potential role of endothelial cells as targets for IL-1 in TB. Collectively, these findings hint at a role played by the IL-1R signaling not only in the hematopoietic, but also in stromal compartments during the establishment of TB, as it is the case for other lung infections (138, 139). Consequently, it is possible that the *in vivo* role of IL-1 is not limited to the activation of microbicidal mechanisms focused on the pathogen elimination, but instead also include the modulation of the immune response in a more holistic manner. Analysis of the infection progression, together with a deep characterization of the local

immune response triggered in cell-restricted models of IL-1R deficiency will be important to clarify the role of IL-1 in TB.

2.3.1.4. The regulation and cross-regulation of IL-1 during *M. tuberculosis* infection

The production of IL-1β by myeloid cells is controlled following a two-step model that involves regulation at the transcriptional and post-translational levels (96). Briefly, the transcription of the *Il1b* gene depends on the activation of pattern recognition receptors (PRR) in myeloid cells and the triggering of specific intracellular signaling cascades and transcription factors (96). This first step results in the production of an inactive form of IL-1β (pro-IL-1β). A second step, consisting on the assembly of inflammasome components and on the enzymatic action of caspases (96), is then required to cleave pro-IL-1β and promote the secretion of the bioactive molecule IL-1β.

In the context of *M. tuberculosis* infection, the transcription of the IL-1β-encoding gene is induced in human peripheral blood mononuclear cells (PBMCs), and in human and mouse macrophages through the activation of pathways downstream of TLR2/TLR6 and NOD2 receptors (140) (Fig. 3). These receptors recognize *M. tuberculosis* and engage the transcription of the *Il1b* gene through mechanisms involving the signaling molecules ERK, p38 and Rip2 (140) (Fig. 3). More recently, the transcription of the *Il1b* gene was reported to be also regulated by the glycolytic reprograming of the macrophage upon *M. tuberculosis* infection (141, 142), via a mechanism involving the transcription factor HIF-1α (143) (Fig. 3). Both blockade of the glycolytic shift with the glucose analogue 2DG and genetic abrogation of HIF-1α decreased IL-1β production by *M. tuberculosis* infected macrophages (141, 142). However, it is important to mention that the macrophage metabolic reprogramming induced by *M. tuberculosis* infection is not completely understood. For example, experiments with killed vs live bacteria yielded different metabolic effects (144), as did experiments using macrophages of different origins (61) and using diverse *M. tuberculosis* strains (145). Understanding these processes in more detail will better define the link between IL-1β and immunometabolism in the context of TB.

The second step needed for IL-1β production consists on the processing of pro-IL-1β into active IL-1β (Fig. 3). The nucleotide-binding oligomerization domain-like receptors family pyrin domain containing 3 (NLRP3) inflammasome and its apoptosis-associated speck-like protein containing a CARD (ASC) and CASP1 components (Fig. 3) were implicated in the canonical processing of pro-IL-1β in *in vitro M. tuberculosis* infected BM-

derived macrophages (39, 146, 147) and BM-derived DCs (146, 148). The absent in melanoma 2 (AIM2)-inflammasome was shown to also contribute to *in vitro* IL-1β production by *M. tuberculosis*-infected monocytes and macrophages (39, 149, 150). However, *in vivo* the situation is not so clear. Whereas mice deficient for AIM2 or ASC showed a greater susceptibility to infection with *M. tuberculosis* (149), those deficient for NLRP3, or CASP1 showed both little to no difference in their susceptibility when compared to WT mice and unimpaired IL-1β production (116, 146, 147, 151). The fact that the absence of NLRP3 or CASP1 did not affect IL-1β production hints at the existence of non-canonical mechanisms of IL-1β processing, which may operate *in vivo* independently of the NLRP3. These mechanisms may comprise other CASP, matrix metalloproteases, chymases, elastases, and cathepsins (Fig. 3), all previously proposed as alternatives to CASP1 for IL-1β processing (94). Indeed, induction and maturation of IL-1β in DCs infected with *M. tuberculosis* has been described downstream of Dectin-1 activation through a non-canonical CASP8-dependent inflammasome (152). Another possibility, not yet proven in *M. tuberculosis* infection, is the existence of a two-cell inflammasome-independent model, where invariant natural killer cells instruct APCs to secrete IL-1β via CASP8 activation (153).

Several mechanisms for IL-1 cross-regulation have been reported in the context of *M. tuberculosis* infection (Fig. 4), affirming the importance of maintaining a balance in IL-1 activity. This cross-regulation may be achieved within the IL-1 family itself, through the binding of IL-1RA to the IL-1R1 or of the IL-1R2 to IL-1 (Fig. 4), which both prevent the cell signaling response to IL-1, thus limiting its effects (154). Another IL-1R family member with a role in damping inflammation and tissue damage in *M. tuberculosis* infection is TIR8/SIGIRR (Fig. 4), a negative regulator of TLR/IL-1R signaling (155). In its absence, *M. tuberculosis*-infected mice succumb prematurely and exhibit massive liver necrosis, as well as increased levels of IL-1β and TNF-α in lung mononuclear cells and serum (155).

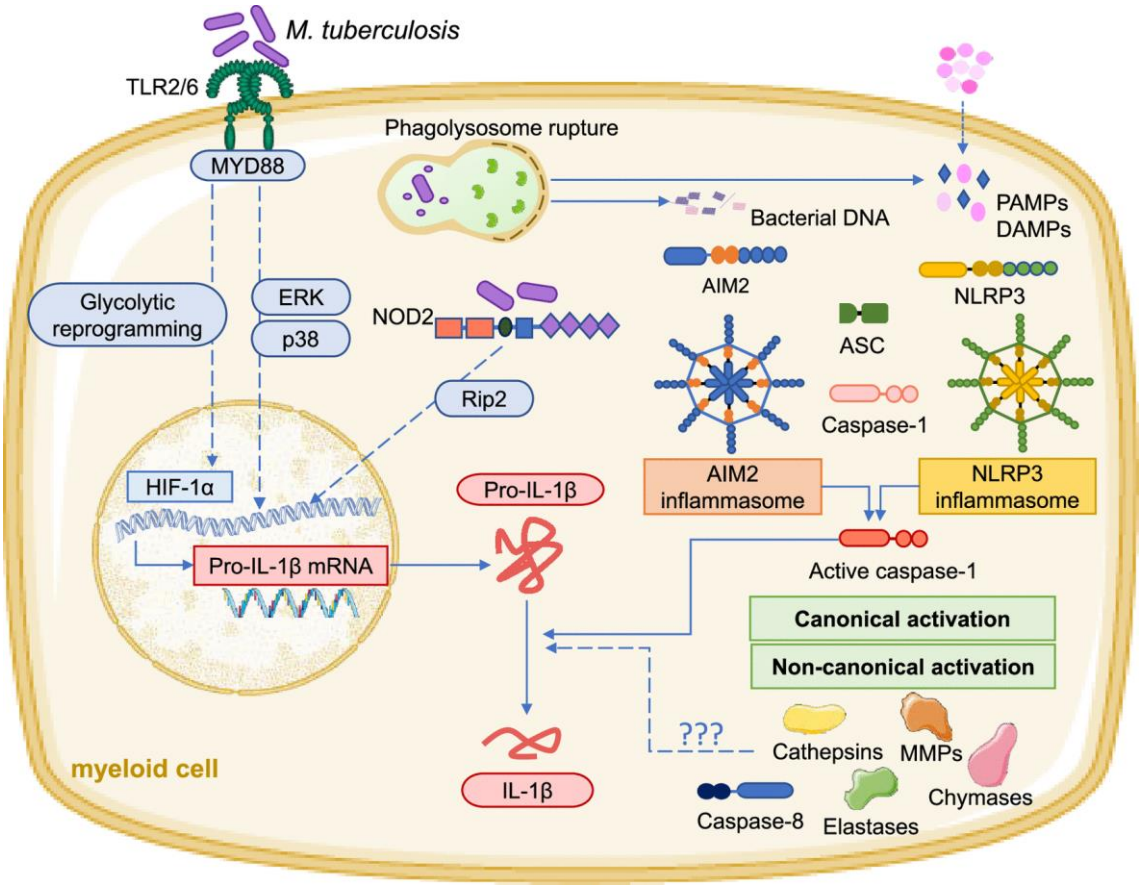

Figure 3. Molecular mechanisms leading to IL-1β production in *M. tuberculosis*-infected cells. The recognition of *M. tuberculosis* molecular patterns by TLR2/6 or by NOD2 induces a series of signaling cascades that culminate in the transcription of the IL-1β mRNA. The glycolytic reprograming of the infected macrophage also enhances *Il1b* transcription. Biological activation of IL-1β requires cleaving of the pro-IL-1β through canonical or non-canonical mechanisms. Canonical activation consists on the assembly of NLRP3 and AIM2 inflammasomes, which are triggered by recognition of PAMPs/DAMPs or bacterial DNA, respectively, resulting from the export of bacterial products from the phagolysosome. The assembly of the inflammasomes leads to the recruitment of CASP1 by ASC. CASP1 becomes activated and cleaves pro-IL-1β into active IL-1β. Non-canonical activation is much less studied in the context of *M. tuberculosis*, but may be mediated by elastases, MMPs, other caspases and chymases.

Although the IL-1RA is also expressed during TB, therefore restraining the IL-1β-induced response, the most notable cross-regulatory mechanism in the context of *M. tuberculosis* infection is possibly the interplay established between IL-1β and type I IFNs (156) (Fig. 4). *M. tuberculosis* induces host production of type I IFNs via different processes, from the activation of TLR4 (157, 158) to the activation of intracellular PRRs, as NOD2 (159), cGAS (160, 161) and RIG-I/MAVS (160). Type I IFNs directly down-regulate the transcription of IL-1β (Fig. 4) in human macrophages infected with *M. tuberculosis* (162). Molecular signals

that negatively regulate type I IFNs in *M. tuberculosis* infected macrophages, such as Tpl2, were shown to positively regulate IL-1β (163). Furthermore, the production of IL-1 by murine inflammatory monocytes, macrophages and DCs during mycobacterial infection was suppressed by type I IFNs *in vivo* (129). Interestingly, this activity of type I IFNs in regulating IL-1 *in vivo* is not entirely direct, being instead mostly dependent on the induction of the anti-inflammatory cytokine IL-10 (129) (Fig. 4). Moreover, type I IFNs also upregulate IL-1RA and IL-1R2 (Fig. 4), thus again indirectly inhibiting IL-1 (115, 129, 164). Interestingly, in turn, IL-1 produced during *M. tuberculosis* infection limits the production of type I IFNs at the translational and transcriptional levels, via the induction of PGE2 (Fig. 4) in myeloid cells, which directly reduced type I IFN-driven bacterial proliferation and mortality (115). Furthermore, an AIM2-IL-1β signaling pathway has been reported to down-regulate the production of *Ifn* genes by inhibiting the STING/TBK1 pathway (165). The crosstalk between type I IFNs and IL-1 in TB is likely implicated in the detrimental effects of the former, as illustrated by the observation that the highly susceptible phenotype of the IL-1R deficient mice is abrogated in mice deficient for both IL-1 and IFN receptors (115).

In addition to type I IFN, other immune mediators have been shown to cross-regulate IL-1 responses (Fig. 4). T-cell derived IFN-γ down-regulates the production of IL-1, although only on monocytes and macrophages, implying that the IL-1 production may be differentially regulated within different myeloid subsets by different IFNs (129). Furthermore, a role for NO in regulating the production of IL-1 (Fig. 4) has been proposed, via nitrosylation and subsequent inhibition of the NLRP3 inflammasome (166). At the functional level, NO halts the neutrophil recruitment cascade driven by IL-1, which if uncontrolled becomes detrimental to the host (167). Other molecules that might also intervene in similar processes of inflammatory regulation are reactive oxygen species (ROS), as demonstrated by an increased IL-1β production and uncontrolled inflammation of the lung in NADPH-deficient mice infected with *Mycobacterium marinum* (168). However, it is important to note that the IL-1 signaling cascade is necessary for the production of ROS and subsequent pathogen control (113), illustrating the importance of regulating IL-1-driven inflammation so that it does not become disadvantageous for the host.

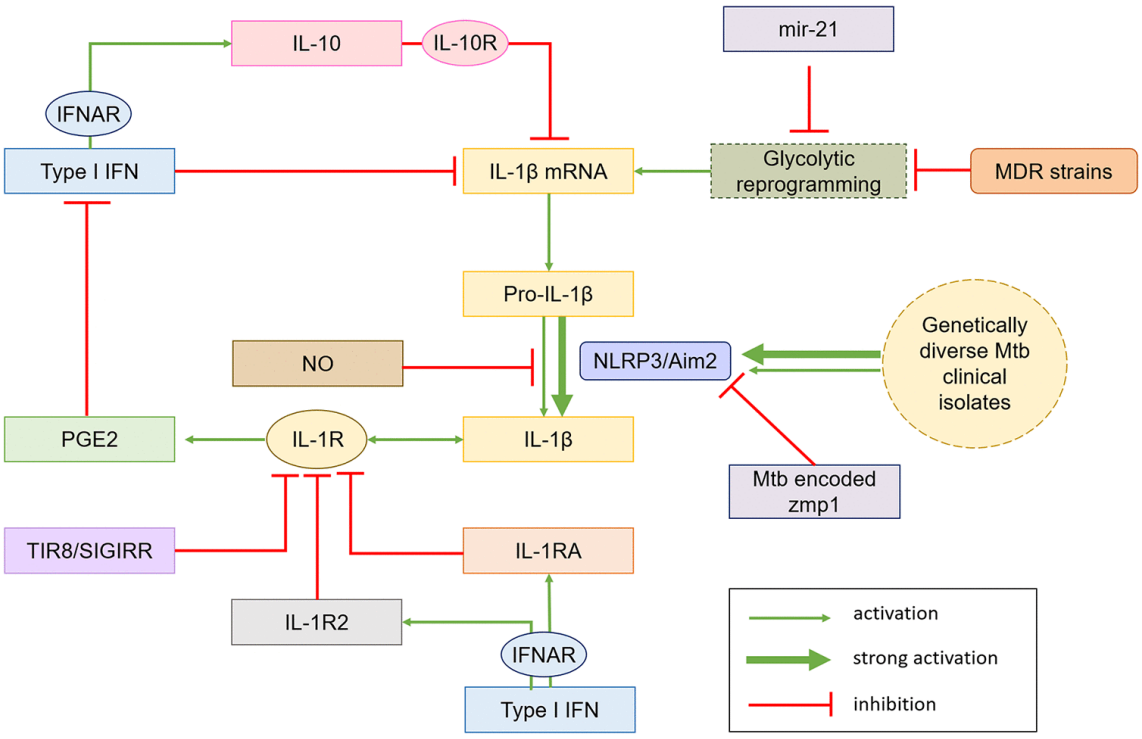

Figure 4. Mechanisms of host and pathogen cross-regulation of IL-1 during *M. tuberculosis* infection. On the host side, type I IFNs are major regulators of IL-1 responses, acting directly by inhibiting the transcription of pro-IL-1β, or indirectly by inducing IL-10, which downregulates the expression of pro-IL-1β, or by upregulating IL-1R2 and IL-1RA. TIR8/SIGIRR also blocks IL-1R responses. PGE2 induced by IL-1β counter-regulates the action of type I IFN, by blocking its transcription. At the post-transcriptional level, nitric oxide (NO) regulates the production of IL-1 by inhibiting the NLRP3 inflammasome. On the pathogen side, *M. tuberculosis* has evolved multiple mechanisms to downregulate IL-1β, from blocking the macrophage glycolytic reprograming, to blocking the inflammasome. Genetically diverse clinical isolates of *M. tuberculosis* have been shown to modulate the production of IL-1β via impacting on the inflammasome activity.

2.3.1.5. Manipulation of IL-1 responses by *M. tuberculosis*

A competent bacterial ESX-1 secretion system is determinant for the induction of IL-1 in *M. tuberculosis*-infected cells (161, 169). This system, encoded by genes belonging to the region of difference 1 (RD1) locus, is a virulence factor present in virulent *Mycobacterium* (e.g. *M. tuberculosis*, *M. bovis*) and necessary for bacterial phagosome evasion (170). Evasion of the phagosome places several bacterial components in the cell cytoplasm, notably ESAT-6, which has been described to activate the NLRP3 inflammasome (171, 172) and the canonical CASP1 processing of IL-1β through a mechanism that involves the induction of potassium ion efflux in infected phagocytes (169, 173). Both RD1- and ESAT6-

deficient *M. tuberculosis* mutants fail to induce the production of IL-1β (and IL-18) in infected macrophages (169, 173). That a virulence factor contributes to the activation of NLRP3 may seem counterintuitive. However, the ESX-1 secretion system also mediates the export of bacterial DNA and RNA from the phagosome into the cell cytosol, thus triggering the production of type I IFNs through cGAS and RIG-I recognition, respectively (160, 161). Thus, the bacterial mechanisms leading to IL-1 production potentiate, at the same time, the synthesis of type I IFN. The pathogen may be able to take advantage of this cross-regulation by manipulating IL-1 levels, assuring its survival and progression of disease (161, 162, 174). Moreover, NLRP3 activation may contribute to necrotic cell death, favoring further bacterial escape from the phagosome (175), a process that also involves the TLR2-MyD88 pathway (172, 176). Considering the links between IL-1, TLR and NLRP3, it is tempting to speculate that IL-1 itself may play a part in this subversion mechanism. *M. tuberculosis* also upregulates the production of LXA4 by human macrophages, which in turn downregulates the synthesis of PGE2 and promotes necrotic cell death, favoring bacterial dissemination (134). Given that IL-1 is necessary for the upstream production of PGE2 (115), one could hypothesize that IL-1 manipulation by *M. tuberculosis* could also be related to the outcome of cell death and consequent bacterial dissemination.

Several mechanisms interfering with the inflammasome activation, thus ultimately modulating the secretion of IL-1β, have been described for *M. tuberculosis* (Fig. 4). The *M. tuberculosis* protein encoded by the *zmp1* gene was found to prevent inflammasome activation and IL-1β production (177) (Fig. 4). Mice infected with bacteria lacking *zmp1* displayed higher production of IL-1β, lower bacterial burden in the lungs and enhanced bacterial clearance, resulting in a positive outcome of the disease (177). More recently, differential induction of IL-1β by genetically distinct *M. tuberculosis* clinical isolates was found to associate with disease severity (39). Clinical isolates recovered from patients with mild TB disease consistently induced high secretion of IL-1β in human PBMCs, THP-1 cells and mouse BM-derived macrophages, which related to a stronger activation of the inflammasome (39) (Fig. 4). Furthermore, *M. tuberculosis* clinical isolates of the modern lineage 4 were shown to induce a higher profile of cytokine production, including IL-1β, when compared to those from ancient lineages, such as lineage 1 and lineage 5 (178). Of note, the modulation of IL-1β production by isolates of *M. tuberculosis* may also occur through hitherto unknown mechanisms (179, 180).

Another potential mechanism used by *M. tuberculosis* to regulate the production of IL-1β relates to the modulation of the metabolic reprogramming of phagocytic cells (181) (Fig. 4). As discussed above, *M. tuberculosis* infection drives a metabolic shift in the macrophage towards glycolysis, which is linked to IL-1β production (142). By upregulating the host production of miR-21 (Fig. 4), *M. tuberculosis* interferes with *PFK-M*, preventing the

glycolytic shift and lowering the production of IL-1β, a process that culminates with enhanced bacterial replication within macrophages (141). Interestingly, IFN-γ was shown to reverse this mechanism and to augment the production of pro-inflammatory cytokines like IL-1β (141). These results may seem in contrast to others previously mentioned, where IFN-γ suppressed the production of IL-1β (129), but these differences could be explained by the specific cellular context, as well as the use of different *M. tuberculosis* strains in these studies. Indeed, bacterial diversity was shown to both impact the metabolic modulation of the macrophage (181), and the production of IL-1β (39, 178-180). Another example of cross-modulation between metabolism and IL-1 is seen in macrophages infected with *M. tuberculosis* strains carrying a rifampicin drug resistance mutation (Fig. 4), which altered the expression of *M. tuberculosis* lipid wall components, impacting the macrophage metabolic reprograming, bypassing the IL-1 signaling, driving the induction of IFN-β and inhibiting glycolysis (145). Given that during *M. tuberculosis* infection *in vivo*, macrophages of different ontogenies were shown to follow distinct metabolic reprograming (61), it will be of interest to understand how that may correlate to IL-1β regulation by AMs vs IMs.

2.3.1.6. The clinical potential of IL-1 in tuberculosis

Given the role for IL-1 in TB pathogenesis, the development of IL-1-based novel TB interventions is an exciting possibility. A challenge will be to pinpoint the individuals who will benefit more from such therapies, namely whether those latently infected or with active TB, with or without comorbidities. Currently, commonly employed IL-1-based therapies are aimed at treating inflammation, based on blocking the effect of IL-1 signaling. These include the administration of the synthetic IL-1RA, Anakinra, soluble decoy receptors for IL-1 or anti-IL-1 neutralizing antibodies (100). Since immune-suppressive treatments, including Anakinra (101-103), associate with increased risk of developing TB, it is tempting to speculate that enhancing IL-1 signaling without causing excessive inflammation may be helpful in protecting the host from TB disease progression. A host-directed therapy (HDT) that is intrinsically linked to IL-1 is the targeting of the host eicosanoid network (115). As previously mentioned, PGE2 is a downstream product of the IL-1R signaling that promotes apoptotic cell death and limits bacterial proliferation (115, 134). Since type I IFNs downregulate the production of PGE2, balancing the immune response between IL-1 and type I IFNs is a promising HDT to manipulate PGE2 production, thus favoring bacterial containment (115). Another interesting HDT based on IL-1 in TB relates to the potential role of IL-1 in trained immunity (182). A recent study has shown that mice trained with β-glucan

showed enhanced protection against *M. tuberculosis* infection, a phenotype that depended on a competent IL-1R and that associated with the expansion of hematopoietic stem and progenitor cells (HSPCs) in the BM and increased myelopoiesis (183). Exploring a possible role for IL-1 in myelopoiesis and the development of trained immunity during TB may be an important HDT to further pursue in the future. Importantly, excessive IL-1 is detrimental in TB and so, the therapeutic control of this cytokine at certain stages of infection, and possibly anatomical locations, may benefit the host. A recent study showed that the side effects of linezolid, a potent antibiotic effective in TB treatment, were abrogated upon IL-1 blocking, without affecting host resistance to TB in mice and non-human primates (126). Also, a recent study suggested that, for *M. tuberculosis* strains that elicit a high IL-1β response, NLRP3-inflammasome inhibition may reduce bacterial survival in macrophages (180). However, increased amounts of IL-1 production elicited by clinical isolates of *M. tuberculosis* may reflect on better outcomes of TB severity (39). Altogether, the modulation of IL-1 responses in TB requires a detailed understanding of the mechanisms through which IL-1 affords protection or pathology, and will also likely need to take into consideration the levels of IL-1 induction provided by different host-*M. tuberculosis* pairs, exposed to different life-style and epidemiologic conditions.

While IL-1-based HDTs may hold promise, there is also evidence that the IL-1 family could be useful as a biomarker in TB. A higher amount of IL-1β produced *ex vivo* by *M. tuberculosis*- stimulated macrophages from LTB as compared to active TB individuals has been reported (184). High production of IL-1β by monocytes and DCs upon TLR stimulation was associated with lower incidence of TB recurrence, while IL-1β production upon stimulation with BCG correlated with higher risk of TB relapse (185). Furthermore, higher IL-1β levels measured in the peripheral blood were associated with higher bacterial loads in the sputum, granuloma cavitation and elevated disease severity in TB patients, which decreased upon TB treatment (186, 187). IL-1α, in combination with EGF, sCD40L, VEGF, TGF-α produced upon antigen-stimulation of whole blood, was also used to distinguish between active and LTB cases (188). Finally, some studies place IL-1RA measured in antigen-stimulated whole-blood and Quantiferon assays as a potential marker to distinguish cases of active versus latent infections (189-192). IL-1RA was also reported as a potential surrogate marker to monitor the efficacy of TB treatment in HIV patients diagnosed with TB (193). A major limitation of these studies, and indeed of using IL-1 family members as potential biomarkers in TB, relies on the difficulty in drawing the threshold of their production relevant to each clinical scenario, an issue that warrants further investigation.

3. Hematopoiesis and myelopoiesis in tuberculosis

3.1. The hematopoietic process

It is evident that the development of an adequate immune response is crucial for fighting TB infections. To better understand how the immune response is influenced, we should also consider the hematopoietic process of immune cell development and how infection and inflammation can impact this process (194), particularly in the case of TB (195).

Hematopoiesis is the process of generating all blood cell types. This is a constant process that renews blood cells throughout an individual's lifetime (196). Most immune cells, both from myeloid and lymphoid origin, are originated in this process through the differentiation of quiescent and pluripotent hematopoietic stem cells (HSCs) located in the BM (196).

HSCs can be divided into long term (LT)-HSCs and short term (ST)-HSCs. LT-HSCs present a longer reconstitution capacity, and are able to differentiate into ST-HSCs, with a shorter reconstitution ability (197). ST-HSCs will then differentiate into multipotent progenitors (MPPs), which no longer possess a self-renewing ability. Together, these 3 types of cells form the HSPCs, and are classically identified by surface makers, such as CD34, CD48, CD150 and Flt3, within the lineage $(Lin)^{-}Sca\text{-}1^{+}cKit^{+}$ (LSK) fraction of the BM (197, 198). MPPs can be further classified into subgroups – MPP2, MPP3 and MPP4. MPP4 will eventually differentiate into the progenitors of the lymphoid lineage, termed common lymphoid progenitors (CLPs), and MPP2/3 will differentiate into progenitors of the myeloid lineage, generally known as common myeloid progenitors (CMPs) (199). The latter can originate granulocyte-monocyte progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs) (197). CMPs, GMPs and MEPs can be found within the $Lin^{-}cKit^{+}$ (LK) compartment of the BM, distinguishable by surface markers such as CD34 and FcγR (200). Eventually, GMPs will originate mature cells of the myeloid lineages, a process globally known as myelopoiesis (Fig. 5) (197).

In recent years, the classical view of the hematopoietic process has been challenged. The idea of compartmentalizing the hematopoietic process into well-defined and stable structures is challenged by the high heterogeneity of the HSC population and differentiation processes (Fig. 5) (201). It is now believed that the acquisition of lineage-specific fates by HSCs derives from a continuous process of differentiation from a pool of low-primed undifferentiated HSCs that gradually acquire lineage biases along multiple directions, eventually progressing into unipotent progenitors (202).

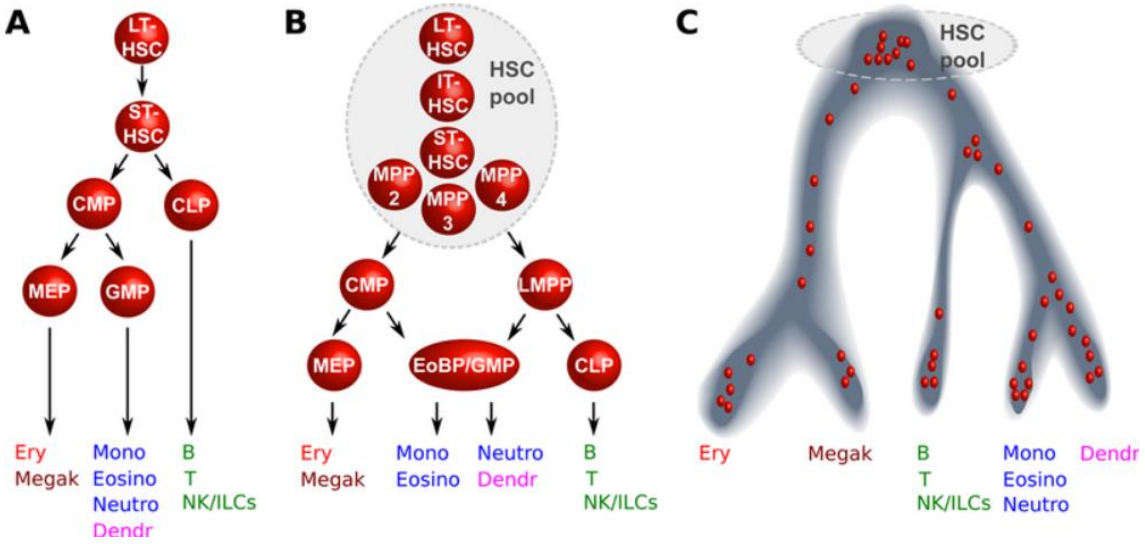

Figure 5. Hierarchical models of hematopoiesis. A: Classic model of hematopoiesis, where a homogeneous population of HSCs will branch into myeloid (CMPs) and lymphoid (CLPs) precursors. B: The HSC and MPP pool is seen as more heterogeneous, which will eventually branch into the myeloid and lymphoid precursors. C: Single cell transcriptomics indicate a continuum of differentiation into mature cells from a highly heterogeneous HSC cell pool. Ery – Erythrocyte; Megak – Megakaryocyte; Mono – Monocyte; Eosino – Eosinophil; Neutro – Neutrophil; Dendr – Dendritic cell; B – B cell; T – T cell; NK – Natural killer cell; ILCs – Innate lymphoid cells; EoBP – Eosinophil/Basophil progenitor; LMPP – lympho-myeloid primed progenitor. From (201).

The hematopoietic process is of extreme importance, thus being tightly regulated. The hematopoietic niche also comprises other non-immune cells that contribute to the regulation of this process, such as perivascular cells or endothelial cells (203). Such cells are responsible for the production of factors that are crucial for HSC maintenance and retention. Examples of such factors are stem cell factor (SCF) or cytokines chemokine C-X-C ligand 12 (CXCL12) (203). Indeed, studies performed on mice lacking CXCL12 signaling led to decreased number of HSCs due to a disturbance in the quiescence of the BM niche (204). Other factors reported to have a role in the hematopoietic process include the notch pathway (involved in early HSC differentiation); the Wnt pathway (involved in the maintenance of immature HSCs); or TGF-β (HSC maintenance in the BM niche) (203).

3.2. Hematopoietic adaptation to infection: emergency myelopoiesis

While in homeostatic conditions hematopoiesis constantly supplies the blood with its cellular components, under physiological stress, this process may be affected and shifted in order to reestablish blood homeostasis (205). Hematopoiesis actively produces cells of the myeloid and lymphoid lineage to ensure a steady state condition of this compartment (206). However, during disturbances of the immune system, such as infection, myelopoiesis can be prioritized to accommodate the need for a higher output of myeloid cells that are the early responders to infection (205). This process results in an increased output of neutrophils, monocytes and DCs and is referred to as emergency myelopoiesis (EM) (207, 208). Indeed, neutrophilia is a common process associated with systemic infections. The high number of neutrophils recruited to the site of infections and their subsequent death enhances the shift towards myelopoiesis usually seen in infections (209). Conversely, increases in myelopoiesis often seen in infection are accompanied by a low lymphopoiesis output (205, 209).

The shift towards EM can occur after direct activation of HSCs by binding of pathogen-associated molecular patterns to their toll-like receptors (TLRs). Specifically, activation of TLRs 2 and 4 promote HSC differentiation and proliferation into EM (210). Studies using lipopolysaccharide (LPS), which binds to TLR4, have shown that its administration impacts the replication and repopulation of HSCs, which can result in shifting the destiny of HSCs towards EM (211, 212). Furthermore, TLR activation also leads to the differentiation of circulating HSCs into tissue-resident myeloid cells upon recruitment to the local of infection (213).

Given the importance of EM for the host immune response, it is not surprising that some cytokines are crucial for this process. Granulocyte colony-stimulating factor (G-CSF) has an essential role in this development, even at the steady-state myelopoiesis, demonstrated by the marked decrease of circulating neutrophils in mice lacking G-CSF or its receptor molecule (207). Furthermore, G-CSF-deficient mice infected with *Listeria monocytogenes* showed increased mortality and a markedly decreased myeloid output (214). Similarly, GM-CSF is also deemed crucial for the myelopoietic process. This cytokine is crucial for the development of neutrophils, monocytes and macrophages, as seen by the growth stimulation of these myeloid lineages upon GM-CSF administration (207). While mice deficient for GM-CSF show no impaired defects in steady-state myelopoiesis (215), if infected with *L. monocytogenes* GM-CSF deficient mice also show decreases in myeloid cell production and increased mortality (216). Macrophage colony-stimulating factor (M-CSF) has also been shown to directly act on HSCs, driving them towards myelopoiesis by

activating PU.1, a master regulator of myelopoiesis (217). Nonetheless, mice deficient for the 3 main myeloid growth factors (G-CSF, GM-CSF and M-CSF) are still able to produced neutrophils under normal conditions, as well as mount a myelopoietic immune response, indicating that other factors may be involved in the process. IL-1 is also linked to increased proliferation and differentiation of HSCs, mainly through the induction of PU.1 in HSCs, linked to GM-CSF and M-CSF signaling. IL-1 administration also resulted in the alteration of the quiescence state of HSCs towards myelopoiesis, while its suppression led to reconstitution of the BM through myelosuppression (218).

Chronic mycobacterial infections with *M. avium* have been shown to promote the differentiation of HSC towards myelopoiesis, resulting in impaired self-renewal of HSCs (219-222). Mice lacking GM-CSF chronically infected with *M. avium* displayed no changes in output of hematopoietic cells and displayed deficiencies in macrophage activation (220). IFN-γ was also found to promote the bias towards myelopoiesis, as mice with deficient IFN-γ signaling did not increase their myelopoietic output upon *M. avium* infection (219, 222). IFN-γ administration in naïve mice and in cultured HSCs induced HSC proliferation both *in vitro* and *in vivo*, showing that IFN-γ can directly impact HSC proliferation (219). Furthermore, transcriptomic analyses also attribute myelopoiesis depletion to IFN-γ signaling defects in mice with chronic *M. avium* infections (221). A role for IFN-γ in myelopoiesis was also attributed in the case of bacille Calmette-Guerin (BCG) infections in mice (223).

In the context of TB, it has been shown that *M. tuberculosis* is able to infect the BM of patients and persist in the HSC niche (224-226). More importantly, once in the BM, *M. tuberculosis* is able to alter the homeostatic environment and influence the differentiation of HSCs into mature immune cell lineages (195, 227). Both in humans and mice, the administration of BCG, the classical vaccine for TB, has resulted in skewing of the hematopoietic process towards myelopoiesis (223, 227). In the mouse model, the increased myelopoiesis triggered by BCG administration resulted in higher protection of monocytes and macrophages which were efficient against M. *tuberculosis* (223). Despite the evidence for an increased hematopoietic output observed in active TB patients or even in the mouse model (38, 205, 228), a recent study with *M. tuberculosis* H37Rv aerosol infection in mice argued that, while the reprogramming of HSCs still occurred, the bacteria severely limited EM to favor the course of infection (229).

3.3. Emergency myelopoiesis and trained immunity

Interestingly, EM has been linked to trained immunity, the capacity of myeloid cells of the innate immune system to acquire “memory”, showing enhanced capability to fight off infections after previous exposure to a primary challenge (230). This is the result of a long-term reprogramming of innate immune cells by a first stimulus, which may vary according to its origin. Indeed, the reprogramming caused by LPS, β-glucan or BCG may have different outcomes in terms of immune cell activation (231). In mice, different microbial ligands have shown to confer not only specific resistance to re-infection, but also non-specific protection against other types of immunological challenges (231). In humans, live vaccines such as BCG have been linked to non-specific protection trough higher activation of innate immune cells (231, 232).

The BCG vaccine has also been linked to early clearance of *M. tuberculosis* trough trained immunity mechanisms in innate immune cells (233, 234). PBMCs from humans previously vaccinated with BCG showed increased activity *ex vivo* against *M. tuberculosis*, as well as changes in DNA methylation in response to BCG exposure when compared to PBMCs from non-vaccinated individuals (235). Additionally, monocytes trained with BCG *in vitro* underwent epigenetic changes, with increased production of ROS that promoted bacterial killing up until 3 months after initial exposure (236). Blocking of autophagy impeded epigenetic reprogramming of monocytes, which hints at a pivotal role for this process in the acquiring of trained immunity (237, 238). Furthermore, as mentioned above BCG was able to reach the BM and reprogram HSCs towards EM. This resulted in increased protection against *M. tuberculosis* due to epigenetic changes towards trained immunity by monocytes and macrophages with long lasting effects (223). β-glucan also showed to be effective in conferring protection against *M. tuberculosis*. This effect was associated with the expansion of HSCs and a shift towards EM. Curiously, lack of IL-1R inhibited the protective effect conferred by β-glucan, which hints a role for IL-1 signaling not only in EM but also in training innate immune cells (183).

M. tuberculosis is also capable of inducing trained immunity in humans. PBMCs from patients with recent cases of TB performed better at controlling the growth of BCG *in vitro* when compared to long-term TB patients, which was correlated to higher expression of CXCR3 ligands (239). Nonetheless, it is still unclear if this innate immune training would be sufficient to prevent a re-infection or new infection with *M. tuberculosis* (238).

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CHAPTER II – AIMS

As previously discussed, the initial events of the immune response to TB are essential for the outcome of disease. In fact, infections with *M. tuberculosis* may be cleared by some hosts only due to their innate immune response, without even needing to initiate an adaptive immune response. This evidences the need to further understand how *M. tuberculosis* infections shape the mounting of the immune response. In this context, my thesis was dedicated to the study of how *M. tuberculosis* may impact the innate immune response in two key parts of the infection. My specific aims are to:

- Understand the interactions of *M. tuberculosis* with the first responding immune cells in the lung (AMs) and the role for the IL-1R signaling in the outcome of disease. IL-1 is a fundamental player in the immune response to *M. tuberculosis*, but it may be both protective and detrimental, depending on the stage of disease. Following on previous work from our group showing that genetically diverse *M. tuberculosis* clinical isolates may lead to differential induction of IL-1β by infected macrophages, in the first part of my PhD, we used the experimental mouse model of infection to investigate how the modulation of IL-1 responses may impact the initial immune events and how that may lead to different outcomes in susceptibility to TB.

- Investigate how *M. tuberculosis* infection may impact the hematopoietic niche, and consequently the production of innate immune cells that will migrate into the sites of infection. It is clear that during infection *M. tuberculosis* is able to reach the bone marrow and persist in HSCs, however there is conflicting evidence for how that may impact myelopoiesis. To address this, in the second part of my PhD, we used the experimental mouse model of infection to investigate if the hypervirulent *M. tuberculosis* HN878 isolate is able to reach and modulate the HSC niche, by inducing emergency myelopoiesis in the bone marrow, and if that translates to enhanced protective immunity from bone marrow derived cells.

CHAPTER III – THE IL-1 RECEPTOR SIGNALING IS ESSENTIAL FOR THE INITIATION OF THE IMMUNE RESPONSE BY ALVEOLAR MACROPHAGES IN *MYCOBACTERIUM TUBERCULOSIS* INFECTIONS

This chapter is in preparation for publication.

The IL-1R signaling is essential for the initiation of the immune response by alveolar macrophages in *Mycobacterium tuberculosis* infections

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Abstract

The fight against tuberculosis (TB) was severely impacted in the last years. To tackle the TB epidemic, one crucial aspect is to better understand the immune interaction between the host and *Mycobacterium tuberculosis*. Previously, our group stratified clinical isolates of *M. tuberculosis* that caused mild to severe cases of TB in patients. Higher disease severity was associated with decreased induction of IL-1β and evasion from cytosolic surveillance systems in macrophages infected *in vitro*. Here, using *M. tuberculosis* isolate "4I2", a mild TB/high IL-1β inducer, and isolate "6C4", a severe TB/low IL-1β inducer, we have recapitulated the differential induction of IL-1β by these clinical isolates in other myeloid immune cells, as well *as in vivo* using the mouse model of experimental infection. Given the known role of the IL-1 receptor (R) signaling in the early stages of *M. tuberculosis* infections, we performed infections with these isolates in mice lacking the IL-1R. Bacterial loads in the lungs were increased in both infections, but this increase was particularly prominent in *M. tuberculosis* 4I2 infections. When looking at the transcriptional profile of alveolar macrophages (AMs) from 4I2-infected IL-1R deficient (IL-1R$^{-/-}$) mice, we observed a drastic decrease in activation when compared to AMs from 4I2-infected wild-type mice. This lack of activation translated into an impaired immune response, as observed by lower amounts of cytokines produced by AMs from IL-1R$^{-/-}$ mice when infected with isolate 4I2 also *in vitro*. These results point at a crucial role for the IL-1 pathway in the transcriptional reprogramming of 4I2-infected AMs. Furthermore, we aimed at understanding the role of IL-1R signaling in different cellular compartments pertaining to the initial immune response, such as the myeloid compartment. We showed that IL-1R signaling in the myeloid compartment had no effect on controlling the growth of *M. tuberculosis* isolate 6C4 infections, while infections of these mice with isolate 4I2 resulted in an aggravated state of disease, likely caused by overt activation of the inflammatory response. Our study demonstrates the importance of *M. tuberculosis* diversity to differentially impact host resistance, and its potential as a tool for better understanding the host-pathogen dynamics in TB.

Keywords

- Tuberculosis
- IL-1
- Alveolar macrophages
- Innate immune response

Introduction

Despite several decades of declining tuberculosis (TB) cases and deaths, the progress made against this severe disease has been halted, and even worsened, since the COVID19 pandemic (1, 2). The spectrum of TB can vary from asymptomatic individuals (who either clear the infection, or may progress to latent or subclinical TB) to active TB patients who present several different clinical manifestations (3). The diversity in the clinical manifestation of TB appears to be at least partly linked to the genetic diversity within the MTBC (4, 5), which hints at the modulation of the immune response by different *Mycobacterium tuberculosis* clinical isolates.

We have previously shown that the severity of *M. tuberculosis* isolates corelated to their ability to induce IL-1β production *in vitro* (6). A clinical isolate representative from severe cases of TB (*M. tuberculosis* 6C4) was shown to evade cytosolic surveillance pathways in macrophages, which led to low IL-1β production (6). On the other hand, clinical isolate *M. tuberculosis* 4I2, representative of mild TB cases, actively induced the inflammasome-dependent IL-1β production, regardless of the host TB status (6). However, it is still unknown if and how IL-1β modulation by these different *M. tuberculosis* isolates impacts the course of disease *in vivo*.

While IL-1β seems to be mostly protective in TB, its role may be a dynamic one, possibly also leading detrimental effects (7). In humans, polymorphisms in the *IL1B* gene have been associated as either protective or detrimental in TB (8-15), showing discrepancies likely depending on the population they were conducted in. In macaques and a susceptible mouse model, administration of the IL-1 receptor antagonist (IL-1Ra) between 14 and 28 days post-infection combined with the antibiotic Linezolid improved TB outcomes due to lower inflammation and lung pathology (16). Nonetheless, IL-1β is also necessary for the host response to TB, as previously demonstrated by the increased pathology and mortality in mice lacking IL-1β or the IL-1R (17-23). Rather than having a direct microbicidal effect on infected cells, the IL-1R signaling-dependent control of infection appears to be provided by trans-protection of both hematopoietic and non-hematopoietic origin (23). It was recently shown that alveolar macrophages (AMs), the first cells to uptake *M. tuberculosis* in the alveoli, produce IL-1β when infected (24) and require IL-1R signaling to initiate dissemination into the lung (24, 25). Blocking of IL-1R signaling through intraperitoneal administration of anti-mouse IL-1R reduced AM capacity to disseminate into the lung, which resulted in slower adaptive immune response and consequently lower control of infection (25). However, it is still unclear how the lack of IL-1R signaling directly affects the early events of TB onset and the implication for tissue-restricted IL-1R signaling in hematopoietic

and non-hematopoietic compartments. Moreover, whether the impact of IL-1R signaling is similar to different *M. tuberculosis* isolates is also unclear.

Here, we show that the clinical isolate *M. tuberculosis* 4I2 consistently induced the production of high levels of IL-1β not only in macrophages, as we previously published (6), but in all other mouse myeloid cells tested *in vitro*, including AMs, while the clinical isolate 6C4 consistently induced low IL-1β production. Infections of C57BL/6 wild type (WT) mice and mice lacking IL-1R signaling (IL-1R$^{-/-}$) resulted in increased bacterial burden at later stages of infection, particularly in the case of the high IL-1β inducing 4I2 clinical isolate. RNA sequencing of AMs from WT and IL-1R$^{-/-}$ mice infected with 4I2 revealed transcriptional differences between them, with AMs from IL-1R$^{-/-}$ mice strikingly showing an impaired immune response. We further expanded the role of the IL-1R signaling to different cellular compartments by infecting cell-restricted IL-1R$^{-/-}$ deficient mice in myeloid and non-hematopoietic compartments. While lack of IL-1R signaling in non-hematopoietic compartments showed no differences in the course of disease, myeloid ablation of IL-1R resulted in increased susceptibility to disease early on, with high bacterial burden and extensive lung pathology. With this study, we hope to shed light on how the modulation of IL-1β can impact the host at early stages of infection, particularly in cases where IL-1R signaling is blocked, and how that may affect the transcriptional profile of AMs upon being infected with *M. tuberculosis*.

Results

Differential IL-1β modulation by clinical isolates 4I2 and 6C4 is consistent *in vitro* and *in vivo*

Infections with *M. tuberculosis* clinical isolates 4I2 consistently induced higher IL-1β production in AMs (Fig. 1A), BM monocytes (Fig. 1B) and BM-DCs (Fig. 1C) than those with clinical isolate 6C4 *in vitro*, similarly to what we had been previously reported in human peripheral blood mononuclear cells (PBMCs) and monocytes and mouse BM-derived macrophages (6). The same pattern was not observed for the induction of TNFα upon infection of the same cells with either isolate of *M. tuberculosis* (Fig. 1A, 1B, 1C), a cytokine that unlike IL-1β does not depend on the inflammasome activation. The induction of IL-1β upon infection with *M. tuberculosis* 4I2 was dependent on both the presence of active bacteria and inflammasome activation in either AMs (Fig. 1D), BM monocytes (Fig. 1E) or BM-DCs (Fig. 1F), as bacteria previously treated with rifampicin or inhibition of the

inflammasome by MCC950 drastically reduced IL-1β production by these cells. These findings are also in keeping with our published report (6), thus suggesting that these isolates of *M. tuberculosis* exploit common mechanisms across a variety of innate immune cells all of which play relevant roles during TB. The fold change of bacterial growth across 4 days of infection was also assessed in these cells, with clinical isolate 4I2 presenting higher colony forming units (CFUs) fold change in AMs (Supp. Fig. 1A) and DCs (Supp. Fig. 1C), but not on BM monocytes (Supp. Fig. 1B), when compared to clinical isolate 6C4. When we infected WT mice with either clinical isolate, we also observed higher amounts of IL-1β in the broncho-alveolar lavage (BAL) of mice infected with clinical isolate 4I2 than for 6C4-infected mice 30 days post-infection. This was not the case for TNFα, which was detected at higher levels post-infection with *M. tuberculosis* 6C4 (Fig. 1G) or IL-1α, which was detected at similar levels for the two infections (Supp. Fig. 1D). Of note, on day 12 post-infection the levels of all tested cytokines were similar to those detected for non-infected mice, with no differences observed between the two infections (Fig. 1G and Supp. Fig. 1D). These results corroborate and expand our knowledge on the differential IL-1β induction by clinical isolates 4I2 and 6C4 both *in vitro* and *in vivo*. To further understand the impact of this modulation in the early immune events, we resorted to infecting WT and IL-1R$^{-/-}$ mice with either clinical isolate.

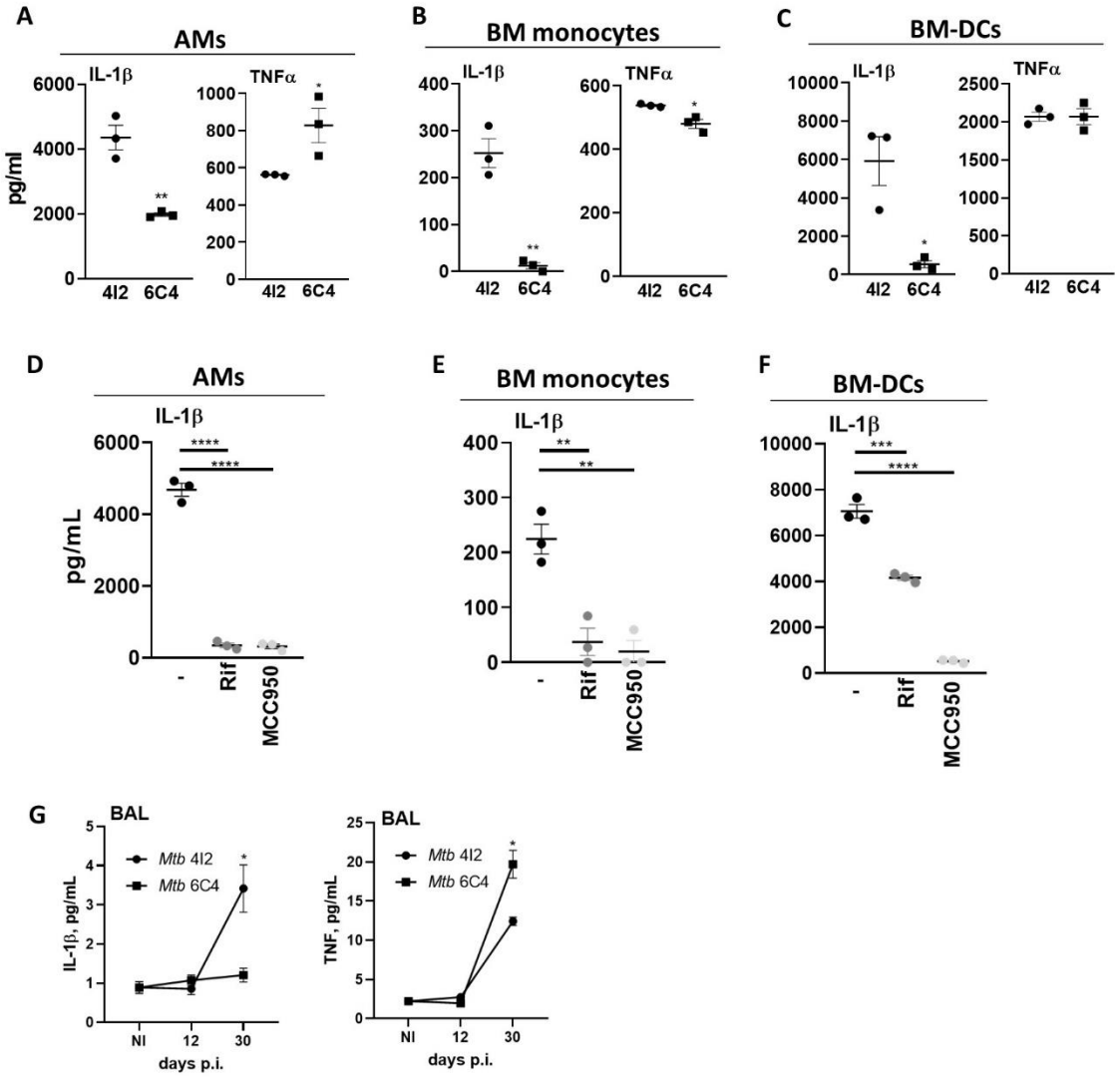


Figure 1. Modulation of IL-1β induction by *M. tuberculosis* clinical isolates 4I2 and 6C4 across different myeloid cells *in vitro* and during *in vivo* infection.

(A, B, C) Quantification of IL-1β and TNFα 24h post-infection of alveolar macrophages (AMs) **(A)**, bone marrow (BM) monocytes **(B)** and BM derived dendritic cells (BM-DCs) **(C)** with *M. tuberculosis* (*Mtb*) isolates 4I2 and 6C4. **(D, E, F)** Quantification of IL-1β induced by infections with isolate *M. tuberculosis* 4I2 in AMs **(D)**, BM monocytes **(E)** and BM-DCs **(F)** in standard infection conditions (-), infections with bacteria previously treated with rifampicin (Rif) and infections done in the presence of the inflammasome inhibitor (MCC950). **(G)** Quantification of IL-1β and TNFα at the indicated time points in the BAL of mice infected by aerosol with high doses of *M. tuberculosis* isolates 4I2 and 6C4. Represented are Mean±SEM. Each dot represents cells in an individual culture well, except for **(G)**, where 3 animals are pooled together. Data represents individual experiments. Statistical differences between groups are indicated as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; and **** $p \leq 0.0001$.

Lack of IL-1R signaling has a differential impact on infections with *M. tuberculosis* isolates 4I2 and 6C4

At day 12 post infection, IL-1R$^{-/-}$ mice infected with isolates 4I2 or 6C4 displayed no differences in bacterial load in the lung (Fig. 2A) and BAL (Fig. 2B) for infections with either isolate, when compared to WT mice. However, at day 30 post infection, we detected an increase in bacterial loads in the lung (Fig. 2A) and BAL (Fig. 2B) of IL-1R$^{-/-}$ mice when compared to WT mice. This increase in bacterial load was much more pronounced for isolate 4I2 than for isolate 6C4, which hints at a more protective role for IL-1R signaling in the case of isolate 4I2 infections. Despite this increase, no differences were seen in terms of mouse weight variation during the course of infection (Supp. Fig. 2A, 2B). The lack of IL-1R signaling did not increase pathological damage to the lung on day 30 post-infection with either isolate (Fig. 2C), as also quantitively shown by similar percentages of lung lesion (Fig. 2D), number of lesions (Fig. 2E) or lesion score (Fig. 2F). Of note, the histopathological analysis performed for infections with either isolate showed a higher amount of histopathological damage in mice infected with isolate 6C4 than with isolate 4I2 (Fig. 2C, 2D, 2E, 2F), which is in concordance with the higher clinical severity of the patient infected with isolate 6C4 than the patient infected with isolate 4I2. Infections of WT or IL1R$^{-/-}$ mice with low doses of either isolate of *M. tuberculosis* largely reflected the findings seen above, with a similar pattern for a higher increase in lung CFUs for infections with isolate 4I2 30 days post-infection over the time course of the experiment (Supp. Fig. 2C) than 6C4 (Supp. Fig. 2D) and no differences in terms of weight variation (Supp. Fig. 2E, 2F). Therefore, the higher protection afforded by a functional IL-1R to infections with *M. tuberculosis* isolate 4I2 was dose independent. The effects of lack of IL-1R signaling were also observed when infecting mice with high doses of the hypervirulent HN878 strain, with IL1R$^{-/-}$ mice displaying increased lung bacterial load at 30 days post-infection (Supp. Fig. 2H) and more pronounced weight loss (Supp. Fig. 2G) when compared to WT mice. Nonetheless, the increase in CFUs was still less than for infections with isolate 4I2, suggesting that IL-1R signaling is likely most important in *M. tuberculosis* isolates inducing high amounts of IL-1β.

Dynamics of immune cell recruitments to lung of IL-1R deficient mice

We then looked at the cellular composition of myeloid cells in the lung at day 12 (Fig. 2G) and day 30 (Fig. 2H) post infection in both infections resorting to flow cytometry (Supp. Fig. 2I, for the gating strategy). For infections with isolate 4I2, there was a higher percentage of myeloid cells in WT mice when compared to IL-1R$^{-/-}$ mice, at day 12 post infection (Fig. 2G). The difference was due to a lower percentage of both AMs and eosinophils in IL-1R$^{-/-}$

mice. In infections with isolate 6C4, the lung myeloid cellular composition was identical at the same time point (Fig. 2G). At day 30 post infection, there was still a higher percentage of myeloid cells in 4I2-infected WT mice when compared to IL-1R$^{-/-}$ mice, mostly explained by the lower percentage of AMs, neutrophils and CD11b+ DCs in IL-1R$^{-/-}$ mice (Fig. 2H). In the case of infections with isolate 6C4, the percentage of myeloid cells remained similar at day 30 post infection, with lower percentage of neutrophils and higher percentage of CD11b+ DCs in IL-1R$^{-/-}$ mice (Fig. 2H). Regarding absolute numbers of myeloid cells in the lung, the main difference observed in infections with isolate 4I2 was the lower number of AMs and neutrophils in IL-1R$^{-/-}$ mice as compared to WT ones at day 30 post infection (Supp. Fig. 2J). As for infections with isolate 6C4, in addition to the lower number of AMs and neutrophils, there was also a decreased number of lung eosinophils in IL-1R$^{-/-}$ mice as compared to WT mice, at day 30 post infection (Supp. Fig. 2J). No significant differences in cell numbers were found on day 12 post-infection for the various myeloid cell populations analyzed between mice (Supp. Fig. 2J). These results seem to indicate a lower infiltration of specific myeloid immune cells into the lungs of infected IL-1R$^{-/-}$ mice, particularly AMs.

Given the previously described role of IL-1R in AM dissemination into the lung (24, 25), we also compared the myeloid cellular composition in the BAL of WT or IL-1R$^{-/-}$ infected mice. At day 12 post infection, there were similar numbers and percentages of AMs in infections with both isolates when comparing WT and IL-1R$^{-/-}$ mice (Fig. 2I). In fact, most of the cells in the BAL were AMs (>94%), which limits the analysis of the other cellular populations at this time point. At day 30 post infection, other immune cells infiltrated into the BAL (Fig. 2J). In 4I2-infected mice, there was a higher percentage of myeloid cells infiltrating into the BAL of WT mice, with a higher percentage of AMs (30%) when compared to IL-1R$^{-/-}$ mice (14%) (Fig. 2J). In 6C4-infected mice, there was also a higher percentage of myeloid cells infiltrating into the BAL of WT mice, mainly explained by lower neutrophil percentages presented in IL-1R$^{-/-}$ mice (Fig. 2J). Looking at absolute numbers, there was a lower number of AMs, neutrophils and eosinophils when comparing WT and IL-1R$^{-/-}$ mice in infections with either isolate (Supp. Fig. 2K).

Put together, these results indicate that, while a lower infiltration of myeloid immune cells into the lung of IL-1R$^{-/-}$ mice is observed independently of the infecting isolate, the susceptibility of IL-1R$^{-/-}$ mice is higher in the case of infections with the higher IL-1β inducing isolate 4I2. The more pronounced increase in bacterial load over the course of infection, combined with the higher induction of IL-1β both *in vitro* and *in vivo*, suggests a differential protective role of IL-1β. Furthermore, while the number of AMs in the lung (Supp. Fig. 2J) and BAL (Fig. 2J) of WT mice increased over time in infections with isolate 4I2, a decrease is observed in IL-1R$^{-/-}$ mice, which hints at possible alterations in the functioning and/or survival of alveolar macrophages when lacking IL-1R signaling. This observation led us to

further dissect what is the impact of lack of IL-1R signaling in AMs upon infections with isolate 4I2.

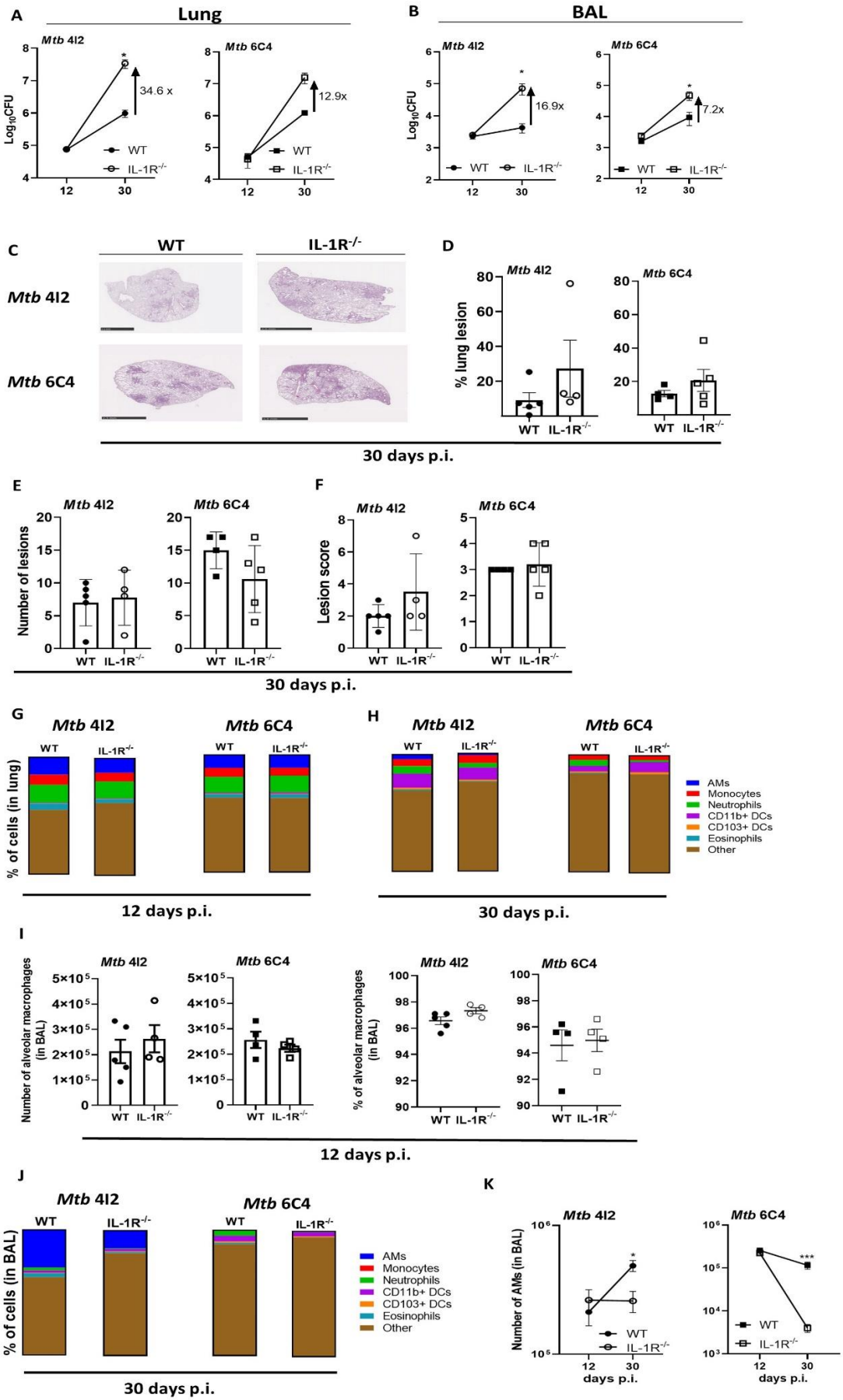

A
Lung
Mtb 4l2
Mtb 6C4
Log10CFU
34.6 x
12.9x
WT
IL-1R-/-
12
30
B
BAL
16.9x
7.2x
C
WT
IL-1R-/-
Mtb 4l2
Mtb 6C4
D
% lung lesion
30 days p.i.
E
Number of lesions
F
Lesion score
30 days p.i.
G
% of cells (in lung)
12 days p.i.
H
AMs
Monocytes
Neutrophils
CD11b+ DCs
CD103+ DCs
Eosinophils
Other
30 days p.i.
I
Number of alveolar macrophages (in BAL)
% of alveolar macrophages (in BAL)
12 days p.i.
J
% of cells (in BAL)
30 days p.i.
K
Number of AMs (in BAL)
days p.i.

Figure 2. IL-1R signaling affords higher protection against infections with *M. tuberculosis* isolate 4I2

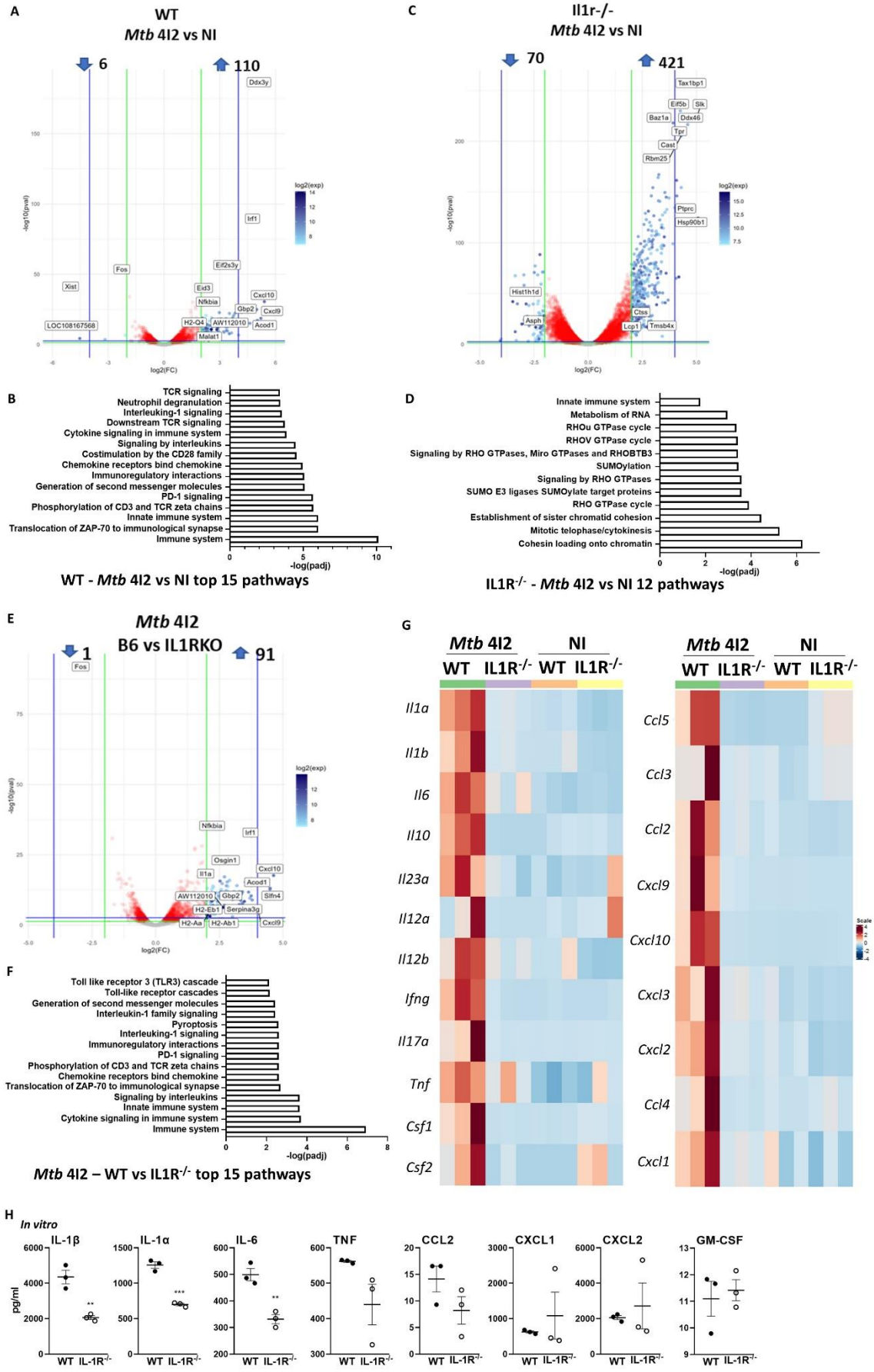
(A, B) Bacterial burden in the lungs **(A)** and broncho-alveolar lavage (BAL) **(B)** of wild type (WT) and IL-1R$^{-/-}$ mice determined 12 and 30 days post-infection with *M. tuberculosis* (*Mtb*) isolates 4I2 and 6C4. **(C, D, E)** Representative histological images of Hematoxylin and Eosin (H&E)-stained lung sections **(C)** from infected mice. Darker areas corresponding to tissue lesions were quantified to obtain the percentage of area of lesion **(D)** and number of legions **(E)** in the lungs. **(F)** Histopathological score attributed to each lesion. **(G, H)** Frequency of cells in the lung of infected mice at day 12 **(G)** and day 30 **(H)** post infection. **(I)** Total number (left) and frequency (right) of alveolar macrophages (AMs) in the BAL on infected mice at day 12 post infection. **(J)** Frequency of cells in the BAL of infected mice at day 30 post infection. **(K)** Dynamics of total number of AMs in the BAL of infected mice at days 12 and 30 post infection. Represented are Mean±SEM. Each dot represents an individual mouse, except for: **(A)** and **(B),** where 8-9 animals are pooled together; and **(K)**, where 5-6 animals are pooled together. Data represents individual experiments, except for **(A)** and **(B)** which are distributed in two independent experiments. Statistical differences between groups are indicated as: * $p \leq 0.05$ and *** $p \leq 0.001$.

Alveolar macrophages lacking the IL-1R present a diminished immune response when infected with clinical isolate 4I2

Transcriptome analysis of BAL cells (>94% AMs) was performed in naïve or *M. tuberculosis* 4I2-infected WT and IL-1R$^{-/-}$ mice. Principal component analysis (PCA) suggested that the main variation of gene expression was explained by the lack or IL-1R (Supp. Fig. 3A). When we analyzed the differentially expressed genes (DEGs; defined as log2 fold change of 2 or above/below and p<0.05) in AMs of naïve and infected WT mice, we observed an upregulation of 110 genes and a downregulation of 6 caused by infection (Fig. 3A). Reactome pathway analysis revealed that these genes were mostly grouped in pathways linked to the immune system activation (Fig. 3B). Of note, IL-1 signaling appears in the top 15 detected pathways, further confirming the IL-1 modulation caused by infection with isolate 4I2 (Fig. 3B). Surprisingly, when comparing gene expression of AMs from naïve and 4I2-infected IL-1R$^{-/-}$ mice, we observed a much higher amount of DEGs, of which 421 were upregulated and 70 downregulated in AMs from infected mice (Fig. 3C). However, these genes mostly belonged to pathways that are not immune related, but rather related to cell cycle (Fig. 3D). These findings suggest that the infection of AMs from IL-1R$^{-/-}$ mice induced transcriptional changes in genes associated with the core functioning of AMs. Next, we compared the impact of the infection with *M. tuberculosis* 4I2 in the transcriptome of WT versus IL-1R$^{-/-}$ AMs and found 91 upregulated and 1 downregulated DEGs in WT cells (Fig. 3E), which were mostly related to pathways of the immune response (Fig. 3F). Thus,

whereas WT AMs responded to infection with a vigorous immune response, those lacking IL-1R mainly upregulated homeostatic pathways. This was further confirmed by interrogating our RNA-Seq dataset for the expression of genes encoding common cytokines and chemokines with relevance to the immune response in TB. While AMs from 4I2-infected WT mice exhibited a strong genetic expression of the tested immune mediators, AMs from 4I2-infected IL-1R$^{-/-}$ mice exhibited a similar immune response to AMs from non-infected animals (Fig. 3G). These findings led us to hypothesize that in the absence of the IL-1R, AMs are hyporesponsive to *M.* tuberculosis. To test this hypothesis, we collected AMs from either WT and IL-1R$^{-/-}$ mice and performed *in vitro* infections with isolate 4I2. The seemingly deficient immune response was corroborated by this experiment, where AMs from WT mice showed a higher production of IL-1β, IL-1α, IL-6 and TNFα than AMs from IL-1R$^{-/-}$ mice (Fig. 3H). These results indicate that AMs lacking IL-1R signaling are less responsive to infection.

The RNA-seq data also suggest that the infection of AMs from IL-1R$^{-/-}$ mice induced transcriptional changes to the core functioning of AMs. Since this was not observed in AMs from WT mice, we next investigated the transcriptional differences existing in AMs from naïve WT versus naïve IL-1R$^{-/-}$ mice. As compared to WT AMs, we found 28 genes upregulated and 270 genes downregulated in cells from IL-1R$^{-/-}$ mice (Supp. Fig. 3B). AMs lacking IL-1R presented downregulation of genes related to AM identity and self-renewal (Supp. Fig. 3C), which hints at a crucial role from IL-1R signaling in the maintenance and activity of AMs. To test this, we next verified if there were any disturbances in the homeostasis of AMs in the BAL of WT and IL-1R$^{-/-}$ regarding their frequency, total number and surface markers. While the frequency and number of AMs in the BAL of WT and IL-1R$^{-/-}$ mice remained similar (Supp. Fig. 3D), flow cytometry analysis revealed a decrease in fluorescence intensity for AM surface markers CD64 and SiglecF, but not MerTK (Supp. Fig. 3E), which further supports that IL-1R signaling in AMs might be needed for their normal functioning.


A
WT
Mtb 4I2 vs NI
6
110
Ddx3y
Irf1
Fos
Eif2s3y
Xist
Eid3
Nfkbia
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Gbp2
Cxcl9
LOC108167568
H2-Q4
AW112010
Acod1
Malat1
log2(FC)
-log10(pval)
log2(exp)
B
TCR signaling
Neutrophil degranulation
Interleuking-1 signaling
Downstream TCR signaling
Cytokine signaling in immune system
Signaling by interleukins
Costimulation by the CD28 family
Chemokine receptors bind chemokine
Immunoregulatory interactions
Generation of second messenger molecules
PD-1 signaling
Phosphorylation of CD3 and TCR zeta chains
Innate immune system
Translocation of ZAP-70 to immunological synapse
Immune system
-log(padj)
WT - Mtb 4I2 vs NI top 15 pathways
C
Il1r-/-
Mtb 4I2 vs NI
70
421
Tax1bp1
Eif5b
Slk
Baz1a
Ddx46
Tpr
Cast
Rbm25
Ptprc
Hsp90b1
Hist1h1d
Asph
Ctss
Lcp1
Tmsb4x
log2(FC)
-log10(pval)
log2(exp)
D
Innate immune system
Metabolism of RNA
RHOu GTPase cycle
RHOV GTPase cycle
Signaling by RHO GTPases, Miro GTPases and RHOBTB3
SUMOylation
Signaling by RHO GTPases
SUMO E3 ligases SUMOylate target proteins
RHO GTPase cycle
Establishment of sister chromatid cohesion
Mitotic telophase/cytokinesis
Cohesin loading onto chromatin
-log(padj)
IL1R-/- - Mtb 4I2 vs NI 12 pathways
E
Mtb 4I2
B6 vs IL1RKO
1
91
Fos
Nfkbia
Irf1
Osgin1
Il1a
Cxcl10
Acod1
AW112010
Gbp2
Slfn4
H2-Eb1
Serpina3g
H2-Aa
H2-Ab1
Cxcl9
log2(FC)
-log10(pval)
log2(exp)
F
Toll like receptor 3 (TLR3) cascade
Toll-like receptor cascades
Generation of second messenger molecules
Interleukin-1 family signaling
Pyroptosis
Interleuking-1 signaling
Immunoregulatory interactions
PD-1 signaling
Phosphorylation of CD3 and TCR zeta chains
Chemokine receptors bind chemokine
Translocation of ZAP-70 to immunological synapse
Signaling by interleukins
Innate immune system
Cytokine signaling in immune system
Immune system
-log(padj)
Mtb 4I2 – WT vs IL1R-/- top 15 pathways
G
Mtb 4I2
NI
WT
IL1R-/-
Il1a
Il1b
Il6
Il10
Il23a
Il12a
Il12b
Ifng
Il17a
Tnf
Csf1
Csf2
Ccl5
Ccl3
Ccl2
Cxcl9
Cxcl10
Cxcl3
Cxcl2
Ccl4
Cxcl1
Scale
H
In vitro
IL-1β
IL-1α
IL-6
TNF
CCL2
CXCL1
CXCL2
GM-CSF
pg/ml
WT
IL-1R-/-

Figure 3. Lack of IL-1R signaling affects the homeostasis of AMs with consequent deficiency in their immune response.

(A) Differentially expressed genes (DEGs) of alveolar macrophages (AMs) from *M. tuberculosis* (*Mtb*) 4I2-infected and non-infected wild type (WT) mice. **(B)** Pathway analysis of the DEGs of AMs from 4I2-infected and non-infected WT mice. **(C)** DEGs of AMs from 4I2-infected and non-infected IL-1R deficient (IL-1R$^{-/-}$) mice. **(D)** Pathway analysis of the DEGs of AMs from 4I2-infected and non-infected IL-1R$^{-/-}$ mice. **(E)** DEGs of AMs from 4I2-infected WT and IL-1R$^{-/-}$ mice. **(F)** Pathway analysis of the DEGs of AMs from 4I2-infected WT and IL-1R$^{-/-}$ mice. **(G)** Heatmap representing the relative expression of cytokine and chemokine genes by either infected and non-infected WT and IL-1R$^{-/-}$ mice. **(H)** Quantification of cytokines produced by AMs from WT and IL-1R$^{-/-}$ mice infected with isolate 4I2 *in vitro*. Represented are Mean±SEM. Each dot represents cells in an individual culture well. Data represents individual experiments. Statistical differences between groups are indicated as: ** $p \leq 0.01$ and *** $p \leq 0.001$.

Lack of IL-1R signaling restricted to myeloid cells leads to a drastic increase in disease severity

Since AMs are the first responders to *M. tuberculosis* infections, a deficient IL-1R signaling may lead to a deficient immune response from the start, with may have implications for the subsequent steps of the immune response. Using a mouse model that lacks IL-1R signaling is crucial to determine the importance of IL-1 in TB; however, these mice constitutively lack IL-1R signaling, meaning that cells from the adaptive immune response may also be hampered in their normal functioning. As we wanted to understand the impact of IL-1R signaling when it is abrogated only in the early immune events, we resorted to use mouse models of cell-restricted IL-1R signaling deficiency in myeloid cells, as well as in non-immune compartments that may impact the early immune response.

Mice with myeloid-specific deficiency in IL-1R signaling (mIL-1R$^{-/-}$) (Supp. Fig. 4A) are more susceptible to infection with isolate 4I2 when compared to their control group (mIL-1R$^{+/+}$). There was an increase in bacterial loads in the lungs (Fig. 4A) and BAL (Fig. 4B) of mIL-1R$^{-/-}$ mice when compared to controls, which, in contrast with the previous results with constitutive IL-1R deficient mice, was already noticeable at day 12 post infection and continued up to 30 days post infection. mIL-1R$^{-/-}$ mice did not increase in weight as much as mIL-1R$^{+/+}$ mice over the course of infection and started losing weight towards day 30 post infection (Fig. 4C). mIL-1R$^{-/-}$ mice also exhibited extensive lung pathology (Fig. 4D), presenting higher percentage of lung lesion (Fig. 4E), higher number of legions (Fig. 4F) and a more severe disease score (Fig. 4G) than mIL-1R$^{+/+}$ mice. These results indicate that

myeloid IL-1R signaling is essential for the early immune response in infections with isolate 4I2.

Infections with isolate 6C4 suggested a specific role for myeloid IL-1R in infections with isolate 4I2, likely linked to the differential induction of IL-1β by isolates 4I2 and 6C4. No differences were found after infection of myeloid competent or deficient IL-1R mice with isolate 6C4 in terms of bacterial load in the lung (Supp. Fig. 4B) or BAL (Supp. Fig. 4C) at either time point of infection, although mIL-1R$^{-/-}$ mice gained less weight when compared to mIL-1R$^{+/+}$ mice (Supp. Fig. 4D). In keeping with similar bacterial burdens in the lung infected with isolate 6C4, the lung pathology was also alike (Supp. Fig. 4E), with similar percentage of lung lesion (Supp. Fig. 4F), number of lesions (Supp. Fig. 4G) and lesion score (Supp. Fig. 4H) between mIL-1R$^{-/-}$ and mIL-1R$^{+/+}$ mice.

Using the same gating strategy as before (Supp Fig. 2I), we observed a decreased percentage of AMs but an increased percentage of monocytes and neutrophils in the lungs of mIL-1R$^{-/-}$ mice when compared to controls, already at day 12 post infection (Fig. 4H). The pattern of decreased cellular percentage appeared to be maintained in AMs up to 30 days post infection, but there appeared to be a higher percentage of CD11b+ DCs at the same time point in mIL-1R$^{-/-}$ mice than mIL-1R$^{+/+}$ mice (Fig. 4H). In the BAL, at day 12 post infection, the majority of cells were AMs, with similar numbers and percentage between mIL-1R$^{-/-}$ and mIL-1R$^{+/+}$ mice (Fig. 4I). At day 30 post infection, the percentage of AMs in the BAL was reduced for mIL-1R$^{-/-}$ mice, but there appeared to be a higher percentage of neutrophils and CD11b+ DCs entering the alveolar space (Fig. 4J). When we accounted for total numbers of cells, there seemed to be a bigger infiltration of immune cells into the lung and BAL of mIL-1R$^{-/-}$ mice when compared to mIL-1R$^{+/+}$ mice (Supp. Fig. 4I).

Curiously, the decrease in AMs over time previously observed for IL-1R$^{-/-}$ did not occur as drastically in the lung (Supp. Fig. 4I) or BAL (Fig. 4K) of mIL-1R$^{-/-}$ mice, hinting that IL-1R signaling from other cellular compartments may be key in maintaining the homeostasis of AMs. Indeed, the expression of the surface markers CD64 and siglecF appeared similar between AMs from mIL-1R$^{-/-}$ and mIL-1R$^{+/+}$ mice (Supp. Fig. J).

Considering the extensive lung pathology and higher immune cell infiltration in mIL-1R$^{-/-}$ mice infected with isolate 4I2, we speculated that there could be a more inflammatory environment in the lungs of these mice when compared to mIL-1R$^{+/+}$ mice. Cytokine measurement in the BAL confirmed this, where a generalized increase of cytokines were quantified in the BAL of mIL-1R$^{-/-}$ mice, particularly IL-1β, TNFα and IL-6 (Fig. 4L). This was further confirmed by the increased expression of TNFα, IL-1β and IL-17 in the lung, which further supported an increased inflammatory environment in mice lacking myeloid IL-1R signaling when infected with isolate 4I2.

Non-hematopoietic IL-1R signaling is dispensable for protection against infections with isolate 4I2

Finally, taking into account previous reports of the IL-1R signaling-dependent control of infection also provided by cells of non-hematopoietic origin (23), we performed infections with isolate 4I2 in mice lacking IL-1R in non-hematopoietic compartments. Both type II AECs and endothelial cells are needed for maintaining lung homeostasis and proper functioning of AMs (26). Thus, we wanted to understand if the lack of IL-1R in these specific compartments could lead to an increase of disease severity. We observed that lack of IL-1R in endothelial cells (Supp. Fig. 4K) did not result in changes in bacterial loads over time (Supp. Fig. 4L). Similarly, neither did the lack of IL-1R in type II AECs (Supp. Fig. 4M) lead to changes in CFUs (Supp. Fig. 4N), which indicates that the contribution of IL-1R signaling of these non-hematopoietic compartments is negligible in infections with isolate 4I2. Nonetheless, it will be interesting in the future to understand if the lack of IL-1R signaling in these compartments would affect the homeostasis of AMs.

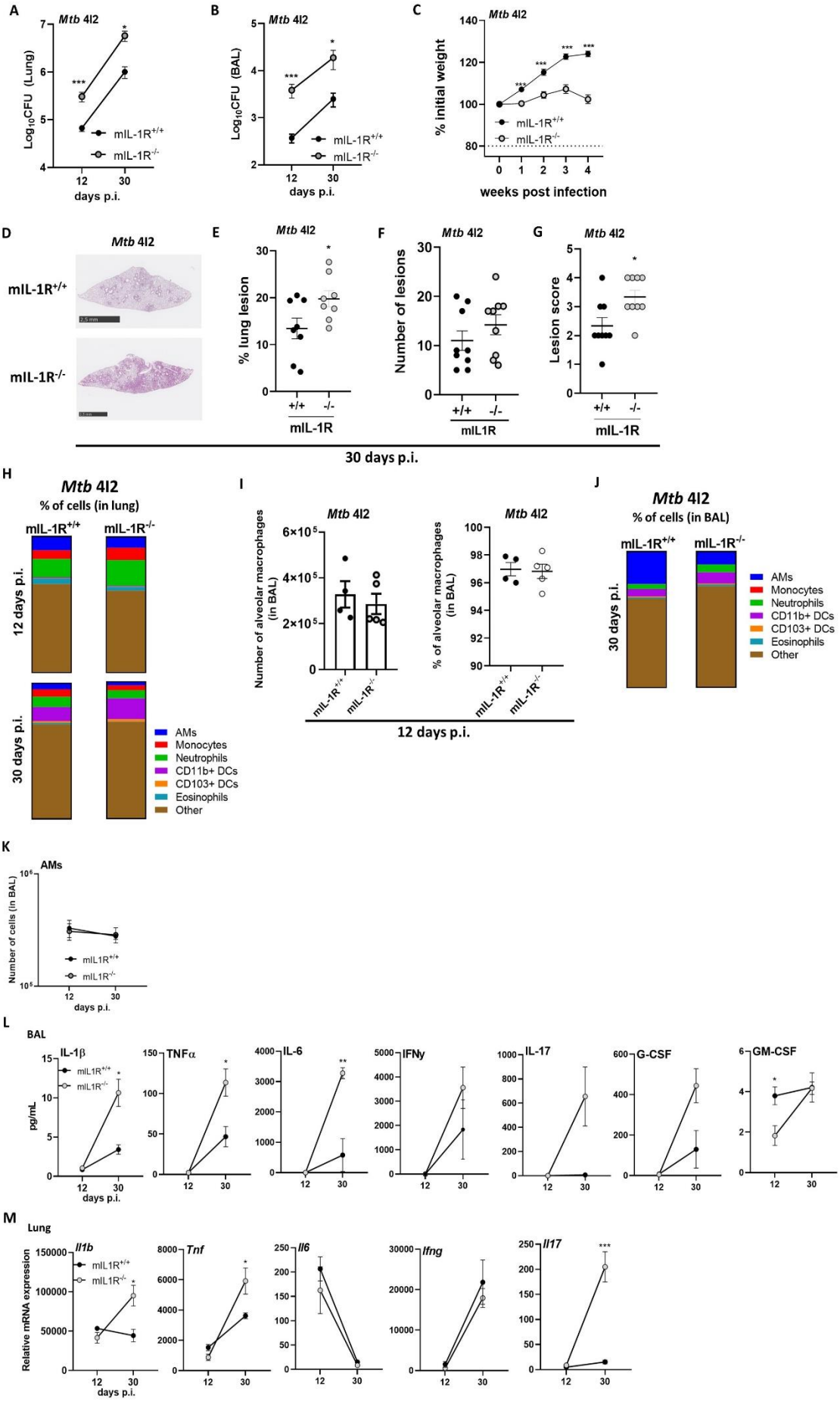
A
Mtb 4l2
Log10CFU (Lung)
mIL-1R+/+
mIL-1R-/-
days p.i.
B
Mtb 4l2
Log10CFU (BAL)
C
Mtb 4l2
% initial weight
weeks post infection
D
Mtb 4l2
mIL-1R+/+
mIL-1R-/-
E
Mtb 4l2
% lung lesion
mIL-1R
F
Mtb 4l2
Number of lesions
mIL1R
G
Mtb 4l2
Lesion score
mIL-1R
30 days p.i.
H
Mtb 4l2
% of cells (in lung)
12 days p.i.
30 days p.i.
AMs
Monocytes
Neutrophils
CD11b+ DCs
CD103+ DCs
Eosinophils
Other
I
Mtb 4l2
Number of alveolar macrophages (in BAL)
% of alveolar macrophages (in BAL)
12 days p.i.
J
Mtb 4l2
% of cells (in BAL)
30 days p.i.
K
AMs
Number of cells (in BAL)
mIL1R+/+
mIL1R-/-
L
BAL
IL-1β
TNFα
IL-6
IFNγ
IL-17
G-CSF
GM-CSF
pg/mL
M
Lung
Il1b
Tnf
Il6
Ifng
Il17
Relative mRNA expression

Figure 4. Lack of myeloid IL-1R signaling drastically increases the severity of infections with isolate 4I2

(A, B) Bacterial burden in the lungs **(A)** and BAL **(B)** of mIL-1R$^{-/-}$ and mIL-1R$^{-/-}$ mice determined 12 and 30 days post-infection with *M. tuberculosis* (*Mtb*) isolate 4I2. **(C)** Weight variation of mIL-1R$^{+/+}$ and mIL-1R$^{-/-}$ mice during the course of infection with *M. tuberculosis* isolate 4I2. **(D, E, F)** Representative histological images of Hematoxylin and Eosin (H&E)-stained lung sections **(D)** from infected mice. Darker areas corresponding to tissue lesions were quantified to obtain the percentage of area of lesion **(E)** and number of legions **(F)** in the lungs. **(G)** Histopathological score attributed to each lesion. **(H)** Frequency of cells in the lung of infected mice at days 12 and 30 post infection. **(I)** Total number (left) and frequency (right) of alveolar macrophages (AMs) in the broncho-alveolar lavage (BAL) on infected mice at day 12 post infection. **(J)** Frequency of cells in the BAL of infected mice at day 30 post infection. **(K)** Dynamics of total number of AMs in the BAL of infected mice at days 12 and 30 post infection. **(L)** Quantification of cytokines produced in the BAL of infected mice at days 12 and 30 post infection. **(M)** Relative mRNA expression in the lungs of infected mice at days 12 and 30 post infection. Represented are Mean±SEM. Each dot represents an individual mouse, except for: **(A)** where 10 animals from 2 independent experiments are pooled together; **(B)** and **(C)**, where 8-9 animals from 2 independent experiments are pooled together**; (K)**, where 5 animals are pooled together; **(L)** where 3 animals are pooled together; and **(M)**, where 5 animals are pooled together. Data represents individual experiments, except for **(A)**, **(B)** and **(C)**. Statistical differences between groups are indicated as: * $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$.

Discussion

With this study, we have demonstrated that the differential induction of IL-1β by *M. tuberculosis* clinical isolates can shape the immune response to infection, and that a functioning IL-1R signaling is crucial for the initial response to TB.

It is well established that mycobacteria are able to modulate the host immune response by manipulating the secretion of cytokines by host cells (27). Maintaining a balance in IL-1 responses is crucial for the development of an adequate immune response to TB (7). Indeed, while lack of IL-1R signaling leads to TB exacerbation in the mouse model (17-23), it is also clear that it can be detrimental by causing excessive immune-pathology in the lungs (16, 28, 29). Thus, it is tempting to speculate that the initial manipulation of IL-1 responses by different bacteria may shape the subsequent events of the immune response. The clinical isolates 4I2 and 6C4 greatly differ in their ability to induce IL-1β in a variety of human (6) and mouse cells *in vitro*, and this pattern also appears to apply for mouse infections. Isolate 4I2, which was representative of mild cases of TB, consistently induced high levels of IL-1β in infected cells or mice. One could hypothesize that the initial induction of IL-1β by this isolate could be conferring increased protection to the host. That seems to

be the case, as the blocking of IL-1R signaling led to a severe increase in bacterial burden over the course of infection. Although an increment in bacterial burden was also observed for infections with isolate 6C4, it was much lower than for isolate 4I2. This is not surprising, as the lower induction of IL-1β by isolate 6C4 hints that the host immune response is likely to depend less on IL-1R signaling. The ability for isolate 6C4 to evade cytosolic surveillance pathways (6) is probably linked to this differential induction of IL-1β, and a lesser activation of the immune response. It is thus tempting to speculate that we could improve the outcome of infections with isolate 6C4 by increasing the effects of IL-1R signaling in the host response. Mechanisms have been described on how IL-1 can impact the immune response to TB. For instance, IL-1 has been linked to the production of PGE2, which favors apoptotic cell death. Low PGE2 production leads to necrotic cell death of macrophages, which increases dissemination of *M. tuberculosis* (21). Furthermore, production of IL-1β after infections with *M. tuberculosis* has been linked to metabolic shifts in macrophages towards glycolysis, which limits bacterial survival (30), and *M. tuberculosis* was shown to prevent this glycolytic shift to enhance bacterial replication (31).

Despite being the first responders to infection, AMs are poorer at containing bacterial growth than interstitial or recruited macrophages, which is linked to a compromised immune metabolism (32-34). In human cases of TB, AMs from patients display an increase in the expression of pro-inflammatory genes (35). Our study confirms that, without IL-1R signaling, AMs from 4I2-infected mice are transcriptionally impaired in the expression of genes related to pathways of the immune response. Not only that, but even non-infected AMs already present transcriptional differences in pathways related to cell cycle, self-renewal and maintenance, indicating that a functioning IL-1R signaling is crucial for the homeostasis of AMs in their steady state. It has been demonstrated that the production of GM-CSF by type II AECS is critical for the development and maintenance of AMs (36), and the production of IL-1 by AMs infected with *Legionella pneumophila* induced the release of GM-CSF by type II AECs (37). Thus, the lack of IL-1R could be impairing the production of GM-CSF by type II AECs, which in turn may result in perturbances in the homeostasis of AMs.

Furthermore, the expression of several cytokine and chemokine genes is severely hampered in 4I2-infected AMs from IL-1R$^{-/-}$ mice, and the lower production of immune mediators *in vitro* further confirms that lack of IL-1R signaling leads to an undermined initial immune response, that can further impact the onset of the adaptive immune response. While it has been reported that AM migration into the lungs is IL-1 dependent and affects bacterial migration into the lung with consequences for T cell priming (25), here we show that AM maturation itself, and consequently their proper immune activation is already hampered by the lack of IL-1R signaling. Of note, in this study, we performed transcriptional analysis of AMs from infected mice, but we did not distinguish the transcriptional profiles

between AMs that had internalized the bacteria and those that did not. It would be interesting to see what differences could occur between both sets of AMs, as it has been previously shown that there are transcriptional differences in AMs that contain *M. tuberculosis* and bystander AMs, with the latter invoking a more pronounced immune response (33).

While the use of constitutive IL-1R deficient mice is useful for studying the effects of lack of IL-1R signaling in the early immune response, we may have other confounding effects present by the generalized lack of IL-1R signaling, as presented above. Thus, using myeloid-specific gene deletion could be useful for exploring the actual initial impact of the lack of IL-1R signaling in the innate immune response (38). Myeloid abrogation of IL-1R signaling led to an evident increase of severity as early as 12 days post infection, which was surprising as this effect surpassed that observed for the full IL-1R abrogation. The increased bacterial load was also evident when compared to controls at day 30 post infection, and there was an increase in lung pathology for mIL-1R$^{-/-}$ mice. This is in contrast to infections of the constitutive IL-1R$^{-/-}$ mice, where bacterial load was only increased at day 30 post infection. This increase in pathology may be linked to the increased infiltration of immune cells, particularly neutrophils, that are known to be detrimental in lung pathology in TB (39, 40). Additionally, analysis of cytokines produced in the BAL and higher expression of cytokine genes in the lung indicate that an excessive immune response may be occurring in mIL-1R$^{-/-}$ mice, and an exacerbated immune response is likely to result in more severe lung pathology in TB (41). These two hypotheses may not be mutually exclusive, as indicated by our findings. Curiously, it seems that while disease outcome is worse whether the lack of IL-1 signaling is constitutive or limited to myeloid cells, the former seems to be linked to an initial under activation of the immune response that likely fails in controlling bacterial growth, while the latter appears to be due to excessive immune response that leads to pathological lung damage and also to lower ability to control bacterial replication. Indeed, this is in concordance with the notion that a balance between pro- and anti-inflammatory responses is crucial for resolution of infection (42).

While a role for IL-1R signaling in the non-hematopoietic compartment has been shown crucial in other lung infections (43, 44), there is conflicting evidence for the role of non-hematopoietic signaling in TB protection. While it is suggested that IL-1R signaling is most important in immune cells (22), another study implicates that IL-1R signaling in the stromal compartment may confer some degree of resistance to infection (23). Curiously, when using IL-1R deficient mice specifically in hematopoietic cells, they did not show increased susceptibility to disease (23), which hints at a possible role for the non-hematopoietic compartment, at least for the H37Rv strain used in that study. Here, we verified that IL-1R signaling in either endothelial or type II AECs did not confer protection to infections with

isolate 4I2. However, the mouse models used in this study that lacked IL-1R in either endothelial cells or type II AECs were both inducible Cre mouse models. Thus, it is possible that the outcome of infection could be more variable in each mouse, as the induction of the phenotype could lead to variability in the deletion of IL-1R that would reflect in different outcomes of disease. Furthermore, it would be interesting to understand if the lack of IL-1R in type 2 AECs would affect the maturation of AMs, seeing that the activity of type II AECs are crucial for the maturation of AMs (45). Therefore, these results do not necessarily mean that non-hematopoietic IL-1R signaling is irrelevant to the immune response in TB. Another interesting observation is that, while hematopoietic deletion of IL-1R signaling was reported to not result in increased bacterial growth (23), we show that myeloid-restricted ablation of IL-1R signaling greatly increases bacterial load early on and even leads to exacerbated lung pathology in infections with isolate 4I2. This further hints at the crucial role for IL-1R signaling, particularly in the early events of the immune response to TB. However, this role may be more crucial for isolates that induce high amounts of IL-1β, such as isolate 4I2. All in all, this study highlights the necessity of taking into account the differential modulation of IL-1 induction by different clinical isolates and how that will impact the initial response to TB. We have further confirmed that an adequate IL-1R signaling is crucial for the host defense in TB, where it can lead to either an insufficient or excessive immune response, both with detrimental outcomes in *M. tuberculosis* infections.

Material and methods

Animal housing

C57BL/6 (B6) WT mice were bred at the i3S Animal House Facility. B6.IL-1R deficient (IL-1R$^{-/-}$), B6.IL-1R^{flox}, B6.LyzM^{Cre} and B6.Sftpc$^{Cre/ERT2}$ mice were bought from the Jackson Laboratory (Bar Harbor, ME, USA). B6.Cdh5$^{Cre/ERT2}$ mice were kindly provided by Ralf Adams, through Cláudio Franco. B6.IL-1R^{flox} mice were crossed with B6.LyzM^{Cre} mice to generate B6.IL-1R^{flox}.LyzM^{Cre+} (mIL-1R$^{-/-}$) and B6.IL-1R^{flox}.LyzM^{Cre-} (mIL-1R$^{+/+}$) mice. B6.IL-1R^{flox} mice were crossed with B6.Cdh5$^{Cre/ERT2}$ mice to generate B6.IL-1R^{flox}.Cdh5$^{Cre+/ERT2}$ (Cdh5IL-1R$^{-/-}$) and B6.IL-1R^{flox}.Cdh5$^{Cre-ERT2}$ (Cdh5IL-1R$^{+/+}$) mice. B6.IL-1R^{flox} mice were crossed with B6.Sftpc$^{Cre/ERT2}$ mice to generate B6.IL-1R^{flox}.Sftpc$^{Cre+/ERT2}$ (SftpcIL-1R$^{-/-}$) and B6.IL-1R^{flox}.Sftpc$^{Cre-/ERT2}$ (SftpcIL-1R$^{+/+}$) mice. All mice were housed at the i3S Animal House Facility, and infected under ABSL3 conditions. Mice from both sexes were used, ranging between 8 and 12 weeks of age at the time of infection. Animals were kept under controlled

humidity (45–65%), temperature (20–24°C) and light cycle (12h light/dark) in specific-pathogen free conditions. Water and food were provided *ad libitum*.

Validation of cell-restricted IL-1R$^{-/-}$ mice

The phenotype for Cdh5IL-1R and SftpcIL-1R mice was induced by administering 100 µl of tamoxifen to each animal, dissolved in corn oil at 20 mg/ml, for 5 consecutive days. For Cdh5IL-1R mice, animal lungs were retrieved and processed as previously described (39, 46). For SftpcIL-1R mice, lungs were processed as described in (47). Dead cells were excluded from the analyses using ZombieNIR (Cat. #423106, Biolegend) viability dye. Cells were purified through a BD FACSAria II. Markers used to select cells to verify site-specific deletion of IL-1R were: CD45$^-$CD31$^+$ for the endothelial compartment; CD45$^-$ EPCAM$^+$Podoplanin$^-$ for the alveolar epithelial cell (AEC) type II compartment; CD45$^+$ for the immune compartment. RNA from these cells was extracted using the RNeasy Mini Kit (Cat. #74104, Qiagen) and converted to cDNA using a ProtoScript® II First Strand cDNA Synthesis Kit (Cat. #E6560L, NE Biolabs). The deletion of *Il1r* was confirmed using semi-quantitative PCR with SYBR green (Cat. #1725120, Bio-Rad). The intron spanning oligonucleotides specific for *IL1R1* used are in Supplementary Table 2. Normalization of target gene abundance was performed using *Ubiquitin* as the reference gene. Melting and standard curves and RQ values were determined for each gene.

Bacteria growth and quantification

M. tuberculosis clinical isolates 4I2 and 6C4, as well as the hypervirulent HN878 isolate, were grown and stored as described previously (39, 46). Viable bacteria were determined by serial dilution and CFU enumeration after 21 days of incubation at 37°C in 7H11 agar plates (Cat. #212203, BD Biosciences). Bacterial quantification in lungs and broncho-alveolar lavage (BAL) was performed using the same procedure.

In vivo infections

Aerosol infection was performed using an inhalation exposure system (Glas-Col) (39, 46). Infection level was determined based on the lung bacterial burden 3 days post-infection. Infections were all performed with high doses (450-1700 CFUs) unless stated otherwise; low dose infections were also performed (<100 CFUs) as indicated in the text. Infected mice were monitored daily for signs of distress and weighed every week. Weight loss of over 20% or lack of responsiveness to physical stimulation were considered humane endpoints, at which point the experiments were terminated. Otherwise, mice were

euthanized at 12 and 30 days post infection by intra-peritoneal injections of pentobarbital, during which efforts were made to minimize suffering.

Organ processing

Organs were excised and processed in aseptic conditions as previously described (39, 46). BAL fluid was collected as described in (48). Lungs were digested using Collagenase type IV (Gibco, Cat. #17104019) followed by physical disruption and filtering in a 70 µm mesh. Single-cell suspensions were used for bacterial burden determination, flow cytometry and RNA analysis. Cytokines were quantified in the BAL supernatant.

Flow cytometry and cell sorting

Mouse lung and BAL cell suspensions were stained for surface antigens (30 min; 4°C) and fixed for 20 min in 4% paraformaldehyde-PBS after erythrocyte lysis. Dead cells were excluded from the analyses using ZombieNIR (Cat. #423106, Biolegend) viability dye. Cells were acquired on a BD Fortessa II or purified through a BD FACSAria II. Data were analyzed using *FlowJo* (version 10.1.r7). All antibodies used are listed in Supplementary Table 1. Gating strategies are shown in Supplementary Figure 2I. Markers used to determine the site-specific deletion of IL-1R were: $CD45^{-}CD31^{+}$ for the endothelial compartment; $CD45^{-}EPCAM^{+}Podoplanin^{-}$ for the AEC type II compartment; $CD45^{+}$ for the immune compartment.

RNA extraction and qPCR

Total RNA was extracted from mouse lung or target cells using TripleXtractor (Cat. #GB23.0100, GRiSP) and converted to cDNA using a ProtoScript® II First Strand cDNA Synthesis Kit (Cat. #E6560L, NE Biolabs). Gene expression quantification was performed using semi-quantitative PCR with SYBR green (Cat. #1725120, Bio-Rad) and intron spanning oligonucleotides specific for the target genes. Normalization of target gene abundance was performed using *Ubiquitin* as the reference gene. Melting and standard curves and RQ values were determined for each gene. Sequences of the oligonucleotides used are in Supplementary Table 2.

BAL cells transcriptome analysis

Cells were retrieved from the BAL of non-infected mice (Supp. Fig. 3D) and infected mice (Fig. 2I) at day 12 post infection, where AMs make up for >94% of cells. RNA was extracted as described above. Targeted RNA sequencing was performed by i3S Genomics platform

using Ion AmpliSeq Transcriptome Mouse Gene Expression Kit (Cat. #A36554, Thermofisher Scientific). Subsequent analyses were performed in R (version 4.3.0). RNA expression levels in the count matrix were normalized using the quantile method, and denoising was performed with the noisyR package (version 1.0.0) (49). The bulkanalysR package (version 1.12.0) was used for analysis and visualization of the data (49). Differential expression analysis was performed using edgeR (50). Genes with adjusted p value ≤ 0.05 and log2 fold-change ≤ -2 or ≥ 2 were considered differentially and significantly expressed. The Benjamini-Hochberg correction was applied to adjust the p values for multiple testing. Volcano plots and heatmaps were obtained with EnhancedVolcano (51) and gplots (52), respectively. Functional enrichment (pathway analysis) was performed in the identified DEGs using gProfiler (https://biit.cs.ut.ee/gprofiler/gost), with Benjamini-Hochberg false discovery rate, a threshold of 0.05 and REACTOME (https://reactome.org) as data source.

Histological analysis

Mouse lungs were fixed in 10% buffered formalin and embedded in paraffin. Serial 3-µm sections were used for Hematoxylin and Eosin (H&E) staining and analysis. Morphometric analysis of lung pathology areas in H&E-stained histological sections was performed using the software *Interactive Learning and Segmentation Toolkit* (*Ilastik* version 1.3.3) and *CellProfiler Analyst* (version 3.1.5). Probability maps for the whole lung and lesion area were created in Ilastik and calculated and analyzed using *CellProfiler Analyst* software as in (39). Pathological scoring analysis of H&E-stained histological sections was performed in a blind way, and the histopathological features were scored according to the parameters in Supplementary Table 3.

Isolation and purification of mouse AMs, BM monocytes and BM-DCs

AMs were isolated directly from the BAL fluid of mice. Bone marrow (BM) was collected by centrifugation as described in (53). BM monocytes were purified from BM cellular suspensions using an EasySep™ Mouse Monocyte Isolation Kit (Cat. #19861, STEMCELL Technologies), following the manufacturer's protocol. BM-DCs were differentiated from BM cell suspensions by adding GM-CSF to cultures as in (54). AMs (>94% purity), BM-monocytes (>50% purity) and differentiated BM-DCs (>65% purity) were plated in in 96-well plates (100,000 cells per well) with RPMI medium (Cat. #61870036, Gibco), completed with 1% sodium pyruvate (Cat. #S8636, Sigma-Aldrich), 1% HEPES (Cat. #15630106, Gibco), and 10% FBS (Cat. #10500064, Gibco), 1 day prior to infection.

In vitro infections

Mouse AMs, BM monocytes or BM-DCs were infected with *M. tuberculosis* clinical isolates 4I2 or 6C4 at a MOI of 2 (2 bacteria:1 cell). In specific cases, bacteria were pre-treated with rifampicin or the inflammasome inhibitor MCC950 was added to the culture medium prior to infection. Culture supernatants were collected 24 hours after infection 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 after infection.

Cytokine quantification

Cytokine production in the BAL of infected mice or infected cell cultures was quantified by immunoassay, using commercially available ELISA kits (Cat. #88-7013-88; 88-7324-88, Invitrogen) or using the Mouse High Sensitivity T-Cell 18-Plex Discovery Assay (EveTechnologies, Calgary, Canada).

Statistical analysis

Data were plotted and analyzed using *GraphPad Prism* (version 8.1.0). Outliers were removed using the Grubbs method. Data was checked for normality using the Shapiro-Wilk test. Student's *t*-test was used to compared two groups in data that followed a normal distribution. A non-parametric Mann–Whitney test was used to compare two groups in data that did not follow a normal distribution. Significant differences are as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; and **** $p \leq 0.0001$.

Ethics statement

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

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Author contributions

DS, RG, and MS designed the experiments and analyzed the data; DS and MS wrote the manuscript; DS, RG, AIF, MSC performed the experiments; NSO analyzed the RNAseq data; MS obtained funding.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Supplementary Material

The IL-1R signaling is essential for the initiation of the immune response by alveolar macrophages in *Mycobacterium tuberculosis* infections

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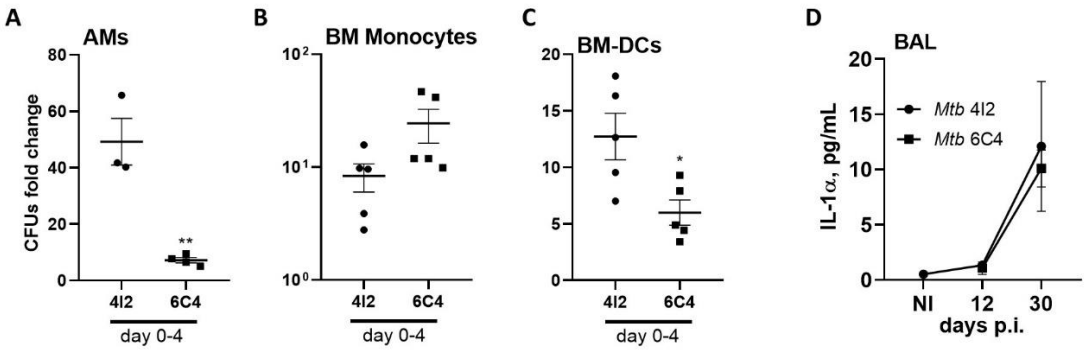
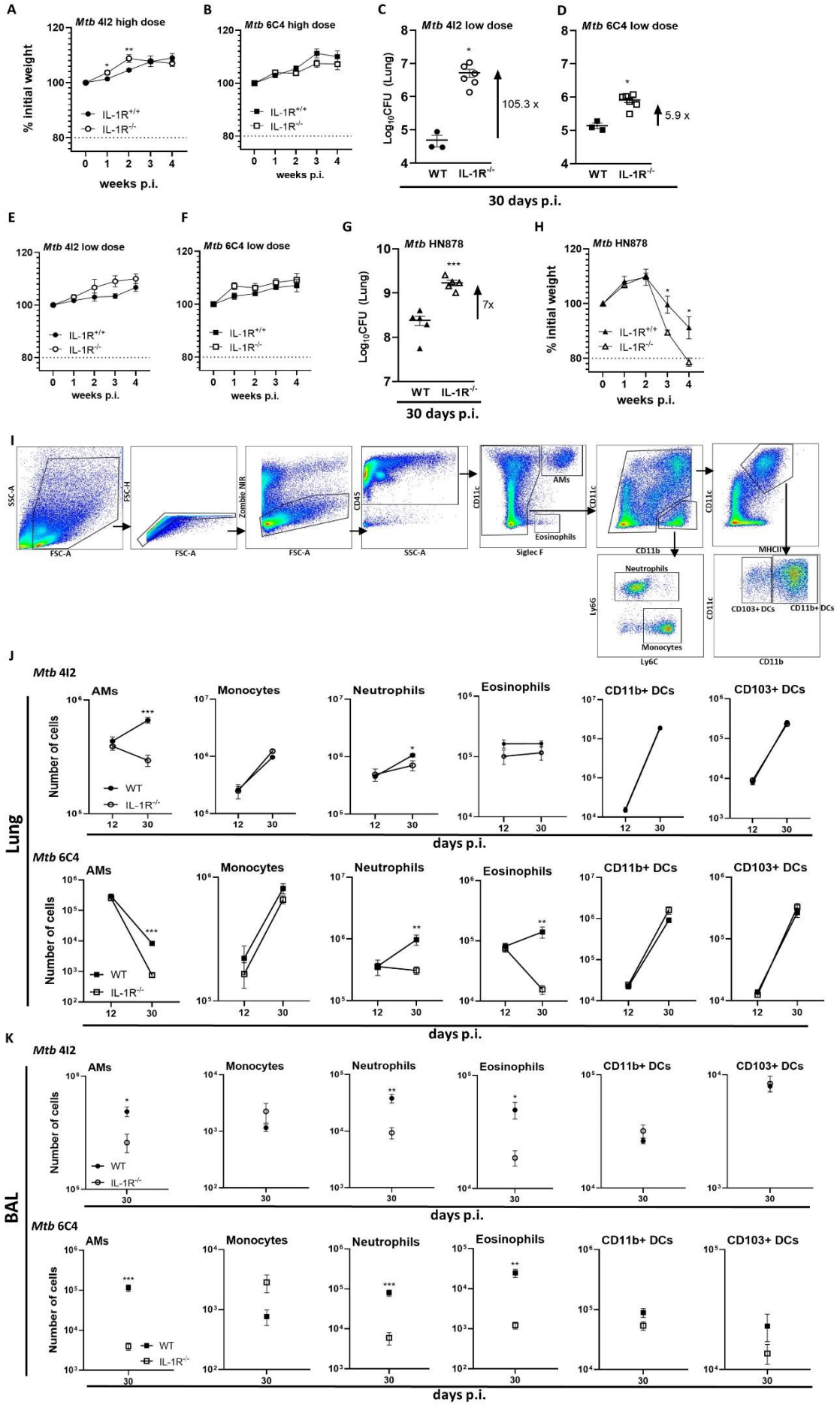


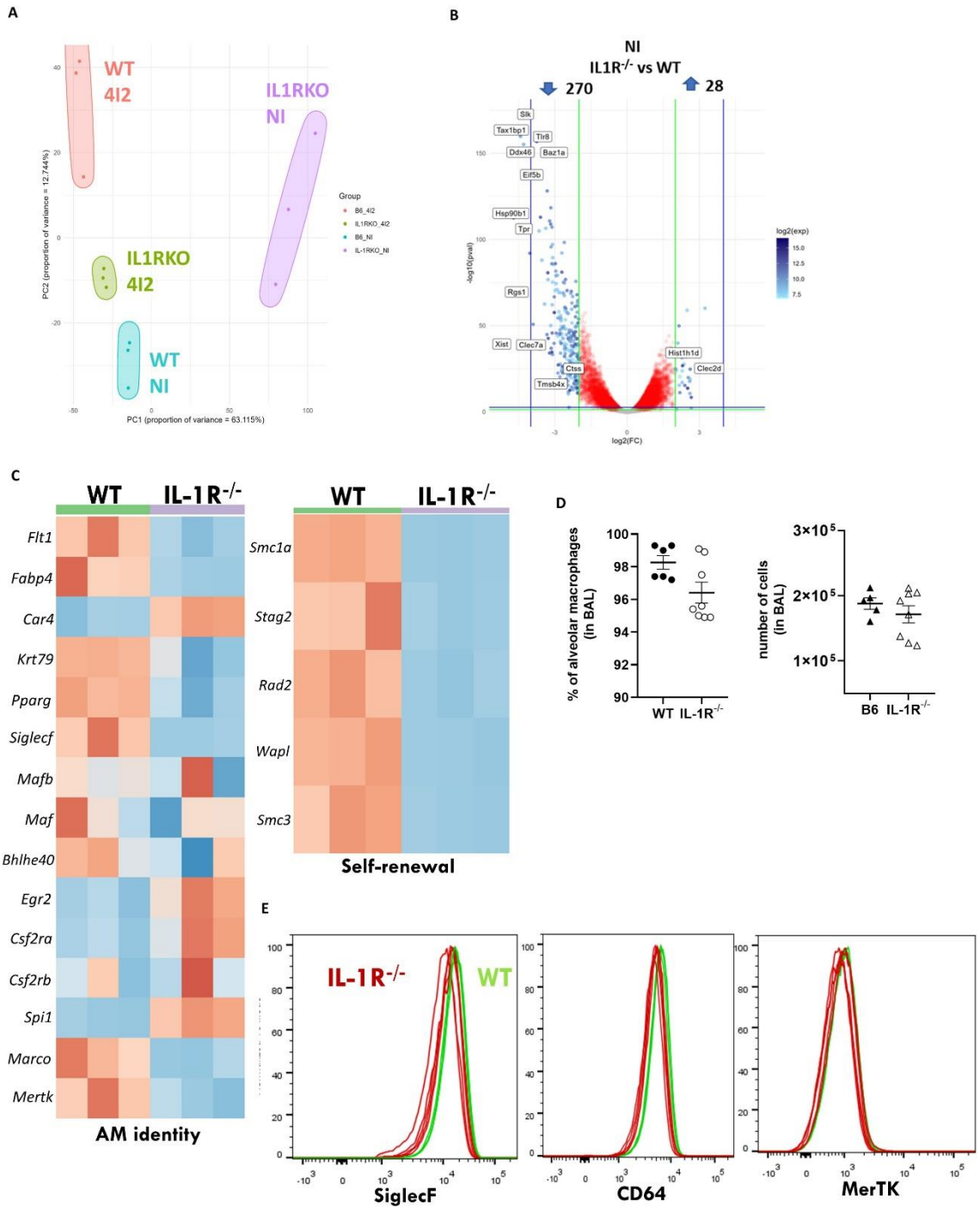
Supplementary Figure 1.

(A, B, C) CFU fold change between 0-4 days in infections with *M. tuberculosis* (*Mtb*) isolates 4I2 and 6C4 in alveolar macrophages (AMs) **(A)**, bone marrow (BM) monocytes **(B)** and BM derived dendritic cells (BM-DCs) **(C)**. **(D)** Quantification of IL-1α induced by infections with isolates 4I2 and 6C4 in the broncho-alveolar lavage (BAL) of infected mice. Represented are Mean±SEM. Each dot represents cells in an individual culture well, except for **(D)**, where 3 animals are pooled together. Data represents individual experiments. Statistical differences between groups are indicated as: * $p \leq 0.05$ and ** $p \leq 0.01$.


A
Mtb 4I2 high dose
% initial weight
IL-1R+/+
IL-1R-/-
weeks p.i.
B
Mtb 6C4 high dose
weeks p.i.
C
Mtb 4I2 low dose
Log10CFU (Lung)
105.3 x
WT
IL-1R-/-
30 days p.i.
D
Mtb 6C4 low dose
5.9 x
E
Mtb 4I2 low dose
F
Mtb 6C4 low dose
G
Mtb HN878
7x
H
Mtb HN878
I
SSC-A
FSC-A
FSC-H
Zombie NIR
CD45
SSC-A
CD11c
Siglec F
AMs
Eosinophils
CD11b
MHCII
Neutrophils
Monocytes
Ly6G
Ly6C
CD103+ DCs
CD11b+ DCs
J
Lung
Mtb 4I2
Mtb 6C4
Number of cells
AMs
Monocytes
Neutrophils
Eosinophils
CD11b+ DCs
CD103+ DCs
WT
IL-1R-/-
days p.i.
K
BAL
Mtb 4I2
Mtb 6C4
Number of cells
AMs
Monocytes
Neutrophils
Eosinophils
CD11b+ DCs
CD103+ DCs
WT
IL-1R-/-
days p.i.

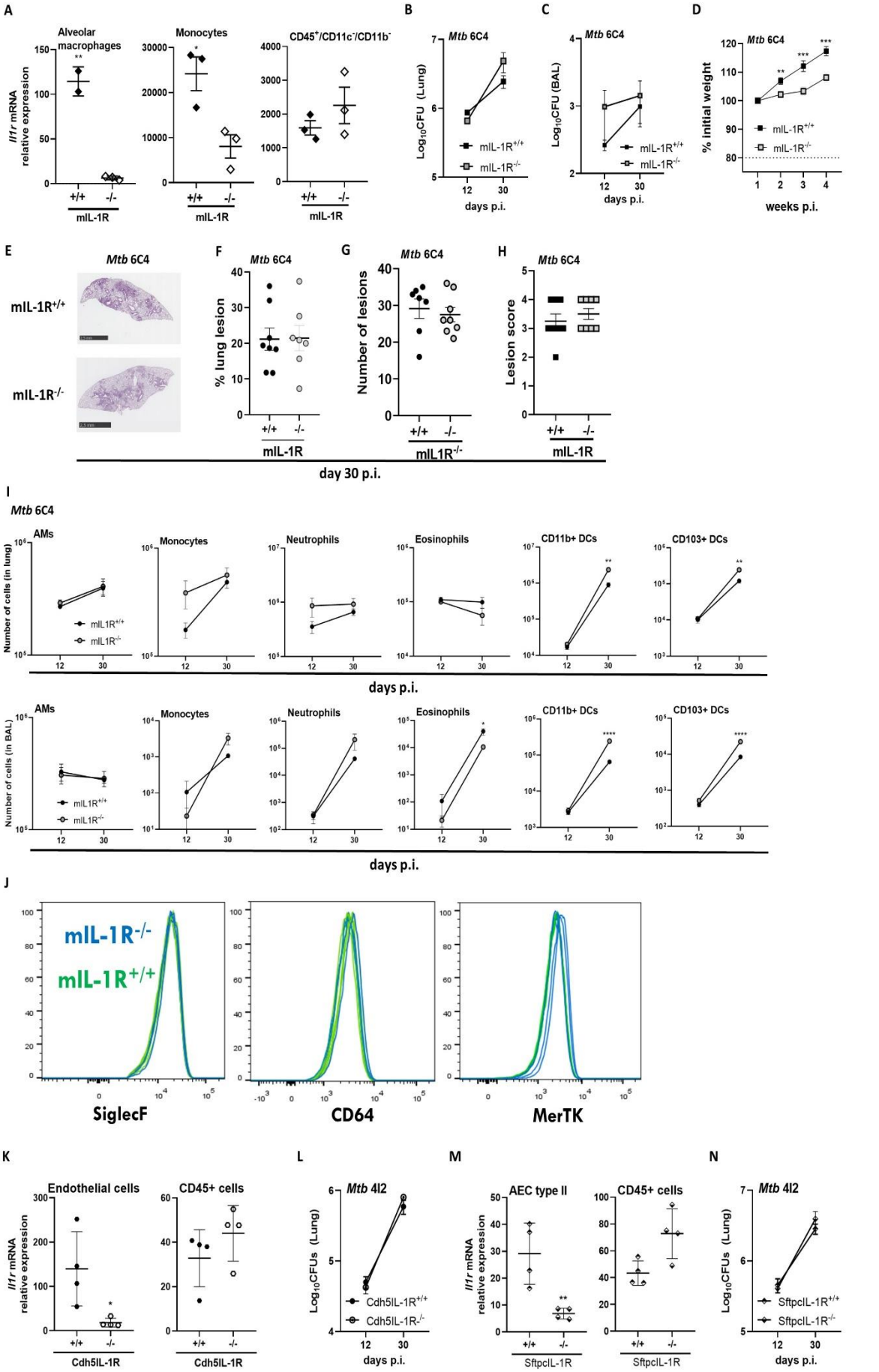
Supplementary Figure 2.

(A, B) Weight variation of wild type (WT) and IL-1R$^{-/-}$ mice during the course of infections with *M. tuberculosis* (*Mtb*) isolate 4I2 **(A)** and isolate 6C4 **(B)**. **(C, D)** Bacterial burden in the lungs of WT and IL-1R$^{-/-}$ determined 30 days post-infection with low doses of isolates 4I2 **(C)** and 6C4 **(D). (E, F)** Weight variation of WT and mIL-1R$^{-/-}$ mice during the course of low dose infections with isolate 4I2 **(E)** and isolate 6C4 **(F)**. **(G)** Weight variation of WT and mIL-1R$^{-/-}$ mice during the course of infections with hypervirulent HN878 isolate. **(H)** Bacterial burden in the lungs of WT and IL-1R$^{-/-}$ determined 30 days post-infection with hypervirulent HN878 isolate. **(I)** Gating strategy used to analyze myeloid cell populations in the lung and broncho-alveolar lavage (BAL) of infected mice. **(J)** Dynamics of total number of myeloid cells in the lung of mice infected with isolates 4I2 (above) and 6C4 (below) at days 12 and 30 post infection. **(K)** Dynamics of total number of myeloid cells in the BAL of mice infected with isolates 4I2 (above) and 6C4 (below) at days 12 and 30 post infection. Represented are Mean±SEM. Each dot represents an individual mouse, except for: **(A)** and **(B)**, where 8-9 animals from 2 independent experiments are pooled together; **(C)** and **(D),** where 5 animals are pooled together; **(G)**, where 5 animals are pooled together; **(J)** and **(K)** where 4-5 animals are pooled together. Data represents individual experiments, except for **(A)** and **(B)**. Statistical differences between groups are indicated as: * $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$.



Supplementary Figure 3.

(A) Principal component analysis (PCA) of gene expression by alveolar macrophages (AMs) from either wild type (WT) or IL-1R$^{-/-}$ mice infected with *M. tuberculosis* (*Mtb*) isolate 4I2. **(B)** Differentially expressed genes (DEGs) in alveolar macrophages (AMs) from naïve WT and IL-1R$^{-/-}$ mice **(C)** Heatmap representing the relative expression of genes related to AM identity and self-renewal by AMs from naïve WT and IL-1R$^{-/-}$ mice. **(D)**. Frequency and number of AMs in the broncho-alveolar lavage (BAL) of naïve WT and IL-1R$^{-/-}$ mice **(E)** Expression of common surface markers in AMs of naïve WT and IL-1R$^{-/-}$ mice. Represented are Mean±SEM. Each dot represents an individual animal from 2 independent experiments pooled together.


A
Alveolar macrophages
Monocytes
CD45+/CD11c-/CD11b-
Il1r mRNA relative expression
+/+
-/-
mIL-1R
B
Mtb 6C4
Log10CFU (Lung)
mIL-1R+/+
mIL-1R-/-
days p.i.
C
Mtb 6C4
Log10CFU (BAL)
D
Mtb 6C4
% initial weight
weeks p.i.
E
Mtb 6C4
mIL-1R+/+
mIL-1R-/-
F
% lung lesion
G
Number of lesions
H
Lesion score
day 30 p.i.
I
Mtb 6C4
AMs
Monocytes
Neutrophils
Eosinophils
CD11b+ DCs
CD103+ DCs
Number of cells (in lung)
Number of cells (in BAL)
days p.i.
J
mIL-1R-/-
mIL-1R+/+
SiglecF
CD64
MerTK
K
Endothelial cells
CD45+ cells
Cdh5IL-1R
L
Mtb 4l2
Cdh5IL-1R+/+
Cdh5IL-1R-/-
M
AEC type II
SftpcIL-1R
N
Mtb 4l2
SftpcIL-1R+/+
SftpcIL-1R-/-

Supplementary Figure 4.

(A) Relative expression of *Il1r* in myeloid and non-myeloid compartments. **(B, C)** Bacterial burden in the lungs **(B)** and BAL **(C)** of mIL-1R$^{-/-}$ and mIL-1R$^{-/-}$ mice determined 12 and 30 days post-infection with *M. tuberculosis* (*Mtb*) isolate 6C4. **(D)** Weight variation of mIL-1R$^{+/+}$ and mIL-1R$^{-/-}$ mice during the course of infection with isolate 6C4. **(E, F, G)** Representative histological images of Hematoxylin and Eosin (H&E)-stained lung sections **(D)** from infected mice. Darker areas corresponding to tissue lesions were quantified to obtain the percentage of area of lesion **(F)** and number of legions **(G)** in the lungs. **(H)** Histopathological score attributed to each lesion. **(I)** Dynamics of total number of cells in the lung (above) and broncho-alveolar lavage (BAL) (below) of infected mice at days 12 and 30 post infection. **(J)** Expression of common surface markers in alveolar macrophages (AMs) of naïve mIL-1R$^{+/+}$ and mIL-1R$^{-/-}$ mice. **(K)** Relative expression of *Il1r* in endothelial and immune compartments. **(L)** Bacterial burden in the lungs of Cdh5IL-1R$^{-/-}$ and Cdh5IL-1R$^{+/+}$ mice determined 12 and 30 days post-infection with isolate 4I2. **(M)** Relative expression of *Il1r* in alveolar epithelial cell (AEC) type II and immune compartments. **(L)** Bacterial burden in the lungs of SftpcIL-1R$^{-/-}$ and SftpcIL-1R$^{+/+}$ mice determined 12 and 30 days post-infection with isolate 4I2. Represented are Mean±SEM. Each dot represents an individual animal, except for: **(B)**, **(C)** and **(D),** where 10 animals from 2 independent experiments are pooled together; **(I)**, where 5 animals are pooled together; **(L)** and **(N)**, where 5 animals are pooled together. Data represents individual experiments, except for **(B)**, **(C)** and **(D)**. Statistical differences between groups are indicated as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ and **** $p \leq 0.0001$.

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

Flow cytometry		
Target	**Clone**	**Company (Catalog #)**
CD45	30-F11	Biolegend (103112)
CD11c	N418	Biolegend (117317)
CD11b	M1/70	Biolegend (101245)
LY-6G	1A8	Biolegend (127616)
LY-6C	HK1.4	Biolegend (128014)
CD64	X54-5/7.1	Biolegend (139304)
MerTK	108928	BD Biosciences (747892)
SiglecF	E50-2440	BD Biosciences (740956)
MHCII	M5/114.15.2	Biolegend (107606)
CD45	30-F11	Biolegend (103106)
CD31	390	Biolegend (102417)
EpCAM	G8.8	Biolegend (118217)
Podoplanin	8.1.1	Biolegend (127409)

Supplementary Table 2. List of oligonucleotides used in this study.

Gene	Forward (5'-3')	Reverse (5´-3')
Tnf	GCCACCACGTCTTCTGTCT	TGAGGGTCTGGGCCATAGAAC
Il1b	ACCTTCCAGGATGAGGACATGA	AACGTCACACACCAGCAGGTTA
Il6	ACACATGTTCTCTGGGAAATCGT	AAGTGCATCATCGTTGTTCATACA
Ifng	CAACAGCAAGGCGAAAAAGG	GGACCACTCGGATGAGCTCA
Il17	CTCAGACTACCTCAACCGTTCCA	TTCCCTCCGCATTGACACA
Il1r	CAGGAGAAGTCGCAGGAAGT	TGGAACAGAGCCAGTGTCAG
Ubiquitin	TGGCTATTAATTATTCGGTCTGCAT	GCAAGTGGCTAGAGTGCAGAGTAA

Supplementary Table 3. Pathological scoring analysis of Hematoxylin and Eosin-stained lung histological sections.

Parameter	
Lesion	**Description**
0	Absence of lesions or alterations in the lung
1	Lung with onset of lesion: • One or few very small infiltrates of inflammatory cells; • Composed of myeloid cells; • Perivascular location; • No other lesion signs present.
2	Lung with minor lesion: • One or few small but well-formed infiltrates of inflammatory cells; • Composed mainly of myeloid cells such as macrophages and some lymphoid cells; • Perivascular location; • No other lesion signs present.
3	Lung with moderate lesion: • One or few medium-sized lesions or small multifocal lesions; • Composed of cells such as macrophages/MN, neutrophils, and lymphocytes; • May present congestion.
4	Lung with extensive lesion: • Several medium-sized lesions or a few very extensive ones; • Composed of various types of inflammatory cells; • Presence of some areas of necrosis or cellular debris and/or congestion.
5	Lung with very extensive lesion: • Lung almost entirely covered in lesions; • Composed of various types of inflammatory cells; • Zones of necrosis in the center of the lesions and other damage elements such as congestion, edema and damaged epithelium.
Necrosis/ Cell death	**Description**
0	Absence of necrotic zones or cellular death.
1	Small/few localized zones with necrosis.
2	Lung with extensive necrosis.

Exudate/ Pulmonary edema	**Description**
0	Lung without edema.
1	Small areas with fluid/pulmonary edema.
2	Lung with vast areas of edema.
Fibrosis/ Calcification	**Description**
0	Absence of fibrosis and calcification.
1	Presence of fibrosis or calcification in an area of the lung.
2	Presence of fibrosis or calcification in more than one area of the lung.
Final Score	**Sum of the score for each parameter**

CHAPTER IV – INFECTION WITH HYPERVIRULENT *MYCOBACTERIUM TUBERCULOSIS* TRIGGERS EMERGENCY MYELOPOIESIS BUT NOT TRAINED IMMUNITY

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Infection with hypervirulent *Mycobacterium tuberculosis* triggers emergency myelopoiesis but not trained immunity

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Infection with hypervirulent *Mycobacterium tuberculosis* triggers emergency myelopoiesis but not trained immunity

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Introduction: During infection, bone marrow (BM) hematopoiesis is reprogrammed toward myeloid cell production, a mechanism named emergency myelopoiesis. In addition to replenishing myeloid cells, emergency myelopoiesis has been linked to trained immunity, a process that allows enhanced innate immune responses to secondary challenges. Although hematopoietic alterations during tuberculosis (TB) have been described and *Mycobacterium tuberculosis* may colonize the BM, studies using the mouse model of infection and the laboratory reference strain *M. tuberculosis* H37Rv have demonstrated limited emergency myelopoiesis and trained immunity.

Methods: To further address this issue, we aerosol- infected C57BL/6 mice with high doses of the hypervirulent M. tuberculosis isolate HN878 and monitored alterations to the BM. This experimental model better resembles the human blood immune signature of TB.

Results and discussion: We found increased frequencies of lineage$^-$Sca-1$^+$cKit$^+$ (LSK) cells and the granulocyte/macrophage progenitor (GMP) population. At the mature cell level, we observed an increase of monocytes and neutrophils in the blood and lung, likely reflecting the increased BM myeloid output. Monocytes or monocyte-derived macrophages recovered from the BM of *M. tuberculosis* HN878-infected mice did not show signs of trained immunity, suggesting an uncoupling of emergency myelopoiesis and trained immunity in the BM. Surprisingly, *M. tuberculosis* HN878-induced emergency myelopoiesis was not fully dependent on IFNγ, as mice lacking this cytokine and infected under the same conditions as wild-type mice still presented BM alterations. These data expand our understanding of the immune response to *M. tuberculosis* and raise awareness of pathogen strain-imposed differences to host responses.

KEYWORDS

tuberculosis, myelopoiesis, trained immunity, interferon-gamma, bone marrow

1 Introduction

The bone marrow (BM) is highly responsive to stress cues, allowing the rapid adaptation of the hematopoietic process to ensure immune cell replenishment. This adaptation is key during infection and inflammation, in which a deviation of hematopoiesis toward the production of myeloid cells, a process also known as emergency myelopoiesis (EM), is well documented (1, 2). Recent evidence supports that, in addition to enhanced myeloid cell differentiation, EM plays a role in trained immunity (3), a mechanism that allows sustained innate immune responses to secondary challenges (4).

Hematopoietic adaptation has been reported in many infections, including those caused by Mycobacteria. Previous studies showed that, in the murine model, *Mycobacterium avium* infections enhanced the proliferation of hematopoietic stem cells (HSCs) (5) and that chronic *M. avium* infection caused pancytopenia (6) and anemia (7, 8). Furthermore, intradermal administration of *Mycobacterium bovis* BCG, the vaccine used against tuberculosis (TB), induced a transcriptomic rewiring of human stem and progenitor cells toward the myeloid cell lineage (9). In mice, intravenous administration of BCG caused emergency hematopoiesis and induced trained immunity in macrophages, which became highly protective against *Mycobacterium tuberculosis* (10). In the case of both *M. avium* and *M. bovis* BCG, interferon (IFN) γ was shown to be a key molecule in driving hematopoietic alterations (5, 6, 10, 11). The impact of IFNγ in myelopoiesis is further acknowledged in different scenarios (12–16). However, IFNγ has also been described as a negative modulator of HSC self-renewal, involved in BM failure (17), indicating that its role as a modulator of hematopoiesis is context dependent.

M. tuberculosis infections caused over 10 million new TB cases and killed over 1.6 million individuals in 2021 alone (18). Several observations support a possible impact of *M. tuberculosis* in the BM and in deregulating hematopoiesis. *M. tuberculosis* may persist in BM mesenchymal stem cells of TB patients even after antibiotherapy (19) and in long-term HSCs in individuals with latent TB infection (20). Additionally, clinical signs of abnormal hematopoiesis, such as anemia and thrombocytopenia, have been reported in TB patients (21, 22). Finally, neutrophilia and altered monocyte/T cell ratios are common in TB and are particularly associated with severe forms of the disease (23). Aerosol infection of mice with *M. tuberculosis* results in BM colonization (19, 24) and the increased recruitment of myeloid cells to the lungs (25), as well as increased numbers of blood monocytes, which has been shown to be caused by increased egress from the BM (26). Altogether, observations from human and mouse studies suggest the occurrence of EM during *M. tuberculosis* infections. However, a recent study showed that although *M. tuberculosis* reprogrammed HSCs in mice infected via aerosol, it also limited EM (24).

Here, we characterize the impact of *M. tuberculosis* infection on the BM using a mouse model previously shown to resemble the human blood immune signature (27). Specifically, we infected C57BL/6 mice with high doses of *M. tuberculosis* HN878 strain and analyzed the immune cell composition of the lung, blood, and BM at the peak of the disease. Our data show BM alterations compatible with the deviation of hematopoiesis toward myelopoiesis, with an increased output of myeloid cells in the blood and lungs of infected animals. Furthermore, we found that IFNγ may be dispensable to this process and despite the BM adaptation to infection, the generated monocytes or macrophages do not acquire an improved capacity to respond to secondary challenges.

2 Materials and methods

2.1 Animal housing, infection, and monitoring

C57BL/6 wild-type (WT) or C57BL/6.IFNγ deficient ($IFN\gamma^{-/-}$) mice were bred and housed at the i3S Animal House Facility, and infected under ABSL3 conditions. Both male and female mice were used, ranging between 8 and 12 weeks of age at the time of infection. Animals were kept under a controlled temperature (20–24°C), humidity (45–65%), and light cycle (12h light/dark) in specific-pathogen free conditions. Water and food were provided *ad libitum*. Aerosol infection was performed using an inhalation exposure system (Glas-Col) (28, 29). Infection level was determined based on the lung bacterial burden 3 days post-infection: <200 CFUs corresponded to a low dose, while >500 CFUs was considered a high dose. Infected mice were weighed every week or every 2 days upon showing signs of disease. Weight loss of over 20% or lack of responsiveness to physical stimulation were considered humane endpoints, at which point the experiments were terminated. Mice were euthanized by CO_2 inhalation, during which efforts were made to minimize suffering.

2.2 Organ processing

Organs were aseptically excised and processed as described previously (28, 29). Lungs were digested using Collagenase type IV (Gibco, Cat. #17104019) followed by physical disruption and filtering in a 70 µm mesh. Blood was collected via cardiac puncture. BM was collected by flushing the tibias and femurs with PBS + 2% FBS (Gibco, Cat. #10500064). Single-cell suspensions were used for bacterial burden determination, flow cytometry, and RNA analysis.

2.3 Bacteria growth and quantification

M. tuberculosis HN878 isolate was grown and stored as described previously (28, 29). Viable bacteria were determined by serial dilution and colony forming unit (CFU) enumeration after 21–28 days of incubation at 37°C in 7H11 agar plates (Cat. #212203, BD Biosciences). Bacterial quantification in infected lungs and BM was performed upon organ homogenization using the same procedure.

2.4 Flow cytometry

Mouse blood, BM, and lung cell suspensions were stained for surface antigens (30 min; 4°C) and fixed for 20 min in 4% paraformaldehyde-PBS after erythrocyte lysis. For the analysis of BM precursors, the mature lineage (Lin^+) was depleted using an EasySep™ Mouse Streptavidin RapidSpheres™ Isolation Kit (Cat. #19860, STEMCELL Technologies) prior to Lin^- staining with specific antibodies for precursor and progenitor markers. Dead cells were excluded from the analyses using ZombieAqua (Cat. #423102, Biolegend) or ZombieGreen (Cat. #423112, Biolegend) viability dyes. Cells were acquired on a BD FACS Canto II or BD Fortessa II. Data were analyzed using *FlowJo* (version 10.1.r7). Dimensionality reduction of concatenated data was performed with the *Uniform manifold approximation and projection* (UMAP) *FlowJo* plug-in (version 3.1) (30). All antibodies used are listed in Supplementary Table 1. Gating strategies are shown in Supplementary Figures 1A, B and Supplementary Figure 2A.

2.5 RNA extraction and qPCR

Total RNA was extracted from mouse lung or BM cell suspensions using TripleXtractor (Cat. #GB23.0100, GRiSP) and converted to cDNA using a ProtoScript® II First Strand cDNA Synthesis Kit (Cat. #E6560L, NE Biolabs). Relative *CSF2* gene expression quantification was performed using quantitative PCR and Taqman probe assay (Cat. #4331182; Assay ID #Mm01290062_m1, Thermo Fisher Scientific). Normalization of target gene abundance was performed using *Hprt* as the reference gene (Cat. #4331182; Assay ID #Mm03024075_m1, Thermo Fisher Scientific). Gene expression quantification for *Csf1*, *Csf3*, *Tnf*, *Il1b*, *Il6*, *Ifng*, and *Ifnb* was performed using quantitative PCR with SYBR green (Cat. #1725120, Bio-Rad) and intron spanning oligonucleotides specific for the target genes. Normalization of target gene abundance was performed using *Ubiquitin* as the reference gene. Melting and standard curves and RQ values were determined for each gene. Sequences of the oligonucleotides used are in Supplementary Table 2.

2.6 Histological analysis

Mouse lungs were fixed in 10% buffered formalin and embedded in paraffin. Serial 3-µm sections were used for Hematoxylin and Eosin (H&E) or immunofluorescence staining and analysis. Morphometric analysis of lung pathology areas in H&E-stained histological sections was performed using the software *Interactive Learning and Segmentation Toolkit* (*Ilastik* version 1.3.3) and *CellProfiler Analyst* (version 3.1.5). Probability maps for the whole lung and lesion area were created in Ilastik and calculated and analyzed using *CellProfiler Analyst* software (28). Pathological scoring analysis of H&E-stained histological sections was performed in a blind way, and the histopathological features were scored according to the parameters in Supplementary Table 3.

2.7 Immunofluorescence

Paraffin-embedded lung sections were stained for myeloperoxidase (Mpo) and nitric oxide synthase (Nos2), as described previously (28, 29, 31). Antigen recovery was performed by incubating slides at 96°C in HIER Buffer L (Cat. #TA135HBL, Epredia) for 45 min. Slides were then incubated overnight with goat anti-mouse Mpo (Cat. #AF3667, R&D systems; 1:40) and rabbit anti-mouse Nos2 (M-19, Cat. #sc-650, Santa Cruz Biotechnology, 1:50) at 4°C followed by incubation with secondary antibodies donkey anti-goat IgG Alexa Fluor 488 conjugated (Cat. #A-11055, Invitrogen) and donkey anti-rabbit IgG Alexa Fluor 647 conjugated (Cat. #A-31573, Invitrogen) for 2 h at room temperature. Slides were stained with DAPI (Cat. #422801, Biolegend; 1:1000 dilution), and glass coverslips were mounted using Fluoroshield (Cat. #F6182, Sigma-Aldrich). Images were acquired using IN Cell Analyzer 2000 (GE Healthcare) and analyzed using *IN Cell Developer software* (version 1.9) and *ImageJ* (version 1.53t) (32, 33).

2.8 Generation of bone marrow-derived macrophages and purification of bone marrow monocytes

BM-derived macrophages (BMDMs) were generated from BM cellular suspensions as previously described (29, 34). Cells were plated in Petri dishes and differentiated into macrophages using DMEM (Cat. #10566016, Gibco), completed with 1% sodium pyruvate (Cat. #S8636, Sigma-Aldrich), 1% HEPES (Cat. #15630106, Gibco), and 10% FBS (Cat. #10500064, Gibco) and supplemented with 20% L-cell conditioned medium (V/V). Once differentiation was complete (7 days), BMDMs were transferred to cell culture plates. BM monocytes were purified from BM cellular suspensions using an EasySep™ Mouse Monocyte Isolation Kit (Cat. #19861, STEMCELL Technologies), following the manufacturer's protocol, and plated in cell culture plates with RPMI medium (Cat. #61870036, Gibco), completed as described above for DMEM. BMDMs were plated in 96-well plates (100,000 cells per well) for CFU determination or in 24-well plates (500,000 cells per well) for supernatant collection. Purified monocytes (purity >95%) were plated in 96-well plates (100,000 cells per well).

2.9 Human $CD14^+$ cell isolation

Peripheral blood was collected and processed to obtain PBMCs as previously described (34). $CD14^+$ monocytes were magnetically isolated using CD14 MicroBeads (Cat. #130-050-201, Miltenyi Biotec), following the manufacturer's protocol. Purified cells (100,000 cells per well; purity >95%) were plated in 96-well plates with complete RPMI medium.

2.10 *In vitro* infections

Human $CD14^+$ cells and mouse BMDMs or BM monocytes were infected with *M. tuberculosis* HN878 isolate at a multiplicity of

infection (MOI) of 2 (2 bacteria:1 cell). Extracellular bacteria were washed away with PBS after 4 h and bacterial growth was quantified by CFU enumeration 4 h (control) or 4 days after infection. The culture supernatants (BMDMs and BM monocytes) were collected 24 h (in the absence of bacterial washing) after infection for protein quantification.

2.11 Cytokine quantification

Cytokine production by BMDM or monocyte cultures infected with *M. tuberculosis* was quantified by immunoassay, using commercially available ELISA kits (Cat. #88-7064-88; 88-7013-88; 88-7324-88, Invitrogen).

2.12 Statistical analysis

Data were plotted and analyzed using *GraphPad Prism* (version 8.1.0): a non-parametric Mann–Whitney test was used to compare two groups and Kruskal–Wallis one-way analysis of variance test was used for the comparison of more than two groups. Multiple comparison correction was performed using a Dunn post-test. Significant differences are as follows: * $p \leq 0.05$; ** $p \leq 0.01$; and *** $p \leq 0.001$. Spearman correlations and correspondent correlation plots were obtained using *R* (version 4.1.2) and *R* package *corrplot* (version 0.92).

3 Results

3.1 Infection with *M. tuberculosis* HN878 strain results in altered cell composition in the lung, blood, and bone marrow

To investigate possible alterations imposed by *M. tuberculosis* infection to the BM hematopoietic output, we aerosol-infected WT C57BL/6 mice with high doses of *M. tuberculosis* HN878. Twenty-seven days post-infection (p.i.), the mice presented a high bacterial burden in the lungs (over 10^9 bacteria) (Figure 1A), which was accompanied by marked tissue pathology, with lesions affecting 14% to 56% of the lung (Figure 1B). Additionally, we observed an increase in lung cellularity, associated with the recruitment and accumulation of $CD45^+$ immune cells (Figure 1C). We next analyzed by flow cytometry the myeloid cell composition within the immune cells present in the lung, which accounted for approximately 45% of the total $CD45^+$ cells (Figure 1D; Supplementary Figure 1A). Although the frequency of alveolar macrophages decreased with infection, resulting in a very low percentage 27 days p.i., both the frequency and numbers of all other myeloid cell types (monocytes, monocyte-derived recruited macrophages, dendritic cells (DCs), and neutrophils) were significantly increased in infected mice (Figure 1D). Neutrophils exhibited the highest increase both in percentage and cell counts of the myeloid populations analyzed. The global frequency of other immune cells (which included eosinophils and NK, T, and B cells) was not altered (Figure 1D). Immunofluorescence staining of lung sections with Mpo, a neutrophil marker, revealed large Mpo-positive areas, which were dominant compared with those corresponding to Nos2-positive activated macrophages (Figure 1E). These results are in line with previous reports showing an accumulation of myeloid cells, especially neutrophils, in the lungs upon infection with *M. tuberculosis* HN878 (28, 31).

We then analyzed the blood and BM compartments at 27 days p.i. (Supplementary Figure 1B). Infected mice presented leukocytosis (Supplementary Figure 1C), which was associated with increased numbers (per µl of blood) of monocytes, neutrophils, and T cells, even though the proportion of T cells in the blood had diminished (Figure 1F). There was a reduction in the frequency of B cells, with no effect on cell numbers (Figure 1F). Thus, the increase in myeloid cells seen in the lungs of infected mice was also observed in the blood, suggesting a possible altered hematopoietic output. Analysis of the BM mature cell populations showed an increase in the frequency and numbers of monocytes, which was accompanied by a decrease in the frequencies and numbers of B cells (Figure 1G). Both neutrophils and T cells remained mostly unaltered (Figure 1G), as did the total number of BM cells (Supplementary Figure 1D). Importantly, *M. tuberculosis* disseminated into the BM (Figure 1H). Targeted transcriptional analysis of the BM 27 days p.i. showed a modest impact of infection on the expression of cytokine genes involved in hematopoietic reprogramming. *Tnf* and *Csf1* expression was statistically significantly increased and *Csf2* expression was statistically significantly decreased (Figure 1I). The lack of more profound differences in gene expression and the decrease of *Csf2* transcription upon infection was surprising and may reflect the overall exhaustion of the infected BM.

The accumulation of myeloid cells in the lungs of the infected animals, together with the alterations seen in the blood and BM, raise the hypothesis that EM may be happening in this immune compartment.

3.2 *M. tuberculosis* HN878 infection leads to bone marrow myelopoiesis

To investigate whether EM may be occurring during *M. tuberculosis* HN878 infections, we analyzed the BM progenitor populations of naive versus HN878-infected mice 27 days p.i. (Supplementary Figure 2A). UMAP dimensionality reduction analysis of flow cytometry data revealed a different precursor and progenitor cell composition profile between infected and non-infected mice (Figure 2A). Closer analysis of the different precursor populations revealed a statistically significant increase of LSK ($Lin^-Sca^+c\text{-}Kit^+$) cells in infected mice (Figure 2B). Within the LSK population, long-term HSC (LT-HSC) were not affected by the infection, but an increase of the multipotent progenitors (MPP) 2, 3, and 4 was observed (Figure 2B). Moreover, although no statistically significant differences were observed for the frequencies of LK ($Lin^-Sca^-c\text{-}Kit^+$) cells (Figure 2C), there was a statistically significant increase in granulocyte-monocyte progenitors (GMPs) accompanied by a decrease in common

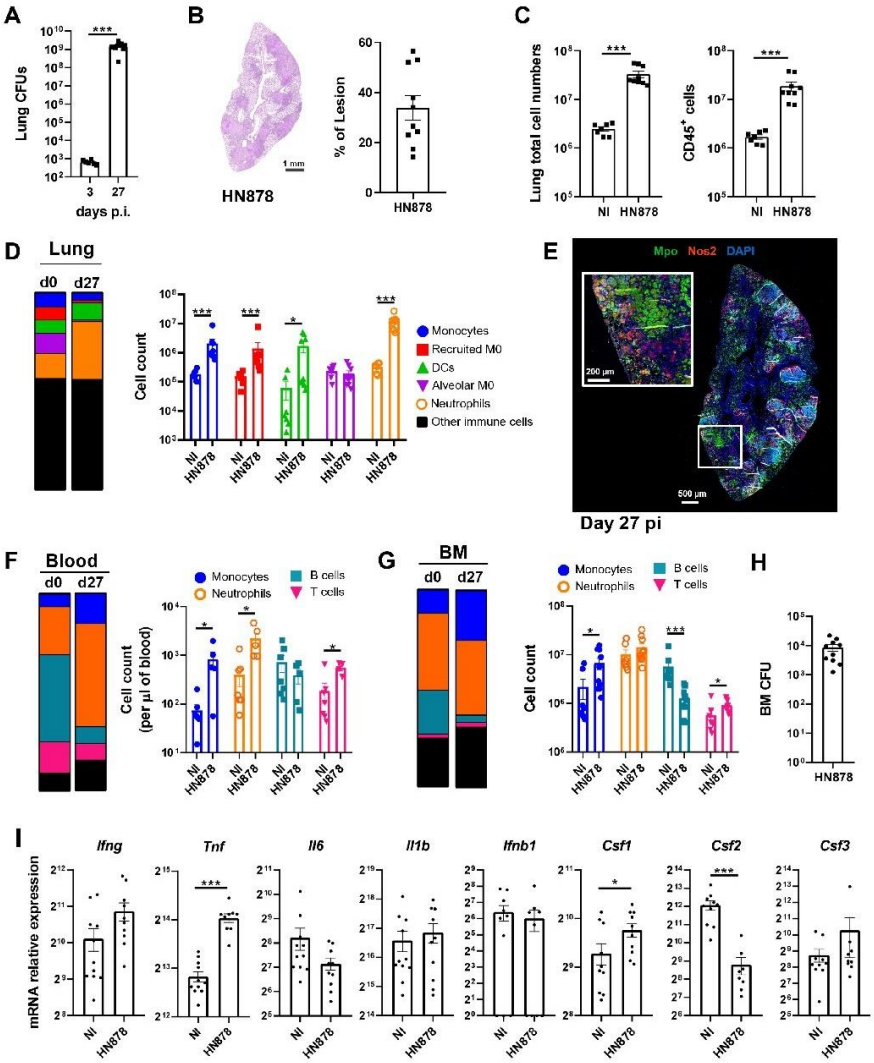

FIGURE 1
Infection with *M. tuberculosis* HN878 is associated with an accumulation of myeloid cells in the lung, blood, and bone marrow. C57BL/6 mice were aerosol-infected with high doses of *M. tuberculosis* isolate HN878. **(A)** Bacterial burden in the lungs determined on day 3 or 27 days post-infection. **(B)** Representative histological images of H&E-stained lung sections from non-infected (NI) or *M. tuberculosis*-infected (HN878) mice. Darker areas corresponding to tissue lesions were quantified to obtain the percentage of area of lesion in the lungs of *M. tuberculosis*-infected mice. **(C)** Number of total cells (left) and immune cells (right) in the lungs of NI or *M. tuberculosis*-infected mice. Immune cells were determined by FACS based on the detection of surface CD45. **(D)** Frequency (left) and total cell counts (right) of distinct myeloid cell populations: monocytes, recruited macrophages (recruited M0), dendritic cells (DCs), alveolar macrophages (alveolar M0), and neutrophils in NI or *M. tuberculosis*-infected mice. Gating strategies are shown in Supplementary Figures 1A, B. **(E)** Representative immunofluorescence image of a lung section from an infected mouse (27 days post-infection) stained for myeloperoxidase (Mpo) and nitric oxide synthase 2 (Nos2). Nuclei are stained with DAPI. **(F, G)** Frequency (left) and cell counts (right) of blood **(F)** or bone marrow **(G)** myeloid cell populations in NI or *M. tuberculosis*-infected mice. **(H)** Bacterial burdens in the bone marrow of mice 27 days post-infection. **(I)** Transcription analysis of the indicated genes detected by qPCR in the bone marrow of NI or *M. tuberculosis*-infected mice. Data are mean ± SEM. Each dot represents an individual mouse, with two independent experiments represented per panel. The infection doses (in CFU ± SEM)/animal ages (in weeks old at the time of infection) for the experiments used in this figure were 683 ± 125/10, 1,093 ± 100/8, and 1057 ± 74/9 and 10. Non-infected animals were 8 weeks old. The statistical analyses were performed using a Mann–Whitney U test to identify statistical differences between groups. p-values inferior to 0.05 were deemed significant: *p-value<0.05, ***p-value<0.001.

myeloid progenitors (CMPs) and megakaryocyte erythrocyte progenitors (MEPs) (Figure 2C). Similar alterations were seen in the frequencies of precursor and progenitor populations (Supplementary Figures 2B, C). Altogether, these data suggest the occurrence of EM as a consequence of aerosol infection with high doses of *M. tuberculosis* HN878.

Given the apparent discrepancy between these data and previously published data (24), we decided to infect mice with

low doses of *M. tuberculosis* HN878. Under these conditions and at day 34 p.i., we still observed colonization of the BM (Supplementary Figure 2D), although the bacterial burden was 2 log lower than that resulting from infections with high doses of bacteria. Aerosol infection with low doses of *M. tuberculosis* HN878 did not significantly remodel BM cell composition, as no changes in mature cell populations were observed (Supplementary Figure 2E), and only an increase in LSK (Supplementary Figure 2F) and a modest decrease in CMPs (Supplementary Figure 2G) were observed. Interestingly, a positive correlation was observed between the BM LSK population and lung bacterial burden, monocytes, and neutrophils, as well as BM bacterial burden (Supplementary Figure 2H).

Collectively, our findings raise the hypothesis that EM may occur during *M. tuberculosis* infections, possibly associated with more severe outcomes of disease, such as those resulting from high doses of infection.

3.3 *M. tuberculosis* impact on the bone marrow does not translate into trained immunity

As we observed the adaptation of the BM towards myelopoiesis in our model of aerosol infection with high doses of *M. tuberculosis* HN878, we next investigated whether the resulting mature cells might be trained. As such, we generated macrophages derived from the BM of non-infected or *M. tuberculosis*-infected mice for 27 days. The resulting BMDMs were infected with *M. tuberculosis* HN878 and the bacterial burden was measured 4 h and 4 days p.i. The bacterial burden 4 h after infection was similarly independent of the BMDM origin. Then, an increase in intracellular bacteria was observed over time and was similarly independent of the BMDM origin (Figure 2D), suggesting that previous infection with *M. tuberculosis* did not enhance the protective capacity of *de novo*-generated BMDM. Additionally, we measured the production of TNFα, IL-6, and IL-1β, cytokines associated with trained immunity in the context of mycobacteria (35, 36), by BMDMs generated from non-infected or infected mice and *in vitro* infected with *M. tuberculosis* HN878. The ability of the generated BMDMs to respond to secondary stimuli was also not enhanced by previous infection across the measured parameters (Figure 2E). Finally, to exclude possible effects imposed on macrophages by the *in vitro* differentiation process, we purified monocytes from the BM of non-infected or *M. tuberculosis*-infected mice and analyzed their microbicidal capacity (Figure 2F) and cytokine response to secondary stimuli (Figure 2G). Monocytes purified from non-infected or infected mice responded similarly, further supporting that despite evidence of EM in the BM, this was not accompanied by training of the mature populations.

As trained immunity was suggested to equip circulating monocytes from individuals recently exposed to *M. tuberculosis* with an increased capacity to control BCG (37), we considered the hypothesis that trained immunity may occur at the periphery. To test this hypothesis, we started by comparing the bacterial growth over a 4-day period in monocytes isolated from IGRA$^-$ or active TB patients. In IGRA$^-$ monocytes the bacteria were able to grow; however, this growth was much more limited in the case of active TB monocytes (Figure 2H). Moreover, when we compared the ability of monocytes from active TB patients to control *M. tuberculosis* before and after antibiotherapy, we found that after treatment the growth of bacteria was superior for all tested donors (Figure 2I).

Altogether, our data demonstrate that in a mouse model mimicking the human transcriptional signature of TB, BM monocytes or macrophages did not acquire trained immunity despite the occurrence of EM. However, untreated active TB in humans appeared to associate with circulating monocytes that display an increased capacity to control *M. tuberculosis* growth in *in vitro* infections.

3.4 Impact of IFNγ deficiency on emergency myelopoiesis upon infection with *M. tuberculosis* HN878

As IFNγ has been widely implicated in EM (38, 39) and we observed an increase of the expression of this cytokine in the BM of *M. tuberculosis*HN878-infected mice (Figure 1I), we sought to investigate whether its presence was required for the EM observed in the C57BL/6 model of infection. Thus, we aerosol-infected C57BL/6 WT and IFNγ$^{-/-}$ mice with a high dose of *M. tuberculosis* HN878. IFNγ$^{-/-}$ mice presented higher lung bacterial burdens than WT mice, in agreement with their known inability to cope with *M. tuberculosis* infections (Figure 3A). In the same line, histological analysis revealed more extensive lesions in IFNγ$^{-/-}$ mice than in WT mice, with damaged tissue and pulmonary exudate visible in areas adjacent to lesions (Figure 3B). Both the percentage of lesion (Figure 3C) and the lesion score (Figure 3D) were increased in infected IFNγ$^{-/-}$ mice compared with WT mice. A lack of IFNγ did not impact the number of total or immune (CD45$^+$) lung cells (Figure 3E). The numbers of monocytes, recruited macrophages, and neutrophils present in the lungs of IFNγ competent or deficient mice were also similar (Figure 3F). Despite this overall similarity in myeloid cell composition in the lungs of infected WT and IFNγ$^{-/-}$ mice, the increase of monocytes and neutrophils seen in the blood of infected WT mice was not observed in the absence of IFNγ (Figure 3G). We then focused on analyzing the BM compartment. As observed in the lung, IFNγ$^{-/-}$ mice presented a higher bacterial burden in the BM than WT mice (Figure 3H), with BM cellularity (Figure 3I) and the variations in the number of monocytes (Figure 3J) similar in the two mouse strains. However, IFNγ$^{-/-}$ mice exhibited a reduction in BM neutrophils compared with WT mice (Figure 3J). These data indicate that, in the absence of IFNγ, the accumulation of monocytes and neutrophils observed in the lung is not fully reflected in the blood and the BM, as seen for WT mice. Although this finding is not fully understood, it may suggest a faster recruitment of neutrophils to the lung, with increased exit rates from the BM, or alternatively an increased lifespan of neutrophils in the lung.

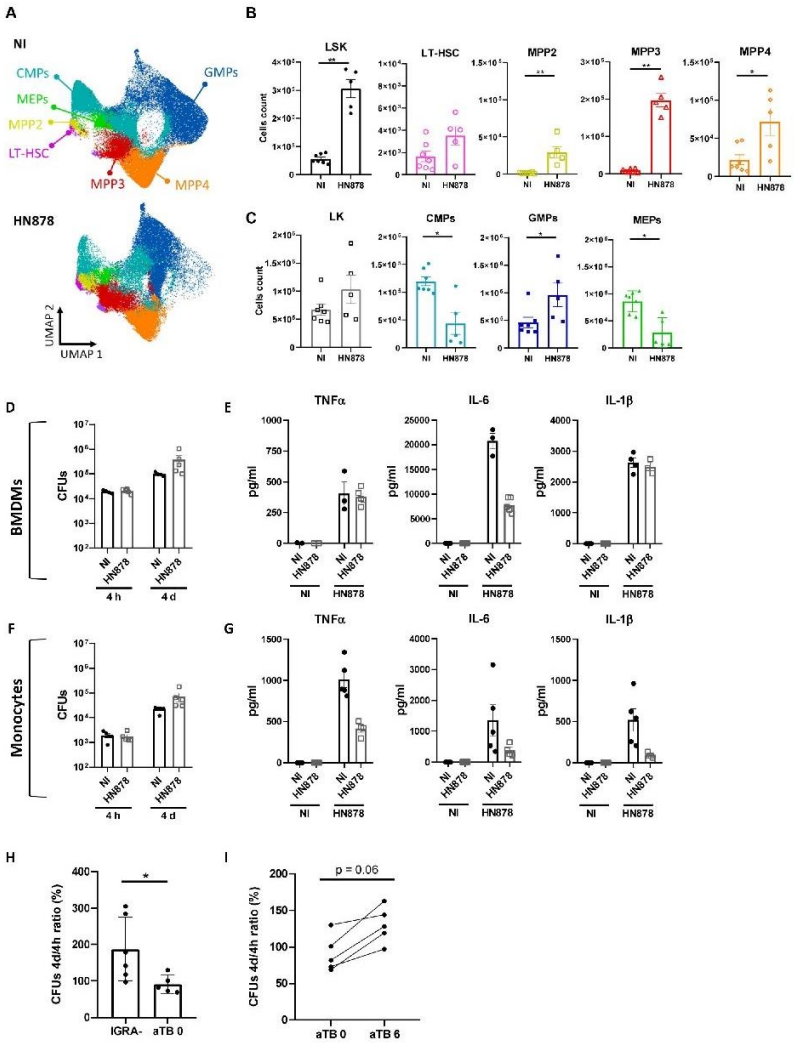

FIGURE 2
Aerosol infection with high doses of *M. tuberculosis* HN878 leads to emergency myelopoiesis but does not result in trained immunity. Bone marrow cells of C57BL/6 mice aerosol-infected with high doses of *M. tuberculosis* isolate HN878 were recovered and analyzed by flow cytometry **(A–C)** or used to investigate trained immunity **(D–G)**. Non-infected (NI) mice (8 weeks old) were used as controls. **(A)** UMAP of flow cytometry data overlaying different immune progenitor populations: CMPs ($CD34^{hi}Fc\gamma R^{-}$), GMPs ($CD34^{hi}Fc\gamma R^{+}$), MEPs ($CD34^{lo}Fc\gamma R^{-}$), LT-HSC ($Flt3^{-}CD150^{+}CD48^{-}$), MPP2 ($Flt3^{-}CD150^{+}CD48^{+}$), MPP3 ($Flt3^{-}CD150^{-}CD48^{+}$), and MPP4 ($Flt3^{+}CD150^{-/int}$). **(B)** Total cell count of LSK ($Lin^{-}Sca1^{+}c\text{-}Kit^{+}$) progenitor and LSK cell subpopulations: LT-HSC, MPP2, MPP3, and MPP4. **(C)** Total counts of LK ($Lin^{-}Sca1^{-}c\text{-}Kit^{+}$) cells and LK subpopulations: CMPs, GMPs, and MEPs, in the bone marrow. Gating strategies are shown in Supplementary Figure 2A. Bone marrow-derived macrophages were generated from NI (closed circles) or *M. tuberculosis* HN878-infected (open squares) mice. Mature cells (day 7 of culture) were infected with the same isolate of *M. tuberculosis* at an MOI of 2. **(D)** Bacterial burdens were determined by CFU enumeration after 4 h and 4 days of infection. **(E)** The production of cytokines TNFα, IL-6, and IL-1β was measured in non-stimulated (NS) or infected (HN878) cell cultures by immunoassay 24 h post-infection. Monocytes were isolated from the bone marrow of NI (closed circles) or *M. tuberculosis*-infected (open squares) mice for **(F)** CFU determination **(F)** or protein analysis **(G)**, as before. **(H)** Monocytes ($CD14^{+}$) were isolated from the blood of $IGRA^{-}$ healthy donors or active TB patients at presentation (aTB 0) or after 6 months of antibiotherapy (aTB 6), and infected with *M. tuberculosis* for 4 h or 4 days, for bacterial burden determination. The percentage increase of CFU from 4h to 4 days of *in vitro* infection is shown. Data are mean ± SEM. Each dot represents an individual mouse, distributed in two independent experiments represented per **(B–G)** or an individual donor **(H, I)**. The infection doses (in CFU ± SEM)/animal ages (in weeks old at the time of infection) for the experiments used in this figure were 1,093 ± 100/8 and 650 ± 110/8. The statistical analyses were performed using a Mann–Whitney U test to identify statistical differences between groups. p-values inferior to 0.05 were deemed significant: *p-value<0.05 and **p-value<0.01.

Next, we questioned whether EM would occur in IFNγ$^{-/-}$ mice upon *M. tuberculosis* HN878 infection. Similar to the WT controls, UMAP reduction analysis of flow cytometry data disclosed differences in precursor and progenitor cell composition profiles between infected and non-infected IFNγ$^{-/-}$ mice (Figure 3K). The analysis of precursor populations revealed an overall similar tendency between WT and IFNγ$^{-/-}$ mice upon infection, with an increase of all populations in infected mice compared with uninfected mice (Figure 3L). However, for some populations, such as LSK, MPP2, and MPP3, this increase was less marked in the case of IFNγ$^{-/-}$ mice, (Figure 3L). A similar profile was observed in progenitor populations, with less pronounced alterations seen in IFNγ$^{-/-}$ mice (Figure 3M). Of note, the most marked change observed in IFNγ$^{-/-}$ mice upon infection was an increase in the GMP population (Figure 3M). Similar findings were observed in terms of cell population frequencies (Supplementary Figure 3). Altogether, these findings suggest that EM in response to severe *M. tuberculosis* infections might occur, at least to some extent, even in the absence of IFNγ. EM has been linked to inflammatory environments in the BM (40). Thus, we questioned whether the lack of IFNγ would alter the inflammatory environment seen in the BM of infected WT mice (Figure 1I). Interestingly, the transcription of several cytokine genes in the BM was decreased in infected IFNγ$^{-/-}$ mice compared with WT or uninfected mice (Figure 3N). However, this was not the case for TNF (Figure 3N), the transcription of which was enhanced by the infection independently of IFNγ (Figure 3N).

Together these data suggest that IFNγ is partly dispensable for the occurrence of EM upon the infection of mice with *M. tuberculosis* HN878, consequently suggesting the occurrence of alternative mechanisms.

4 Discussion

TB remains a major health problem, with increasing numbers of new cases and fatalities reported over the past 2 years (18). Neutrophils have been consistently associated with poor outcomes of TB in humans and experimental models of TB (41, 42). Accompanying the enhanced neutrophil blood signature present in TB patients (43), an under-abundance of B cell, NK cell, and effector T cell signatures was also identified in active TB, progression from latent TB, and TB-susceptible mice (27). Decreased levels of mRNAs associated with lymphocyte lineages and increased expression of those associated with myeloid differentiation were also associated with progression from latent to active TB (44). Thus, a myeloid-biased remodeling of the hematopoietic landscape likely accompanies TB disease progression and establishment, which may at least partially result from the adaptation of the BM to the infection, as seen in other infection and inflammatory settings (2, 45). To experimentally address this hypothesis, we characterized the alterations imposed by *M. tuberculosis* infection on the mature and progenitor cellular populations in the BM. As such, C57BL/6 mice were aerosol-infected with high doses of *M. tuberculosis* isolate HN878. This experimental condition results in severe TB disease and is the one condition to better recapitulate the blood transcriptomic signatures of TB patients in the mouse C57BL/6 genetic background (27). The *M. tuberculosis* HN878 isolate has been described as hypervirulent as a result of enhanced induction of type I IFN and deregulated Th1 immunity (46, 47). More recently, infections with *M. tuberculosis* HN878 were also shown to lead to neutrophil overactivation by type I IFN, with exacerbated NETosis formation and extensive tissue pathology (31).

We observed alterations to the BM cell progenitor composition, namely an increased frequency of LSK and GMP cell populations, together with a decrease in the CMP population. Furthermore, we also observed an expansion of monocytes in the BM, blood, and lung and of neutrophils in the blood and lung. Altogether, our data support the occurrence of EM as a consequence of *M. tuberculosis* infection. Additionally, a previous study identified an increase in the LSK population but not in GMPs upon *M. tuberculosis* infection (24). However, in that study, a different strain of *M. tuberculosis*, the laboratory reference strain H37Rv, was used and the aerosol infection was performed with low bacilli doses, which results in a milder outcome of infection, with the mice surviving for long time periods and presenting lower bacterial burdens in the lungs and BM (24). Most notably, the blood transcriptional signature observed in TB patients was not reproduced in such experimental conditions (27). Therefore, it is likely that the discrepancies seen in terms of GMP dynamics upon infection are reflecting the severity of the infection and contributing to the characteristic TB blood immune signature. In line with this, we show that infections of mice with low doses of *M. tuberculosis* HN878 did result in BM colonization but not in EM. Additionally, it is important to emphasize that infections with the *M. tuberculosis* HN878 isolate, such as those performed in this study, induce a relatively rapid progression to disease and may thus be reflecting the peak of active TB, rather than a long-term chronic infection. The differences seen in the GMP dynamics between high and low doses of infection, or between isolates of distinct virulence, may also reflect BM adaptation to these two different settings: peak of TB disease vs. chronic TB infection.

Independently of the distinct BM adaptation to infections by the different *M. tuberculosis* isolates, a common point is the resulting impaired trained immunity. In fact, we observed decreased cytokine production in response to secondary challenge by monocytes isolated from infected BM. It is possible that the environment the BM is subjected to by hypervirulent strains of *M. tuberculosis* may promote innate cell tolerance. This is an intriguing question worth investigating in the future. Interestingly, trained immunity has previously been suggested to equip monocytes from individuals recently exposed to *M. tuberculosis* with increased capacity to control BCG (37). By comparing the ability of monocytes purified from IGRA^{-} donors or from active TB patients at presentation or post-treatment, we also report that the best controllers of *M. tuberculosis* growth are monocytes from active TB patients before treatment. Therefore, it is tempting to speculate that training of myeloid cells in the context of *M. tuberculosis* may be achieved in the periphery rather than in the BM. Related to this, training of alveolar macrophages has been described in several studies, namely upon viral infection (48–50). Further exploring these questions using genetically distinct isolates of *M. tuberculosis* and strains of

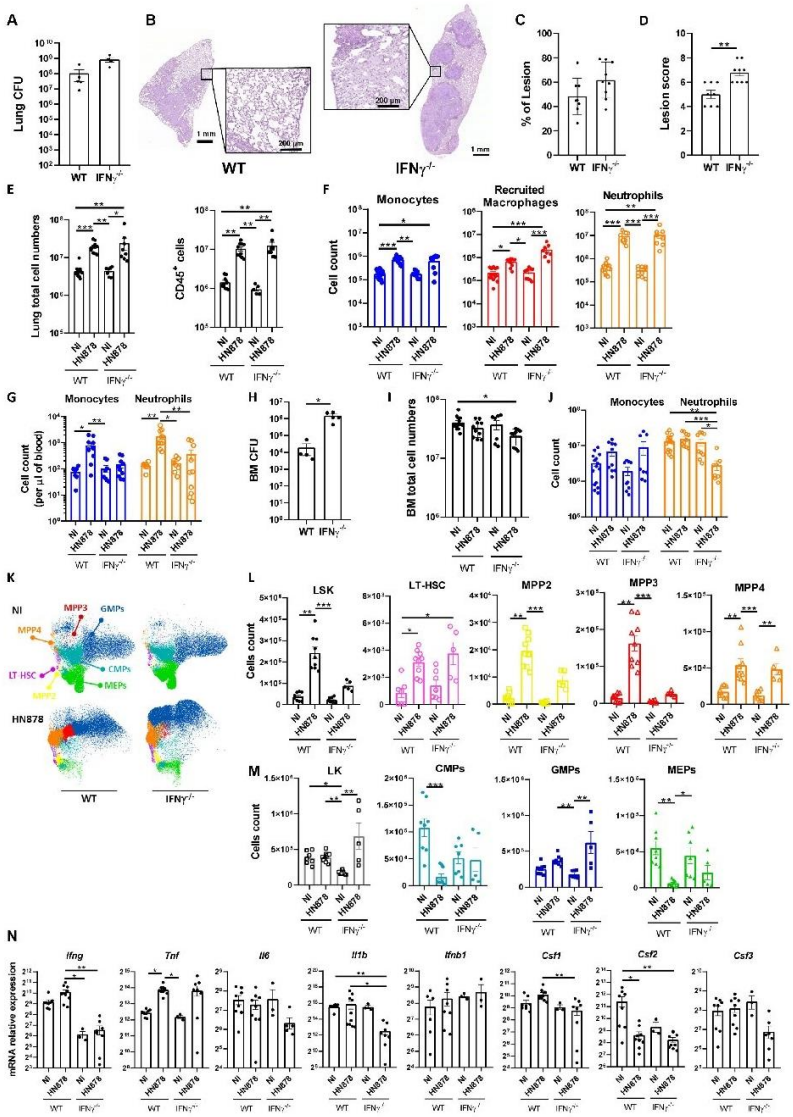

FIGURE 3
Role of IFNγ in *M. tuberculosis*-induced emergency hematopoiesis. C57BL/6 WT or IFNγ$^{-/-}$ mice were aerosol-infected with high doses of *M. tuberculosis* isolate HN878. When animals reached humane endpoints the experiment was terminated. This happened between days 24 and 27 post-infection. At these time points, the bacterial burden in the lungs was determined **(A)**. **(B)** Representative histological images of H&E-stained lung sections from *M. tuberculosis*-infected WT or IFNγ$^{-/-}$ mice. Zoomed areas show lesion-adjacent tissue. **(C)** Morphometric quantification of the percentage of lesion in the lung section. **(D)** Histopathological score attributed to each lesion. **(E)** Number of total cells (left) and immune cells (right) in the lungs of NI or *M. tuberculosis*-infected mice. Immune cells were determined by FACS based on the detection of surface CD45. **(F)** Number of monocytes, recruited macrophages, and neutrophils in NI or *M. tuberculosis*-infected mice. Gating strategies are shown in Supplementary Figures 1A, B. **(G)** Monocyte and neutrophil cell counts per µl of blood of NI or *M. tuberculosis*-infected mice. **(H)** Bacterial burdens in the bone marrow of WT or IFNγ$^{-/-}$ mice 24–27 days post-infection. **(I, J)** Total cell numbers **(I)** and numbers of monocytes and neutrophils **(J)** in the bone marrow of NI and infected mice. **(K)** UMAP of flow cytometry data overlaying different immune progenitor populations: CMPs (CD34^{hi}FcγR^{-}), GMPs (CD34^{hi}FcγR^{+}), MEPs (CD34^{lo}FcγR^{-}), LT-HSC (Flt3^{-}CD150^{+}CD48^{-}), MPP2 (Flt3^{-}CD150^{+}CD48^{+}), MPP3 (Flt3^{-}CD150^{-}CD48^{+}), and MPP4 (Flt3^{+}CD150$^{-/int}$). **(L)** Total cell count of LSK (Lin^{-}Sca1^{+}c-Kit^{+}) progenitor and LSK cell subpopulations: LT-HSC, MPP2, MPP3, and MPP4. **(M)** Total counts of LK (Lin^{-}Sca1^{-}c-Kit^{+}) cells and LK subpopulations: CMPs, GMPs, and MEPs, in the bone marrow. Gating strategies are shown in Supplementary Figure 2A. **(N)** Transcription analysis of the indicated genes detected by qPCR in the bone marrow of NI or *M. tuberculosis*-infected WT or IFNγ$^{-/-}$ mice. Data are mean ± SEM. Each dot represents an individual mouse, distributed in two independent experiments represented per panel. The infection doses (in CFU ± SEM)/animal ages (in weeks old at the time of infection) for the experiments used in this figure were 1,093 ± 100/8 for WT and 11–12 for IFNγ$^{-/-}$ and 1,057 ± 74/9–10 for WT and 9 for IFNγ$^{-/-}$. Non-infected animals were 10–11 weeks old. The statistical analyses were performed using a Mann–Whitney U test when comparing only two groups, or a Kruskal–Wallis one-way analysis of variance to identify statistical differences between groups. p-values inferior to 0.05 were deemed significant: *p-value<0.05, **p-value<0.01, and ***p-value<0.001.

mice is exciting, as uncovering potential mechanisms of trained immunity in circulating monocytes and resident macrophages may unleash novel strategies to tackle TB.

The mechanisms underlying BM adaptation during TB, and how they may articulate with immunity at the site of infection, remain elusive. In line with several previous studies (19, 20, 24), we show colonization of the BM by *M. tuberculosis*. Thus, the presence of the bacteria in the BM may directly impact on the hematopoietic skewing, e.g., by activating pattern recognition receptors expressed on HSCs or progenitor cells (2, 45). On the other hand, the local and systemic inflammatory response caused by the infection may play a key role in modulating the hematopoietic niche and prioritizing myelopoiesis. Indeed, several cytokines have been described to modulate myelopoiesis, including type I and type II IFNs, IL-1, TNFα, IL-6, IL-27, and colony stimulating factors (2, 45). Analysis of the transcription of several of these molecules in the BM upon infection with high doses of *M. tuberculosis* HN878 revealed only modest changes. However, as we are analyzing bulk RNA and one time point only, we cannot exclude an elevation of some of these or other molecules in the BM niche over time. Of note, IFNγ has been shown to play such a role in the context of *M. avium* and *M. bovis* BCG infections (5, 10), but not in other settings (17), which suggests a context-dependent action. In the context of aerosol infection with high doses of *M. tuberculosis* HN878, IFNγ appears to be partly dispensable. It is possible that the excessive inflammation resulting from infections by *M. tuberculosis* HN878 may surpass on one hand the requirement of IFNγ for myelopoiesis, as seen in BCG (10), and on the other hand, the limiting effect of type I IFN, as seen in *M. tuberculosis* H37Rv infections (24). Whether the global alteration to the hematopoietic niche or a particular cytokine are key effectors in our setting remains to be addressed. The most marked alteration in the BM of infected IFNγ$^{-/-}$ mice was the pronounced increase of GMPs. This is interesting because even though neutrophils accumulated in the lungs of these mice, an increase of these cells in the blood was not observed. In addition to the differential recruitment of neutrophils to the lungs of IFNγ$^{-/-}$ mice, it is also conceivable that the full differentiation of GMPs is not achieved in the absence of IFNγ. Finally, it is worth mentioning that compared with WT mice, IFNγ$^{-/-}$ mice show increased lung bacterial burdens and lesion scores in the absence of increased cellularity. It is possible that a lack of IFNγ in infections with *M. tuberculosis* HN878 may not impact the already high amount of neutrophils in the lung described for this isolate (31) but contribute to the worsening of lesions by failing to control neutrophil responses, as described in the context of *M. tuberculosis* H37Rv infections (51).

In conclusion, this study adds to the expanding evidence linking BM alterations to myeloid/lymphoid imbalances in TB. Understanding the crosstalk between central hematopoiesis and local immune responses may provide novel insights into the immune response to TB, which is an important driver of TB disease. This may in turn benefit the design of novel host-directed therapies (52, 53), which are acknowledged as promising strategies to mitigate TB severity and mortality.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The study protocol leading to peripheral blood mononuclear cells (PBMC) isolation was approved by the Health Ethics Committees of the Centro Universitário Hospitalar de S. João (approval number 109-11), the North Health Region Administration (approval number 71-2014) and the Portuguese Data Protection Authority (approval number 12174-2011). To ensure confidentiality, each case was anonymized by the assignment of a random identification number. Participants were enrolled at a TB clinic in Porto and provided informed consent. Experiments were conducted according to the principles expressed in the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study. 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).

Author contributions

AM, DS, and MS designed the experiments, analyzed the data, and wrote the manuscript; AM, DS, RG, and MC performed the experiments; MS obtained funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1211404/full#supplementary-material

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Supplementary Material

Infection with hypervirulent *Mycobacterium tuberculosis* triggers emergency myelopoiesis, but not trained immunity

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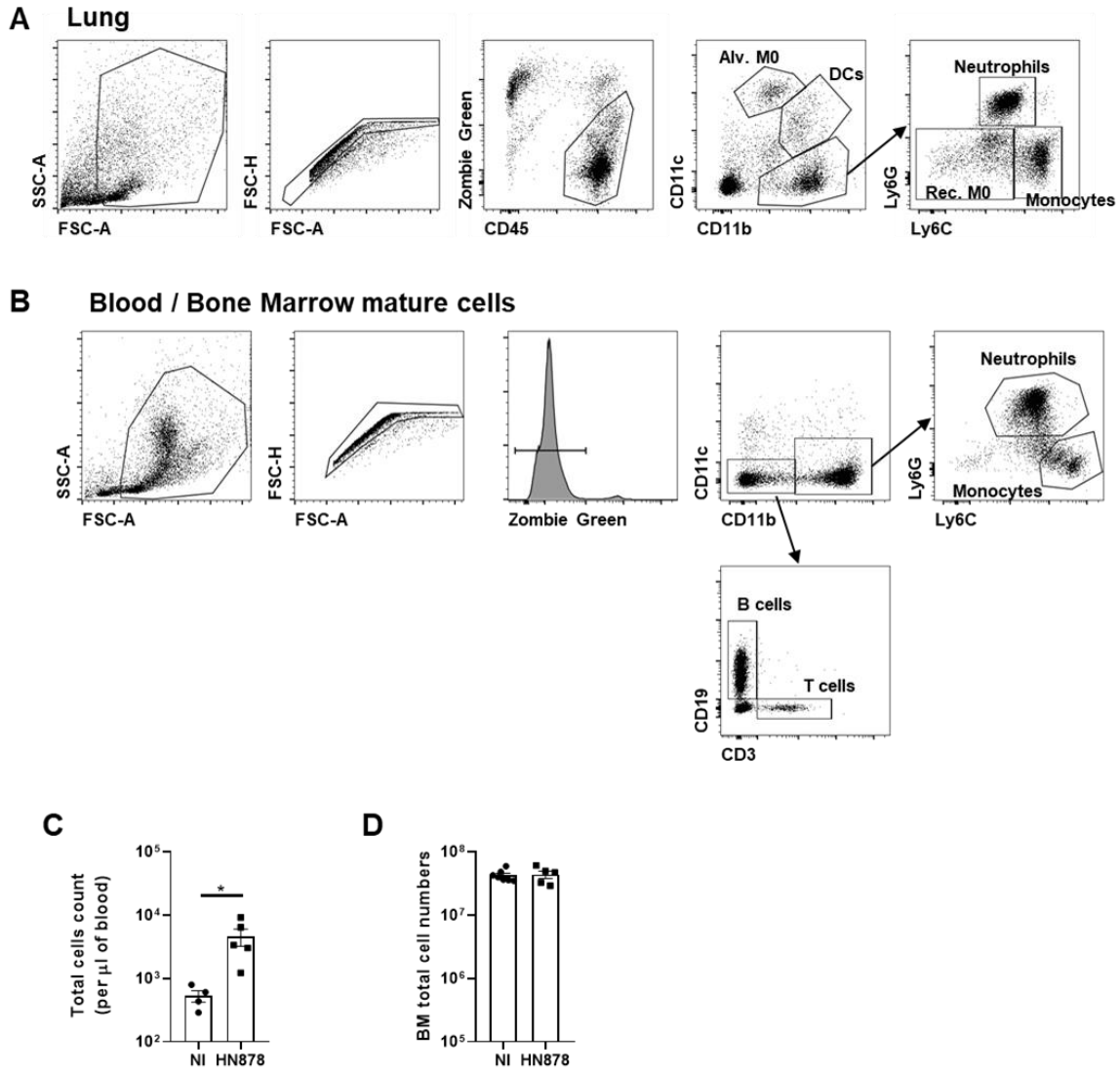
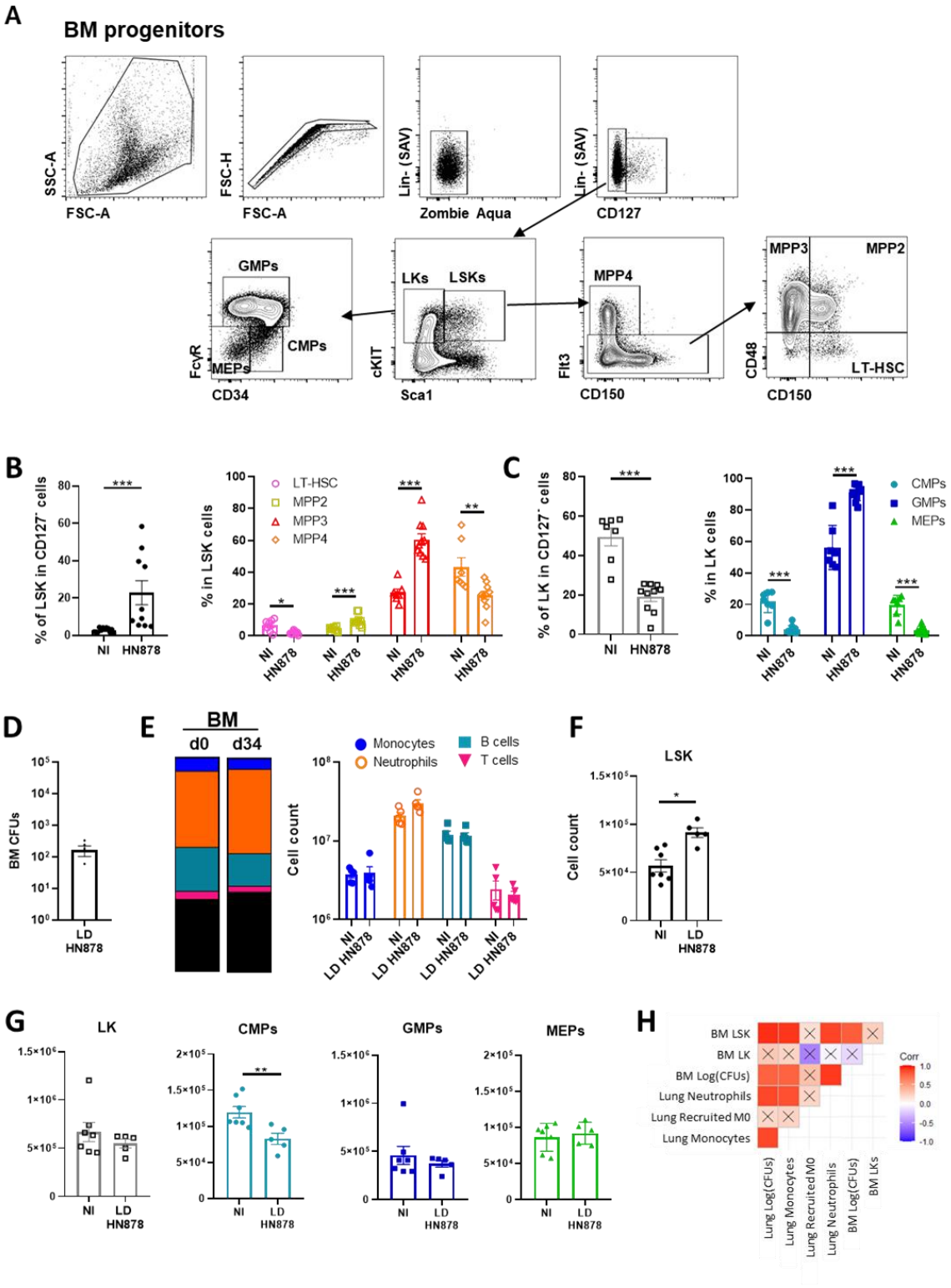
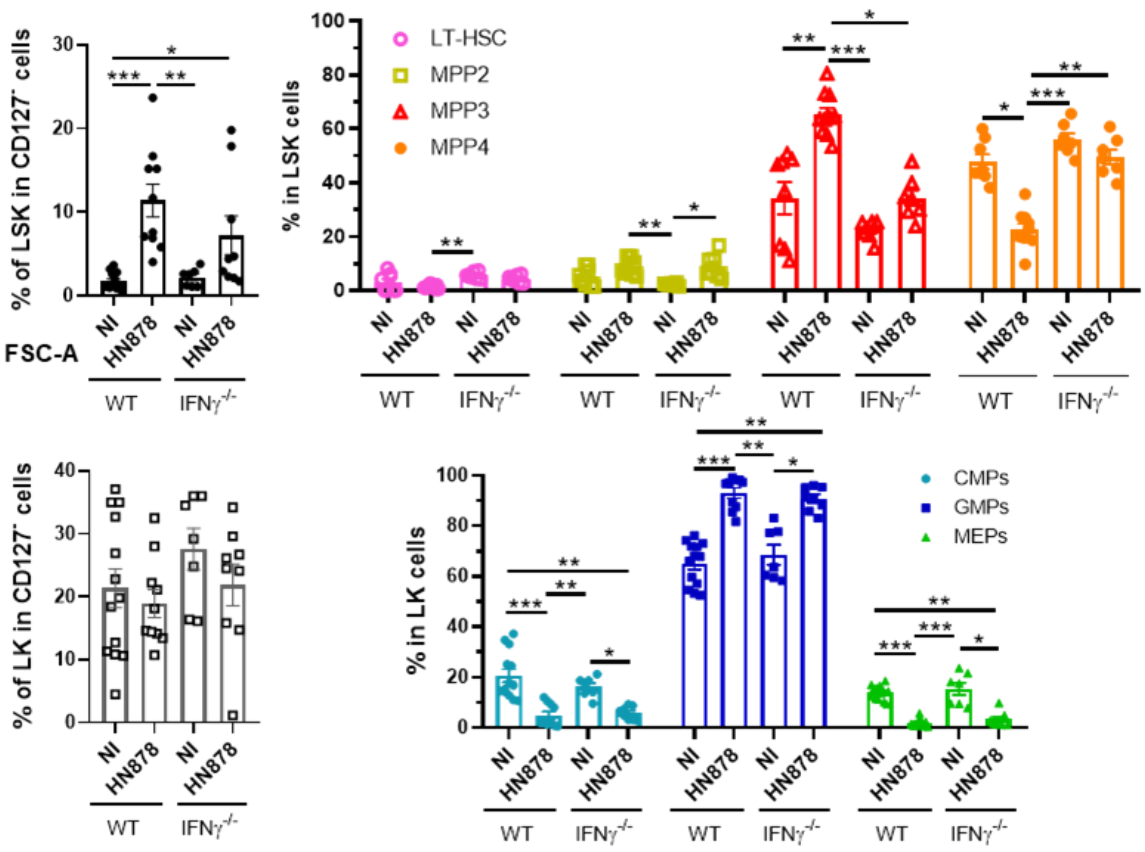


Supplementary Figure 1. Gating strategy used to analyse lung (A) and blood and bone marrow (B) immune mature cell populations. Total cell numbers present in the blood (C) or the bone marrow (D) of non-infected (NI) or *M. tuberculosis*-infected (HN878) mice. (C,D) Represented are Mean±SEM. Each dot represents an individual mouse, distributed in two independent experiments. The infection doses (in CFU±SEM) / animal ages (in weeks old at the time of infection) for the experiments used in thisfigure were 683±125 / 10; 1093±100 / 8; and 1057±74 / 9 and 10. Non- infected animals were 8 weeks old. The statistical analyses were performed using Mann–Whitney U test to identify statistical differences between groups. p-values inferior to 0.05 were deemed significant: * p-value < 0.05. Alv. M0, alveolar macrophages; DCs, dendritic cells; Rec. M0, recruited macrophages.



Supplementary Figure 2. (A) Gating strategy used to analyse bone marrow progenitor and precursor cell populations. (B-C) Frequencies of bone marrow precursor (B) and progenitor (C) cell populations in non-infected (NI) mice or mice infected with high doses of *M. tuberculosis* HN878. (D-G) C57BL/6 mice were infected via aerosol with low doses (LD) of *M. tuberculosis* HN878 isolate. On day 34 post-infection their bone marrow was analysed for bacterial burden (D), mature (E), progenitor (F) and precursor (G) cell populations. (H) Plot of Spearman's correlation between

Log10(CFUs), numbers of lung immune cell populations (monocytes, recruited macrophages and neutrophils), bone marrow CFUs, LSK and LK cell populations. Data used are from mice infected with low dose or high dose of *M. tuberculosis* HN878 for 34 or 27 days, respectively. Non-significant correlations are identified with a cross. (B-G) Represented are Mean±SEM. Each dot represents an individual mouse, distributed in two independent experiments. The infection doses (in CFU±SEM) / and animal ages (in weeks old at the time of infection) for the experiments used in this figure were: 1093±100 / 8 and 650±110 / 8 for high dose infections; 185±10 / 12 for low dose infection. Non-infected animals were 8 weeks old. The statistical analyses were performed using Mann–Whitney U test to identify statistical differences between groups. p-values inferior to 0.05 were deemed significant: * p-value < 0.05; ** p-value<0.01 and *** p-value < 0.001.



Supplementary Figure 3. Frequencies of bone marrow precursor and progenitor cell populations in C57BL/6 WT or IFNγ deficient non-infected (NI) mice or mice infected with high doses of *M. tuberculosis* HN878. Represented are Mean±SEM. Each dot represents an individual mouse, distributed in two independent experiments. The infection doses (in CFU±SEM) / and animal ages (in weeks old at the time of infection) for the experiments used in this figure were 1093±100 / 8 for WT and 11-12 for IFNγ$^{-/-}$; and 1057±74 / 9-10 for WT and 9 for IFNγ$^{-/-}$. Non- infected animals were 10-11 weeks old. The statistical analyses were performed using Kruskal–Wallis one-way analysis of variance to identify statistical differences between groups. p-values inferior to 0.05 were deemed significant: * p-value < 0.05, ** p-value < 0.01 and *** p-value < 0.001.

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

Flow cytometry – Mouse bone marrow, blood, and lung – mature immune cells		
Target	**Clone**	**Company (Catalog #)**
CD45	30-F11	BioLegend (103112)
CD11c	N418	BioLegend (117318)
CD11b	M1/70	BioLegend (101245)
Ly-6G	1A8	BioLegend (127616)
Ly-6C	HK1.4	BioLegend (128014)
CD3	145-2C11	BioLegend (100308)
CD4	GK1.5	BioLegend (100453)
CD8	53-6.7	BioLegend (100741)
CD19	6D5	BioLegend (115530)
Lineage depletion – Mouse bone marrow precursors (Lin^+)		
Target	**Clone**	**Company (Catalog #)**
CD3	145-2C11	BioLegend (100304)
CD4	GK1.5	BioLegend (100404)
CD8	53-6.7	BioLegend (100704)
CD11c	N418	BioLegend (117304)
CD11b	M1/70	BioLegend (101204)
GR1	RB6-8C5	BioLegend (108404)
CD19	6D5	BioLegend (115504)
B220	RA3-6B2	BioLegend (103204)
Nk1.1	PK136	BioLegend (108704)
TER-119	TER-119	BioLegend (116204)
Flow cytometry – Mouse bone marrow progenitors (Lin^-)		
Target	**Clone**	**Company (Catalog #)**
Ly-6A/E (Sca-1)	E13-161.7	BD Pharmigen (553335)

CD16/CD32 (FcγR)	2.4G2	BD Pharmigen (560540)
CD48	HM48-1	BioLegend (103412)
CD117 (c-kit)	2B8	BioLegend (105825)
CD34	RAM34	Invitrogen (48-0341-82)
CD150	TC15-12F12.2	BioLegend (115941)
CD135 (Flt3)	A2F10	BioLegend (135305)
CD127 (IL-7Rα)	A7R34	BioLegend (135014)
Streptavidin (SAV)		BioLegend (405249)

Supplementary Table 2. List of oligonucleotides used in this study.

Gene	Forward (5'-3')	Reverse (5´-3')
Csf1	GTGTCAGAACACTGTAGCCAC	TCAAAGGCAATCTGGCATGAAG
Csf3	ATGGCTCAACTTTCTGCCCAG	CTGACAGTGACCAGGGGAAC
Tnf	GCCACCACGTCTTCTGTCT	TGAGGGTCTGGGCCATAGAAC
Il1b	ACCTTCCAGGATGAGGACATGA	AACGTCACACACCAGCAGGTTA
Il6	ACACATGTTCTCTGGGAAATCGT	AAGTGCATCATCGTTGTTCATACA
Ifng	CAACAGCAAGGCGAAAAAGG	GGACCACTCGGATGAGCTCA
Ifnb	GCACTGGGTGGAATGAGACT	AGTGGAGAGCAGTTGAGGACA
Ubiquitin	TGGCTATTAATTATTCGGTCTGCAT	GCAAGTGGCTAGAGTGCAGAGTAA

Supplementary Table 3. Pathological scoring analysis of Hematoxylin and Eosin-stained lung histological sections.

Parameter	
Lesion	**Description**
0	Absence of lesions or alterations in the lung
1	Lung with onset of lesion: • One or few very small infiltrates of inflammatory cells; • Composed of myeloid cells; • Perivascular location; • No other lesion signs present.
2	Lung with minor lesion: • One or few small but well-formed infiltrates of inflammatory cells; • Composed mainly of myeloid cells such as macrophages and some lymphoid cells; • Perivascular location; • No other lesion signs present.
3	Lung with moderate lesion: • One or few medium-sized lesions or small multifocal lesions; • Composed of cells such as macrophages/MN, neutrophils, and lymphocytes; • May present congestion.
4	Lung with extensive lesion: • Several medium-sized lesions or a few very extensive ones; • Composed of various types of inflammatory cells; • Presence of some areas of necrosis or cellular debris and/or congestion.
5	Lung with very extensive lesion: • Lung almost entirely covered in lesions; • Composed of various types of inflammatory cells;

	• Zones of necrosis in the center of the lesions and other damage elements such as congestion, edema and damaged epithelium.
Necrosis/ Cell death	**Description**
0	Absence of necrotic zones or cellular death.
1	Small/few localized zones with necrosis.
2	Lung with extensive necrosis.
Exudate/ Pulmonary edema	**Description**
0	Lung without edema.
1	Small areas with fluid/pulmonary edema.
2	Lung with vast areas of edema.
Fibrosis/ Calcification	**Description**
0	Absence of fibrosis and calcification.
1	Presence of fibrosis or calcification in an area of the lung.
2	Presence of fibrosis or calcification in more than one area of the lung.
Final Score	**Sum of the score for each parameter**

CHAPTER V – GENERAL DISCUSSION

The TB burden worldwide had been slowly declining since 2003. However, for the first time in several years, there was an increase of the estimated cases and deaths of TB in 2021 (1). While some countries have managed to attain the goals of the End TB Strategy by 2020, there are still countries with very high TB burdens (1, 2). In many of these countries, there is a high association between poverty and TB incidence (3). One way to improve the fight against TB, particularly in lower income countries, is to develop new and cheaper TB diagnostic methods and therapies (4). It is clear now that there is high heterogeneity in clinical progression of TB (5), and there is increasing evidence for the role of genetic diversity within the MTBC in the diverse clinical manifestations of TB that may occur (6, 7). This evidences the need to further dissect the interactions of the pathogen and the host, taking into account both host factors and bacterial factors, as well as how the surrounding environment may shape their interaction (8).

In this thesis, we have enhanced the understanding of the early immune events during host-pathogen interactions in TB and how bacterial diversity may lead to differences in the early immune response, which may in turn shape the course of clinical progression during *M. tuberculosis* infection (9). Building on previous work from our group (10), we have now shown that the differential IL-1β induction by clinical isolates 4I2 and 6C4 is maintained in several innate immune cells, as well as in an *in vivo* setting. Verifying that a generalized lack of IL-1R signaling increased TB severity in infections with either isolate was not surprising, given the known crucial role for IL-1R signaling in the immune response to TB (11-17). However, the extent to which the severity increased in infection with each isolate is revealing of how an adequate IL-1 response in the early immune events is key to determine the balance between an underwhelming or exaggerated immune response.

Seeing that the higher induction of IL-1β by isolate 4I2 was associated with lower severity of disease (10), we speculated that this increased IL-1 response induced by isolate 4I2 benefited the host. Indeed, that appears to be the case, as the lack of IL-1R signaling led to a higher increase in bacterial burden for infections with this isolate when compared to infections with isolate 6C4. The ability for isolate 6C4 to evade cytosolic surveillance pathways was previously associated to the lower induction of IL-1β by this isolate (10). If this induction is already low, the lack of IL-1R signaling would not make as much of a difference as in the case of infections with isolate 4I2. This work further supports the protective role for an adequate IL-1β induction in the mounting of the immune response, as well as evidencing the modulation of IL-1β as an escape mechanism from the host immune response.

The IL-1R signaling was reported to be particularly crucial in the immune response by AMs, the first responders to *M. tuberculosis* infection (18). Indeed, IL-1R signaling is necessary for the migration of infected AMs into the lung interstitium, which affects the

subsequent immune response (18, 19). However, there was little information on how the immune response by AMs themselves was impacted by the lack of IL-1R signaling in TB. Here, we have demonstrated that IL-1R$^{-/-}$ AMs are impaired in their immune response during TB, as shown by the prominent decrease in expression of immune-associated genes in *M. tuberculosis* infected AMs from IL-1R$^{-/-}$ mice. Even at their steady state, the homeostasis of IL-1R$^{-/-}$ AMs seems already impaired, with a much lower induction of genes related to AM fitness and self-renewal than IL-1R$^{+/+}$ AMs. This indicates that IL-1R signaling is necessary not only for fighting infection, but most likely for the full development of AMs in the alveolar niche. Another important aspect observed in this study was the higher depletion of AMs in IL-1R$^{-/-}$ mice in the course of infections with isolate 4I2, which was in contrast with the increase of AMs in IL-1R$^{+/+}$ mice. Indeed, it had been previously observed that IL-1R signaling prevented the depletion of AMs during influenza and *Streptococcus pneumoniae* co-infection (20). Such seems to be the case in *M. tuberculosis* infections as well, which further evidences the role for IL-1R signaling in maintaining AMs during infection. Nonetheless, it is still unclear if this effect is caused by IL-1R signaling directly on AMs, or by the lack of IL-1R signaling in other cells. For instance, the correct development and functioning of AMs is associated with the production of GM-CSF by type II AECs (21, 22). It is possible that the lack of IL-1R signaling in other cells, from either hematopoietic or non-hematopoietic origin (such as type II AECs), could be the culprit for the incorrect development of AMs.

Although investigating this interesting hypothesis will be further developed in new studies, results from our study indirectly hint that this may be a plausible explanation. Indeed, when depleting IL-1R signaling only on myeloid cells, instead of a diminished immune response, the opposite was observed, with a higher expression of immune-coding genes and higher production of immune mediators by mIL-1R$^{-/-}$ mice. Thus, while the lack of a generalized IL-1R signaling led to an under activation of the immune response, the lack of IL-1 myeloid signaling does not result in the same. It appears that IL-1R signaling in other cells, possibly of non-hematopoietic origin, may reflect on the correct development of innate immune cells such as AMs, while the AM intrinsic IL-1R signaling may reflect on their adequate production of immune mediators to fight off infection. Indeed, the overt activation of the immune response, with higher production of pro-inflammatory cytokines and higher infiltration of immune cells into the lung appears to cause a detrimental immune response, leading to increased bacterial burden early in the infection, as well as extensive lung pathology in the lung. Despite the lack of myeloid IL-1R signaling, the production of IL-1β in the lungs was higher for mIL-1R$^{-/-}$ mice. Excessive IL-1 production can be detrimental in the immune response (23), and suppression of IL-1 responses in animal models after two weeks of infection has had positive results in diminishing lung pathology associated with

excess inflammation in TB (24). Thus, this study evidences the need to find the balance between protection or pathology mediated by IL-1R signaling. It would be very interesting to develop an HDT to explore the spatiotemporal dynamics of IL-1β modulation taking into consideration the host, the pathogen and the extrinsic factors that may affect their interaction. Ideally, we would aim at ensuring that the initial IL-1β response would be sufficient to ensue a protective immune response, while its control at later stages of infection or at certain anatomical locations would benefit the host by reducing excessive inflammation and tissue injury. Interestingly, the role of IL-1R signaling has also been associated with the expansion of HSPCs and myelopoiesis (25, 26). While we did not explore the effect on IL-1R signaling directly in the BM of mice infected with these clinical isolates, the lower influx of neutrophils into the lungs of IL-1R$^{-/-}$ mice for infections with both isolates may be the reflection of a lower myelopoietic output in these mice.

As previously mentioned, *M. tuberculosis* is able to colonize and alter the homeostasis of the HSC niche, leading to alterations in the hematopoietic output of the BM (27). During infection, EM may be prioritized to accommodate the need of an initial immune response by innate immune cells. Thus, the way *M. tuberculosis* may alter the hematopoietic niche will reflect on the early immune response to TB. Since in this thesis we were interested in dissecting the early immune events in the host response to TB, we have also assessed how *M. tuberculosis* may impact the production and maturation of myeloid cells and how that reflects on their ability to fight off infection.

Seeing how the genetic diversity of *M. tuberculosis* may lead to different outcomes in the clinical setting (6, 7), it is critical to delve into the immune interactions of other *M. tuberculosis* isolates with the host. So far, studies that showed *M. tuberculosis* being able to colonize the BM used the reference H37Rv strain (28-30). Here, we showed that the hypervirulent isolate HN878 is also able to reach the BM of mice, with impact on the hematopoietic outcome of the HSC niche. Indeed, we observed an increase in the frequency of LSK and GMP cell populations in the BM, coincident with the expansion of monocytes and neutrophils in blood and lungs of mice. Put together, these results indicate that the myeloid output of the BM is increased in infections with isolate HN878, consistent with the occurrence of EM in other infections (31). Curiously, these results are in contrast with previous results from infection of mice with the H37Rv laboratory reference strain, which reported the lack of EM (30). These discrepancies are likely linked to the differential modulation of the immune response by the different *M. tuberculosis* isolates. Indeed, infections with isolate HN878 are characterized by a deregulated Th1 immunity and the induction of type I IFNs, leading to a high neutrophilic influx into the lung with consequent exacerbated lung pathology (32, 33). This influx of neutrophils, which we also reported in our study, must be reflected in alterations to the HSC niche, as these neutrophils must be

produced in the BM. Thus, if *M. tuberculosis* isolates are able to differentially induce EM in the infected host, the lack of EM previously reported in infections with the H37Rv strain (30) may be another example of the modulation of the immune response by different *M. tuberculosis* isolates. Taking into account the differential modulation of the immune response with clinical isolates 4I2 and 6C4, it would be very interesting to also see if there are different modulations of the HSC niche in infections with these isolates, and whether and how the IL-1R signaling may impact the hematopoietic outcome of the BM.

Another important aspect that may influence the hematopoietic output, and consequently the immune response, is the bacterial load upon infection. Here, we used high doses of isolate HN878 to infect B6 mice, which recapitulate the blood transcriptome signatures of TB patients (34). However, when performing low dose infections with the same isolate, the EM feature previously observed did not occur, despite the bacterial colonization of the BM still occurring. The previous study with strain H37Rv also used low doses of bacteria in infections, which resulted in mild outcome of infection and low bacterial burden (30). It would be interesting to see if EM would also occur in infections with higher doses of the H37Rv isolate.

Nonetheless, regardless of the differences observed in EM between the H37Rv strain and isolate HN878, the occurrence of trained immunity does not appear to occur in the BM of either infection. Thus, while the occurrence of EM is a critical process in the immune response against infections (31), it appears that *M. tuberculosis* does not lead to an enhanced immune response against other immune challenges by cells produced in the BM. Curiously, it has been previously demonstrated that BCG was also able to colonize the BM and induce EM (35). However, myeloid cells from BCG-administered mice were capable of increasing protection against subsequent *M. tuberculosis* infection due to epigenetic changes in monocytes and macrophages towards trained immunity (35). Seeing that BCG is an attenuated version of *M. bovis* (36), it is possible that the virulence mechanisms of *M. tuberculosis* are associated with an impairment of inducing trained immunity. BCG has an impaired ability to induce cytolysis of infected cells due to the lack of the RD1 region present in virulent mycobacteria (36). Indeed, in contrast with infections with strain H37Rv, infections with attenuated H37Rv strain, lacking the RD1 region, induced myelopoiesis in a similar manner to BCG (30). Thus, it would be very interesting to see if infections of *M. tuberculosis* isolate HN878 lacking this region would result in different hematopoietic modulations, and if these mutated isolates would be better at protecting against *M. tuberculosis* infections. Nonetheless, here we also show that monocytes from active TB patients are better at controlling *M. tuberculosis* growth, which may hint that trained immunity may be occurring elsewhere than in the BM, or that trained immunity could occur in the human host but not on the mouse host.

While we did not verify how the lack of IL-1R signaling impacted the HSC niche, we did observe that mice lacking the IL-1R were more susceptible to infections with isolate HN878. We have also observed that lack of IFNγ resulted in aggravation of disease in infections with isolate HN878. While IFNγ has been shown to play a role in the hematopoietic reprogramming of the BM in infections with *M. avium* or *M. bovis* (35, 37), here we show that EM still occurs in infections with isolate HN878, at least to an extent, in the lack of IFNγ. It is possible that the higher severity of disease caused by this isolate may circumvent the role of IFNγ in EM, again hinting at a possible role for severity in the modulation of the hematopoietic niche, with consequences for the subsequent immune response. One possible mechanism leading to EM in infections with isolate HN878, even in the absence of IFNγ, is the induction of type I IFN characterized by infections with this isolate (32, 34). Indeed, it has previously shown that type I IFN mediated the expansion of GMPs in a mouse model of systemic lupus erythematosus pathogenesis (38) and in infections with influenza A virus (39). Thus, the overt induction of type I IFN characterized by infections with isolate HN878 could be surpassing the effects on IFNγ in EM.

Put together, the results presented in this thesis aimed at elucidating how bacterial diversity within *M. tuberculosis* modulates the host immune response with consequences for the initial immune events that dictate the subsequent immune response. We have shown that the *M. tuberculosis* clinical isolates 4I2 and 6C4 lead to a differential modulation of the IL-1 responses *in vivo*; that a proper IL-1R signaling is crucial in the immune response of AMs to *M. tuberculosis*; and that the IL-1R signaling may affect the balance of inflammatory response that are essential for the immune response. In parallel, we also demonstrated how hypervirulent *M. tuberculosis* HN878 isolate leads to EM, where the BM is skewed to produce massive amounts of myeloid cells to deal with infection. Despite this increased myeloid output, these cells are not necessarily better at handling *M. tuberculosis*, indicating that *M. tuberculosis* may modulate the innate immune response from as early as the production of innate immune cells. Further studies should be performed in order to understand the impact of genetic diversity in *M. tuberculosis* and how that diversity results in the modulation of immune responses and disease outcome, with the final objective of developing tools to address the TB epidemic that still lingers today.

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