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THE YIN AND YANG OF HOST-PATHOGEN INTERACTIONS IN TUBERCULOSIS

Jeremy Nicolas de Carvalho de Sousa

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THE YIN AND YANG OF HOST-PATHOGEN INTERACTIONS IN TUBERCULOSIS

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O autor esclarece que na elaboração desta tese foram incluídos os artigos publicados ou manuscritos submetidos para publicação abaixo indicados, e declara ter participado ativamente na conceção e execução das experiências que estiveram na origem da mesma, assim como na sua interpretação, discussão e redação.

The author declares that in the elaboration of this thesis published articles or manuscripts submitted for publication were included as listed below, and declares that he participated actively in the conception and execution of the experiments that produced such data, as well as in their interpretation, discussion and in the manuscript writing.

PUBLICATIONS

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***Mycobacterium tuberculosis* associated with severe tuberculosis evades cytosolic surveillance systems and modulates IL-1β production**

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Abstract

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* infection, remains a major global health problem, with around 1.6 million deaths and 10 million new cases annually. Despite all the efforts to eliminate this disease, the lack of an effective vaccine, the increase of cases of drug resistance, and the difficulties to provide efficient diagnosis contribute to the high burden of TB. To further advance in all these areas, a deep understanding of the protective mechanisms associated with the immune response is urgently required. An important factor to consider in this context is the genetic diversity associated with *M. tuberculosis*, which is largely neglected in the literature and has been the main focus of this thesis.

The recognition of *M. tuberculosis* by the immune cells and their subsequent activation are crucial for the development of an efficient immune response, which requires a perfect balance between pro- and anti-inflammatory responses to promote bacteria elimination in the absence of pathology. Unfortunately, some immune molecules, such as type I interferon (IFN), impair the response against *M. tuberculosis*. In the first study presented, we show a novel mechanism where this cytokine plays a protective role during *M. tuberculosis* infection. Using a toll-like receptor 4-activating *M. tuberculosis* strain, we demonstrate that infected macrophages are able to produce type I IFN, which in turn promotes the expression of nitric oxide synthase 2 and downregulation of arginase 1. Most importantly, we demonstrate that in the absence of IFN-γ receptor, IFN-α/β receptor signaling inhibits the expression of T helper 2 cell-associated cytokines and enhances tumour necrosis factor-α expression in infected lungs. This type I IFN-mediated mechanism allows to suppress alternative macrophage activation, thus conferring protection during infection with *M. tuberculosis*.

The regulation of the immune response during TB does not depend exclusively on host regulatory factors. More recently, several studies have shown the contribution of genetic diversity among strains of *M. tuberculosis* in dictating the immune response. In the second study presented in this thesis, we investigated how bacteria diversity can contribute to the immune response and TB severity. We studied 681 pulmonary TB cases and used selected *M. tuberculosis* isolates associated with specific disease severity in patients to study the immune response triggered in immune cells of unrelated donors. Our data show that *M. tuberculosis* isolated from patients with mild TB induced high cytokine production in macrophages, as compared to *M. tuberculosis* isolated from severe TB patients. This lower cytokine induction in macrophages infected with severe *M. tuberculosis* isolates was due to

a diminished activation of cytosolic surveillance systems, such as the NLRP3 inflammasome and cGAS. This capacity to evade the immune response by severe TB-causing isolates may be associated with the accumulation of mutations in genes encoding components or regulators of the ESX-1 secretory pathway. Our findings demonstrate that the modulation of the immune response and TB severity in patients can be regulated by specific *M. tuberculosis* isolates.

Collectively, our studies show that the infection by *M. tuberculosis* is a complex process. On one hand, we demonstrated that an appropriate regulation of molecular signaling pathways is crucial to guarantee protection and elimination of the pathogen, but on the other hand, this regulation can be strongly influenced by *M. tuberculosis* specific characteristic. Therefore, the future of TB research will require the incorporation of both host and pathogen diversity and interactions.

Resumo

A tuberculose (TB), provocada pela infeção pelo agente *Mycobacterium tuberculosis*, continua a ser um problema de saúde pública mundial. Em 2017, foram reportados 10 milhões de novos casos de TB e 1,6 milhões de pessoas morreram da doença. Apesar dos esforços para eliminar a doença, a falta de uma vacina mais eficaz, o aumento de casos de resistência a antibióticos e a falta de diagnóstico eficaz têm contribuído para o aumento dos casos de TB. De forma a ultrapassar estes problemas, é preciso com urgência perceber melhor os mecanismos de proteção associados à resposta imune. Neste contexto, a variabilidade genética do *M. tuberculosis* é uma das variáveis a considerar. A minha tese foca-se então neste aspeto.

A deteção do *M. tuberculosis* pelas células do sistema imune e consequentemente a ativação destas é crucial para o desenvolvimento de uma resposta imune eficiente. Esta última requer um balanço perfeito entre as respostas pro- e anti-inflamatória, de forma a promover a eliminação do bacilo na ausência de patologia. Infelizmente, alguns mediadores imunes, como o interferão do tipo I, compromete o desenvolvimento da resposta protetora contra o *M. tuberculosis*. No primeiro estudo apresentado mostramos um novo mecanismo no qual esta citoquina tem um papel protetor durante a infeção. Usando uma estirpe de *M. tuberculosis* que ativa o recetor do tipo toll 4, demostramos que macrófagos infetados eram capazes de produzir interferão do tipo I, que consequentemente promove a expressão do óxido nítrico sintase 2 e a diminuição da expressão da arginase 1. Demonstramos que na ausência do recetor para interferão-γ, a sinalização através do recetor para interferão α/β é importante para inibir a expressão das citoquinas associadas a resposta celular *T helper* 2 e também para aumentar a expressão do fator de necrose tumoral α em pulmões de ratinhos infetados. Estes mecanismos permitem a supressão da ativação alternativa dos macrófagos e assim consequentemente promovem a proteção do hospedeiro durante infeção com o *M. tuberculosis*.

A regulação da resposta imune durante a TB não depende exclusivamente dos fatores reguladores do hospedeiro. Recentemente, vários estudos mostraram um papel relevante da diversidade genética entre estirpes de *M. tuberculosis* na regulação da resposta imune. No segundo estudo apresentado nesta tese, investigamos como a diversidade das bactérias podem contribuir para o tipo de resposta imune e severidade da TB. Estudamos 681 casos de TB pulmonar e selecionamos isolados clínicos de *M. tuberculosis* associados a manifestação mais ou menos severa da doença em pacientes. Usando os isolados selecionados, estudamos a resposta imune desencadeada por estes

isolados em células do sistema imune de dadores independentes. Os nossos resultados mostraram que isolados clínicos de *M. tuberculosis* de pacientes diagnosticados com uma TB ligeira induzem uma forte produção de citoquinas nos macrófagos comparativamente com isolados clínicos de *M. tuberculosis* de pacientes com formas mais severas de TB. A baixa produção de citoquinas observada em macrófagos infetados com isolados clínicos "severos" de *M. tuberculosis* é devida à diminuição da ativação de sistemas de vigilância citosólicos, como o inflamassoma NLRP3 e o cGAS. A capacidade dos isolados clínicos que causam TB severa em evadir a resposta imune do macrófago parece estar associada com a acumulação de mutações nos genes de codificam componentes ou reguladores da via de secreção do sistema ESX-1. Os nossos resultados demonstram que a modulação da resposta imune e a severidade da TB observada em diferentes pacientes podem ser regulada por características específicas e variáveis da bactéria causadora da doença.

Globalmente, os nossos estudos mostram que a infeção com *M. tuberculosis* é um processo complexo. Por um lado, demonstramos que uma regulação apropriada das vias de sinalização molecular é crucial para garantir proteção do hospedeiro e eliminação do patogénico. Por outro lado, esta regulação pode ser fortemente influenciada pelas características específicas do *M. tuberculosis*. Assim, o futuro da investigação da TB precisará de incorporar diversidade e interação ao nível do hospedeiro, do patogénico e das suas interações.

Thesis planning

Chapter I gives a general introduction about the different players involved during infection with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). It starts with an overview of TB, from its epidemiology to its prevention and treatment, and finishes with the description of the existing spectrum of TB disease. Then, a new section is dedicated to *M. tuberculosis*, with a description of its origin and the role of the different *M. tuberculosis* complex lineages in the disease. Finally, the last section of Chapter I focuses on the immune response during the infection, the role of immune cells and of different molecular effectors during TB.

The main objectives of the thesis are presented in Chapter II.

Chapter III describes the experimental work developed. The first part, entitled “Type I IFN Inhibits Alternative Macrophages Activation during *M. tuberculosis* Infection and Leads to Enhanced Protection in the Absence of IFN-γ Signaling”, has been published in *The Journal of Immunology*. The second part of the experimental work, entitled “*Mycobacterium tuberculosis* Associated with Severe Tuberculosis Evades Cytosolic Surveillance Systems and Modulates IL-1β Production”, is presented under Manuscript Format. This part is now submitted for publication.

Chapter IV summarizes the findings described in Chapter III and presents a final discussion.

List of abbreviations

AIM2	-	Absent in melanoma 2
AKT	-	Protein kinase B
AM	-	Alveolar macrophage
AMP	-	Adenosine monophosphate
ASC	-	Apoptosis-associated speck-like protein
BCG	-	Bacillus Calmette-Guérin
BMDM	-	Bone marrow-derived macrophage
CARD	-	Caspase activation and recruitment domain
CCL	-	C-C chemokine ligand
CCR	-	C-C chemokine receptor
cDMEM	-	Complete DMEM medium
cGAMP	-	Cyclic guanosine monophosphate-adenosine monophosphate
cGAS	-	Cyclic GMP-AMP synthase
cRPMI	-	Complete RPMI-1640 medium
CXCL	-	C-X-C chemokine ligand
DAPI	-	4´,6-diamidino-2-phenylindole
DC	-	Dendritic cell
ds	-	Double-stranded
ELISA	-	Enzyme-linked immunosorbent assay
ER	-	Endoplasmic reticulum
ERK	-	Extracellular-signal-regulated kinase
ESAT-6	-	Early secretory antigen-6
GFP	-	Green fluorescent protein
GMP	-	Guanosine monophosphate
GSDMD	-	Gasdermin D
HEPES	-	4-(2-hydroxyethyl)-1-piperazineethanesulfic acid
HIV	-	Human immunodeficiency virus
IFN	-	Interferon
IFNAR	-	IFN-α/β receptor
IGRA	-	Interferon-γ release assay
IL	-	Interleukin
IM	-	Interstitial macrophage
InDels	-	Insertion and deletion of bases
IRF	-	Interferon regulatory factor

JAK	-	Janus kinase
JNK	-	Jun N-terminal kinase
LAM	-	Latin-American-Mediterranean
LPS	-	Lipopolysaccharide
LTBI	-	Latent tuberculosis infection
MAPK	-	Mitogen-activated protein kinase
MDSC	-	Myeloid-derived suppressor cell
MHC	-	Major histocompatibility complex
MOI	-	Multiplicity of infection
mRNA	-	Messenger RNA
MSMD	-	Mendelian susceptibility to mycobacterial disease
MTBC	-	*Mycobacterium tuberculosis* complex
MyD88	-	Myeloid differentiation primary response protein 88
NF-κB	-	Nuclear factor κ-light-chain-enhancer of activated B cells
NK	-	Natural killer
NLR	-	NOD-like receptors
NLRP	-	NOD, leucine-rich repeat and PYD-containing protein
NO	-	Nitric oxide
NOD	-	Nucleotide-binding oligomerization domain
NOS	-	Nitric oxide synthase
OADC	-	Oleic Albumin Dextrose Catalase
PAMP	-	Pathogen-associated molecular pattern
PBMC	-	Peripheral blood mononuclear cells
PET	-	Positron emission tomography
PI3K	-	Phosphoinositide 3-kinase
Poly I:C	-	Polyinosinic:polycytidylic acid
PPD	-	Purified protein derivative
PRR	-	Pattern recognition receptor
PYD	-	Pyrin domain
RIG-I	-	Retinoic acid-inducible gene I
SNP	-	Single-nucleotide polymorphism
STAT	-	Signal transducers and activators of transcription
STING	-	Stimulator of interferon genes
TB	-	Tuberculosis
TBK1	-	TANK-binding kinase 1
TGF	-	Transforming growth factor
Th	-	T helper

TIR	-	Toll-interleukin 1 receptor
TLC	-	Thin-layer chromatography
TLR	-	Toll-like receptor
TNF	-	Tumour necrosis factor
Treg	-	Regulatory T cell
TRIF	-	TIR domain-containing adapter protein inducing IFN-β
TST	-	Tuberculin skin test
TYK	-	Tyrosine kinase
WHO	-	World Health Organization
WT	-	Wild-type

Chapter I – Introduction

I. Tuberculosis

I.I Epidemiology

The history of tuberculosis (TB) is full of countless human tragedies, and even today TB represents the infectious disease that causes the most victims worldwide. TB was for a long time considered as an inherited condition, until Robert Koch discovered, in 1882, that TB was actually caused by a bacterial pathogen, *Mycobacterium tuberculosis* (Keshavjee and Farmer 2012). TB remains a critical health problem in our modern world. Despite years of coordinated global efforts to reduce the burden of TB, the disease continues to infect and kill millions every year. The latest report from the World Health Organization (WHO) has shown that in 2017, around 10 million new cases of TB were diagnosed and 1.6 million people died from the disease (WHO 2018). Furthermore, 23% of the world population is estimated to have TB in its latent state, and 5-10% of these individuals may be at risk of developing active TB during their lifetime (Houben and Dodd 2016). TB can affect individuals of either sex or age, although a higher number of cases has been observed in men (WHO 2018). Globally, the highest cases of TB have been reported in Asian and Africa, with only 3% in the European region (WHO 2018). Important problematics with TB infection are co-infection with Human immunodeficiency virus (HIV) and the increased numbers of drug-resistant cases. In 2017, around 300 000 TB-related deaths have been reported among HIV-positive people, together with almost 1 million of estimated new cases of HIV-TB co-infection (WHO 2018). Drug-resistant TB cases are a public health crisis, with almost half of the world cases being reported in India, China and the Russian Federation (WHO 2018). In 2017, drug-resistant TB represented around 600.000 cases, among which 82% had multidrug-resistance (WHO 2018). Still, the absolute number of TB deaths has fallen by 29% since 2000, and cases among HIV-positive people have fallen by 44% (WHO 2018).

I.II Transmission and disease symptoms

M. tuberculosis is an intracellular pathogen transmitted in aerosolized droplets generated by coughing from persons with active form of TB. The course of *M. tuberculosis* infection is extremely variable in humans (Lin and Flynn 2018). Once infection is established, most people control, but do not eliminate the bacteria. Individuals under this condition are considered latently infected and present no signs or symptoms of TB and are presumed to be noncontagious (Lin and Flynn 2018). Symptoms of disease evolve slowly, possibly due to the slow growth of *M. tuberculosis*, and include coughing lasting for several

weeks, haemoptysis, chest pain, unintentional weight loss, fatigue, fever, night sweats, chills or loss of appetite. The disease can present in a variety of ways, most commonly as pulmonary TB, but *M. tuberculosis* can infect any organ in the body (Zumla et al. 2013).

I.III Diagnosis, prevention and therapy

Several methods of diagnosis and treatment have been developed or improved in order to better identify TB cases and to recommend the best antibiotic combination to improve bacteria elimination. Depending on the "state" of infection, different methods of diagnosis can be applied. In the case of diagnosis of latent TB, immune-based approaches, as the tuberculin skin test (TST) or the interferon-γ release assay (IGRA) are currently used (Sousa and Saraiva 2019). Both IGRA and TST are based on the measurement of memory T cell responses to antigens of *M. tuberculosis* (Pai and Behr 2016). IGRA measures interferon (IFN)-γ production upon stimulation of whole blood with *M. tuberculosis* specific antigens, deleted from the genome of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and absent in most environmental mycobacteria (Whitworth et al. 2013). On the contrary, TST measures the infiltration of cells to the skin caused by intradermal injection of purified protein derivative (PPD), which is a crude mixture of antigens, many of which shared by *M. tuberculosis*, BCG and several species of environmental mycobacteria. Both tests present several limitations: low accuracy in immune-compromised patients, lack in distinguishing latent from active or subclinical TB, and low prediction values for active TB diagnosis (Diel, Loddenkemper, and Nienhaus 2012; Sester et al. 2014). Furthermore, these tests do not inform on the presence of viable bacilli *in vivo*, as memory T cell responses may persist for years after the infection has been treated. A comparison between IGRA and TST performances to identify latent TB infection (LTBI) that progresses to active disease has shown inconsistent results (Auguste et al. 2017). Thus, it is important to develop alternative methods to better identify individuals with increased risk of developing active forms of TB.

To confirm suspicions of active TB, more tests are performed. Generally, methods such as bacteriological detection through sputum smear-positive microscopy or culture positive have been classically used to diagnose active TB cases (WHO 2018). Radiography screening has made a come-back with the upgrade of digital technology in health services. Digital chest X-rays with computer-aided detection of TB have been increasingly used in various setting, helping as a triage test (WHO 2016). Most advances have been made in the area of molecular testing for the presence of genomic *M. tuberculosis* DNA with or without drug-resistance mutations. The rise of bacteria strains resistant to antibiotics has been a global health problem, and did not spare TB. Besides the need to correctly detect

TB cases, clinicians have to guarantee that TB treatment will be efficient against the bacilli. Among molecular tests, the Xpert MTB/RIF test has been strongly recommended by WHO and widely used in TB screening (WHO 2018; Boehme et al. 2011). This assay can detect *M. tuberculosis* genetic material along with mutations that cause resistance to rifampicin. A new version of this test that can detect resistance to isoniazid, injectable agents, and fluoroquinolones, has been validated in a large study (Xie et al. 2017). The advantages of this test in TB diagnosis include a sensitivity for *M. tuberculosis* detection similar to culture assays classically used to evaluate drug resistant *M. tuberculosis* strains, the need for fewer resources and the faster yield of results (Nguyen et al. 2019). Another tool that has become more used is the whole-genome sequencing (Consortium et al. 2018). This technology is used to identify mutations in the *M. tuberculosis* genome that are associated with phenotypic drug resistance, but it also allows to improve the understanding of TB transmission and the investigation of outbreaks at the molecular level (Consortium et al. 2018; Walker et al. 2013; Cancino-Munoz et al. 2019; Goig et al. 2019). TB diagnosis through transcriptional blood profiling of the host might be in the near future a more reliable method to determine the infection state of individuals (Berry et al. 2010; Singhania, Wilkinson, et al. 2018). The use of transcriptomics as a specific diagnosis for TB also relies on the ability to identify commonalities and differences in the host responses observed in TB patients as compared to those in other infections and diseases (Lin et al. 2016; Bloom et al. 2013; Singhania, Verma, et al. 2018; Elkington, Tebruegge, and Mansour 2016; Clayton et al. 2017; Mourik et al. 2017). Although the distinction between gene signatures to TB and other diseases needs to be improved, several studies have shown potential gene markers to distinguish healthy/LTBI versus active TB, or even the TB progression risk (Singhania, Verma, et al. 2018; Suliman et al. 2018; Zak et al. 2016; Maertzdorf et al. 2016; Roe et al. 2016; Sweeney et al. 2016; Kaforou et al. 2013).

BCG is the only preventive vaccine existing to TB and has been used for almost 100 years (Brosch et al. 2007). This vaccine resulted from the attenuation of *M. bovis* achieved experimentally by Albert Calmette and Camille Guérin. Immunisation with BCG provides a high degree of protection in infants and toddlers against highly lethal meningeal and miliary TB (Fine 1995). However, in the adult population, BCG vaccination provides highly variable protection against pulmonary TB, ranging from 0% to 80%, which appears to correlate with the geographical distribution of the population (Palmer and Long 1966). Therefore, due to the variable protection offered by BCG, new vaccine candidates have been developed. Several vaccine candidates, differing in their composition, are currently in clinical testing. These candidates are based on live, attenuated whole cells (e.g. recombinant BCG vaccine VPM1002, attenuated *M. tuberculosis* vaccine MTBVAC) (Nieuwenhuizen et al. 2017; Loxton et al. 2017; Arbues et al. 2013; Aguilo et al. 2017; Spertini et al. 2015); inactivated

whole cells (e.g. RUTI, DAR-901 vaccine) (Cardona 2006; Nell et al. 2014; Lahey et al. 2010; von Reyn et al. 2010); adjuvanted protein subunits (e.g. M72/AS01$_E$) (Van Der Meeren et al. 2018); and viral vectors (e.g. MVA85A) (Satti et al. 2014; Scriba et al. 2011).

Medical treatment of TB, together with correct diagnosis, is an important feature in the management and control of this disease. In the case of drug-susceptible TB, WHO recommended antibiotic therapy consists in the daily administration of four drugs during the first 2 months (isoniazid, rifampicin, pyrazinamide and ethambutol), and of two drugs during the next 4 months (isoniazid and rifampicin) (WHO 2018). Depending on the pathophysiology of each patient, the treatment period may be extended to more than 6 months to achieve cure (Jo et al. 2014). Treatment for drug-resistant TB requires the combination of new drugs. For cases of isoniazid-resistant TB, WHO recommends the administration of fluoroquinolones, whereas for rifampicin-resistant TB, the administration of bedaquiline and delamanid with increasing use of repurposed agents such as linezolid and clofazimine has been proposed. The treatment period for drug-resistant *M. tuberculosis* strains can take up to 9-24 months (WHO 2019). The thoracic surgery can be applied for TB treatment only in the following cases: when the patient displays an inappropriate healing response to medication, in which clinical and radiological pictures remain unchanged or indicate progression of the disease; when the acid-fast bacilli sputum smears positive even after 3-month treatment period, with a circumscribed radiological lesion or a destroyed lung (Kang et al. 2010). There is a resurgence of the role of surgery in managing TB due to the overall increase in global incidence and the emergence of multidrug-resistant or extensively drug-resistant TB (Sihoe, Shiraishi, and Yew 2009). Thoracic surgery offers highly effective treatment of TB and its sequel with less trauma and morbidity, and with the advance of minimal invasive thoracic surgery, a wide range of TB patients can be considered for effective surgical management (Takeda et al. 2005).

I.IV The spectrum of tuberculosis disease

Infection with *M. tuberculosis* may lead to elimination or persistence of the pathogen (Pai et al. 2016). In the first case, the pathogen is eliminated either because of innate immune responses (in this case, individuals might remain IGRA/TST negative) or because of adaptive immune responses (in this case, individuals become IGRA/TST positive or remain negative, depending on whether memory T cell responses have been primed or not, respectively) (Barry et al. 2009; Esmail et al. 2014). It has been recognized that, even among close household contacts of patients with TB, nearly half of the exposed individuals have negative TST results, with no evidence of infection during a period of 2 years after

case exposure (Morrison, Pai, and Hopewell 2008; Stein et al. 2018). The finding that there is a genetic predisposition to remain persistently TST negative despite ample exposure potentially explains why some people are naturally resistant to TB (Cobat et al. 2009). Additionally, BCG vaccination may contribute to an early clearance of *M. tuberculosis* infection (Verrall, Alisjahbana, et al. 2019; Verrall, Schneider, et al. 2019). If *M. tuberculosis* is not eliminated, the pathogen can persist in a latent state and the individual will develop IGRA/TST positive results, but without showing any symptoms (Esmail et al. 2014). Latently infected individuals may benefit from preventive therapy. Unfortunately, a positive IGRA result does not automatically imply LTBI, since individuals who successfully eliminated the infection upon T cell immunity might still be IGRA positive (Barry et al. 2009; Esmail et al. 2014). This finding partly explains the low predictive prognostic value of IGRA and other tests such as TST (Rangaka et al. 2012). For these reasons, and as discussed above, new methods for diagnosing LTBI, particularly to identify those individuals at risk of progression, or already progressing to active TB, are needed (Sousa and Saraiva 2019).

In a subset of hosts, for reasons that are not completely clear, LTBI can progress to clinical disease, in as early as weeks or as long as decades post exposure (Dutta and Karakousis 2014). Failures in the host immune response or immune dysfunctions seem to be determinant for the progression of latent to active disease. In particular, HIV co-infection, anti-tumour necrosis factor (TNF) treatment and diabetes have been identified as major risk factors (Sonnenberg et al. 2005) (Tobin et al. 2012; Lalvani, Behr, and Sridhar 2012) (Kumar Nathella and Babu 2017). Additionally, before undergoing into an active form of disease, characterised by symptoms, a subclinical TB disease has been recently documented in immune competent individuals (Barry et al. 2009; Ernst 2012). This subclinical disease is described as having detectable *M. tuberculosis* in sputum, but with a normal chest X-ray and no symptoms of TB. Subclinical TB is likely underreported, because sputum examinations are not typically performed on patients without symptoms or with negative chest radiographs (Houk 1980; Berry et al. 2010). Recent studies identified some patients who would have been defined as LTBI but had a whole blood transcriptomic signature compatible with active disease and were found to have positive sputum samples, performed serendipitously for research purposes (Berry et al. 2010). Similarly, features of active TB have been historically observed at autopsy of patients who died from other non-TB causes, suggesting that these patients were otherwise asymptomatic of TB (Opie 1917). More sophisticated imaging technologies such as computed tomography have been used in research and specific clinical settings, resulting in even more sensitive detection of disease. Positron emission tomography (PET) using 2-deoxy-2-[18F]-fluoro-D glucose (a PET probe measuring metabolic activity), combined with computed tomography, has enabled researchers and clinicians to find metabolically active granulomas in either asymptomatic

or LTBI patients (Esmail et al. 2016; Geadas et al. 2018). In fact, a spectrum of image patterns has been observed in subjects with clinically defined LTBI and in patients with symptomatic active disease (Goo et al. 2000; Ankrah et al. 2018). Collectively, these studies support the existence of a full spectrum of *M. tuberculosis* infection beyond the dogmatic and clinically defined binary outcome (Figure 1).

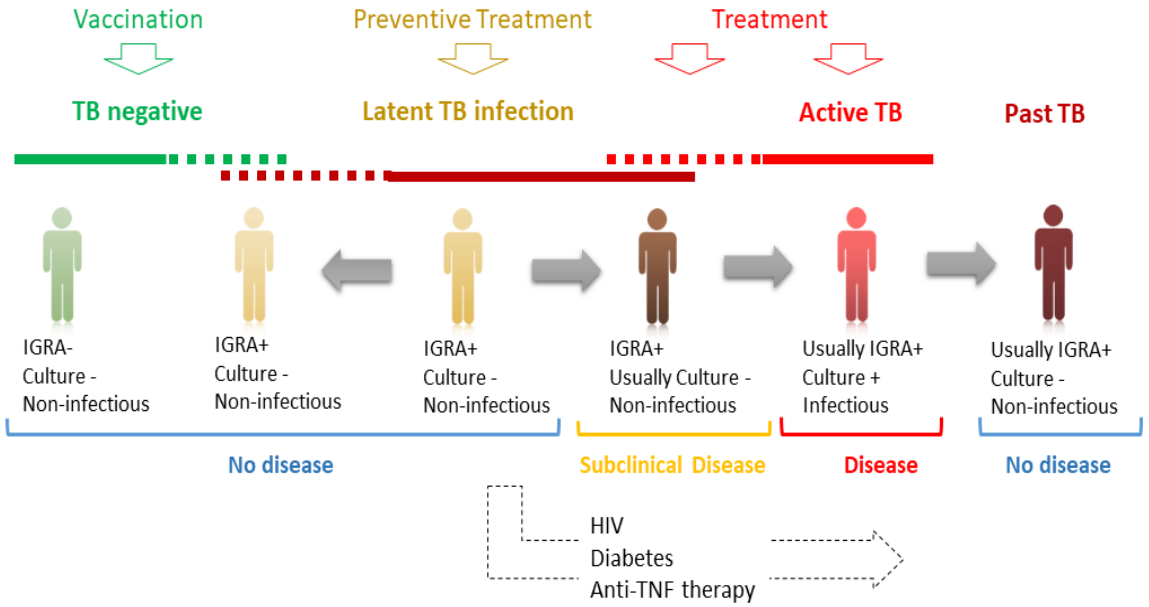

Figure 1 - The different groups of TB.

Several outcomes for infection with *M. tuberculosis* have been described. An unknown number of individuals show no signs of cellular responses to previous contact with *M. tuberculosis*, being therefore considered TB negative. Some of these individuals have never been exposed to the bacteria, whilst others may have eliminated it through innate immune mechanisms. An estimated 1.7 billion individuals control the infection in a latent form, being IGRA+. Among these, some appear to eliminate the bacteria, thus becoming TB negative, although no molecular markers are known to identify this process; others progress to active disease through a reactivation step. Failures in the immune system, such as those promoted by HIV, diabetes and immunosuppressive therapies, precipitate the transition between latency and active TB disease. A stage of subclinical disease is now accepted, which can be followed through clinical analysis by PET- Computed Tomography scan and at the molecular level. Active TB patients show ouvert disease with clinical symptomatology and positive microbiological tests. TB management required by these different groups is different, ranging from vaccination, to preventive therapy to treatment. From (Sousa and Saraiva 2019).

II. Mycobacterium tuberculosis

II.I The origin

The genus *Mycobacterium* includes more than 170 species, most of them are environmental organisms (Fedrizzi et al. 2017). Human TB is mainly caused by members of the *M. tuberculosis* complex (MTBC), a group of closely related acid-fast bacilli, with little sequence variation compared with other bacteria (Achtman 2008). The human-adapted TB causing bacteria within the MTBC are *M. tuberculosis* and *Mycobacterium africanum* (Brites and Gagneux 2015). The MTBC also includes animal-adapted strains that cause disease across a range of mammalian species, such as *M. bovis* (infecting cows), *Mycobacterium caprae* (goats and sheep), *Mycobacterium pinnipedii* (seals and sea lions), *Mycobacterium microti* (voles) and *Mycobacterium orygis* (oryxes) (Smith et al. 2006; van Ingen et al. 2012) (Figure 2). Other Mycobacteria causing disease in humans are *Mycobacterium ulcerans* (the agent of Buruli ulcer) and *Mycobacterium leprae* (the agent of leprosy). Among all of these pathogenic mycobacteria, *M. tuberculosis* has emerged as the most prevalent mycobacterial species and one of the most historically successful human pathogens (Cambier, Falkow, and Ramakrishnan 2014). It is now known that there are seven lineages of human-adapted members of the MTBC, resulting from the evolution and migration of modern humans out of Africa during the Neolithic demographic transition (Brites and Gagneux 2017; Comas et al. 2013). These lineages exhibit a strong phylogeographical population structure, with lineages 2, 3 and 4 being globally distributed, and lineages 1, 5, 6 and 7 showing a geographical restricted distribution (Coscolla and Gagneux 2014) (Figure 2). Characteristics of these lineages will be developed in the next section.

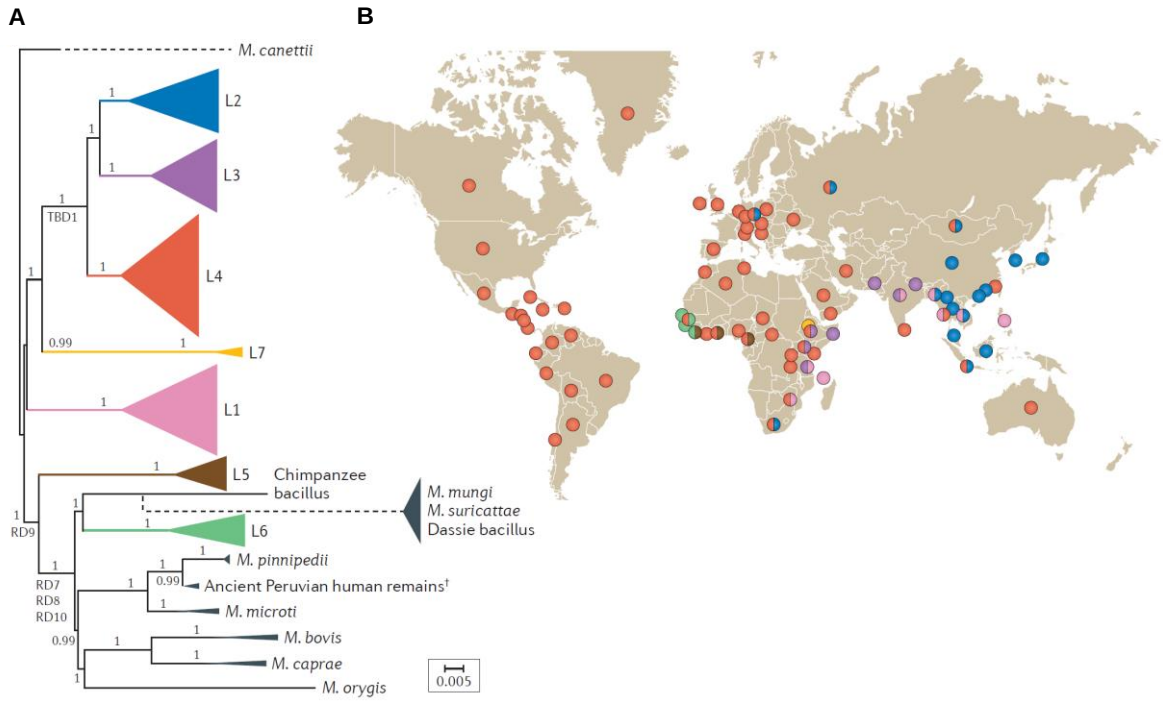

Figure 2 - Global phylogeography of the human-adapted MTBC.

(A) Genome-based phylogeny of the MTBC rooted with *Mycobacterium canettii* based on previously published data (Bos et al. 2014). The MTBC comprises seven human-adapted lineages (in colour) and several lineages adapted to various wild and domestic animals (in grey). Branches of the main lineages are collapsed to improve clarity (indicated by triangles). *M. tuberculosis*-specific deletion 1 (TbD1) indicates that all lineage 2 (L2), L3 and L4 strains share this genomic deletion (Brosch et al. 2002). Similarly, the deletion of the region of difference 7 (RD7), RD8, RD9 and RD10 is indicated under the respective branches. The grey dotted line leading to *Mycobacterium mungi, Mycobacterium suricattae* and the dassie bacillus was added based on previously published genotypic data (Alexander et al. 2010; Dippenaar et al. 2015), and indicates the most likely phylogenetic relationship of these animal-adapted ecotypes with the other members of the MTBC. The dagger indicates genomes generated from around 1,000-year-old MTBC DNA that was recovered from archaeological human remains in Peru (Bos et al. 2014). Bootstrap confidence intervals are indicated. Scale bar represents the number of nucleotide substitutions per site. (**B)** The global distribution of the seven main human-adapted MTBC lineages. Adapted from (Gagneux 2018).

The earliest claim of mycobacterial disease comes from a 500,000 year old fossil of *Homo erectus* that demonstrated lesions characteristic of mycobacterial infection (Roberts, Pfister, and Mays 2009). Since no ancient DNA was recovered from this fossil, it is not possible to determine which species of mycobacteria might have caused the lesions. Based on lipid profiles unique to pathogenic mycobacteria and on the *IS6110* insertion element, only present in members of MTBC, the oldest confirmed sample of mycobacterial disease was found in bison fossils, dating from approximatively 17,000 years ago, while the earliest known association of the MTBC with humans is dating from 9,000 years ago (Gernaey et al. 2001; Thierry et al. 1990; Lee et al. 2015; Lee et al. 2012; Hershkovitz et al. 2008). As the techniques to collect and sequence ancient DNA improve, we may one day better understand the origin and evolution of MTBC and the nature of its interactions with its host. The origin of *M. tuberculosis* has been pointed to around 70,000 years ago, as determined based on the parallels of mitochondrial DNA haplogroups and the lineages of MTBC that

are most commonly found among the corresponding human populations (Comas et al. 2013). However, this estimated date is still not definitive. By sequencing ancient DNA from mummified human remains presenting skeletal lesions indicative of TB, it was revealed that the disease was caused by *M. pinnipedii*, a MTBC member. Comparing ancient DNA from these samples with a current strain of *M. pinnipedii*, the authors estimated that the emergence of MTBC occurred 6,000 years ago (Bos et al. 2014).

II.II Genetic diversity within the *M. tuberculosis* complex

Despite the clonal evolution of human-adapted TB causing bacteria, significant genetic variation exists and, on its basis, relates to the seven existing major lineages. The genetic structure of human-adapted MTBC lineages was firstly defined by lineage-specific deletions, referred to as large sequences polymorphisms (Gagneux and Small 2007). Due to the extreme rarity of ongoing horizontal gene transfer among species of the MTBC, these markers are thought to be largely irreversible and well-suited to lineage classification (Coscolla and Gagneux 2014). Nucleotide polymorphisms are also informative for the MTBC phylogenetic structure because of the lack of ongoing horizontal gene transfer, and help additionally to increase the resolution of relationship among strains within lineage (Comas et al. 2010; Saelens et al. 2015; Saelens et al. 2018).

The human-adapted TB causing bacteria can be grouped into “ancient” and “modern” evolutionary lineages, with the *M. tuberculosis* specific deletion 1 genetic region, TbD1, acting as a genetic marker separating the two groups (Brosch et al. 2002). It is now clear that “ancient” lineages are divided into distinct phylogenetic groups, and are therefore paraphyletic in nature. Conversely, the “modern” lineages are more closely related than “ancient” lineages are with one another and share a more recent common ancestor (Krishnan et al. 2011; Portevin et al. 2011). The “ancient” lineages, that include lineages 1, 5, 6 and 7, demonstrate a high degree of geographic constraint, whereas “modern” lineages, composed of lineages 2, 3 and 4, are found more largely spread in the world (Hershberg et al. 2008; Firdessa et al. 2013; Vasconcellos et al. 2010; Reed et al. 2009) (Figure 2). Lineage 1 mainly circulates in Southeast Asia, lineages 5 and 6 in West Africa, and lineage 7 in the Horn of Africa. Lineage 2 is strongly associated with an East Asian origin, although it causes significant disease burden in Eurasia, South Africa and Peru (Luo et al. 2015). Lineage 3 mostly circulates in India and Central Asia. Lineage 4 causes the most global disease and is the most widely distributed among MTBC lineages (Coscolla and Gagneux 2014) (Figure 2). Interestingly, some discrete sublineages within lineage 4 are more capable of spreading to new host populations (Stucki et al. 2016).

These human-adapted-MTBC lineages have evolved independently in separate human populations, which led to distinct induction of inflammatory phenotypes and differential modulation of innate immune signaling (Krishnan et al. 2011; Portevin et al. 2011; Wiens and Ernst 2016). That different modulation of immune responses could reflect diverse virulence strategies that emerged during the evolution of the “ancient” and “modern” lineages to the evolutionary adaptation of low or high human population densities (Portevin et al. 2011).

II.III *M. tuberculosis* complex diversity and disease presentation

Several studies have demonstrated that there are differences in disease presentation among the different lineages of the human-adapted TB causing bacteria. In the United States, strains belonging to lineages 1, 3 and 4 were found to be more likely to cause extrapulmonary disease compared to strains from lineage 2 (Click et al. 2012). In Florida, lineage 1 strains were associated with higher rates of extrapulmonary disease than strains form lineages 2 and 4 (Seraphin et al. 2017). In Vietnam, lineages 1 and 2 strains were more strongly associated with TB meningitis than lineage 4 strains (Caws et al. 2008). In the United Kingdom, lineages 1 and 2 were also associated with increased likelihood of exclusively extrapulmonary disease compared to strains belonging to lineages 3 and 4 (Pareek et al. 2013). Beside the site of disease, other characteristic such as time of sputum culture conversion and transmissibility also differ between lineages. Unlike strains from lineages 2, 3 and 4, in the United States, lineage 1 strains demonstrated a more rapid time to sputum culture conversion (Click et al. 2013). Additionally, in Gambia, lineage 6 strains (*M. africanum)* progressed to active disease at a significantly lower rate compared to strains from lineages 2, 3 and 4, but without differences in transmission rates (de Jong et al. 2008). However, in the Netherlands, strains from lineages 1, 5 and 6 showed reduced transmissibility than lineage 4 strains (Nebenzahl-Guimaraes et al. 2015).

In a study involving a non-human primate infection model, a strain from lineage 6 was found to develop lower bacterial load in pulmonary tissue than strains from lineages 2 and 4, but disseminated more to extrapulmonary sites than lineage 4 (Via et al. 2013). Interestingly, lineage 2 strains showed the highest burden in all organs assayed, effectively replicating within the lung and disseminating to extrapulmonary sites. This suggests that strains from lineages 2, 3 and 4, i.e. the “modern” strains, are more capable of transmitting by establishing pulmonary disease than the “ancient” lineage 6. Additionally, lineage 2 strains can also spread effectively to extrapulmonary sites (Via et al. 2013).

II.IV *M. tuberculosis* complex diversity and immune responses

Differences in the activation and regulation of the immune response during *in vitro* and *in vivo* infection with *M. tuberculosis* lineages were also reported. Using representative strains of each lineage, it has been shown that strains from lineages 2, 3 and 4 elicited lower early inflammatory responses in human derived macrophages than those from lineages 1, 5 and 6 (Portevin et al. 2011). Furthermore, human peripheral blood mononuclear cells (PBMC) infected with *M. tuberculosis* CDC1551, a lineage 4 strain, produced higher levels of proinflammatory cytokines interleukin (IL) -1α/β, macrophage inflammatory protein 3-α, indicative of a T helper (Th) 1 type immunity and immune protection, as compared to *M. tuberculosis* HN878, a clinical isolate belonging to lineage 2. In contrast, *M. tuberculosis* HN878 infection led to high levels of IL-4 and IL-13 produced by PBMCs, suggesting a Th2 type immune response (Manca et al. 2004). Strain HN878 has also been for a long time associated with increased production of type I IFN, which has been correlated to its hypervirulence in mice (Manca et al. 2001). Despite this high production of type I IFN, a number of studies demonstrated that lineage 2 strains are low-cytokine inducers (Wang et al. 2010; Lopez et al. 2003; Manca et al. 2005). Globally, experimental infections with lineage 2 strains have shown variable immune responses (Aguilar et al. 2010; Dormans et al. 2004; Dunn and North 1995; Lopez et al. 2003; Manca et al. 2001). A study in mice showed that production of IL-17 via a toll-like receptor (TLR) 2-dependent pathway was protective for mice infected with *M. tuberculosis* HN878, but not with *M. tuberculosis* H37Rv, a lineage 4 strain (Gopal et al. 2014). Additionally, it was demonstrated that C-C chemokine receptor (CCR) 2 is critical for protective immunity in mice during infection with *M. tuberculosis* HN878, but dispensable during infection with *M. tuberculosis* H37Rv. CCR2 is required for localization of alveolar macrophages (AMs) within TB granuloma. The absence of CCR2 results instead in the accumulation of neutrophils and the development of necrotic lesions (Dunlap et al. 2018). Our group has also been contributing to a better understanding of the regulation of the immune response by *M. tuberculosis* diversity. It has been shown that differential recognition of *M. tuberculosis* by TLRs was in place, with an impact on the response of phagocytes and cytokines production (Stamm, Collins, and Shiloh 2015; Carmona et al. 2013). We have showed that most of the strains are recognized by TLR2 in macrophages. However, a specific lineage 2 strain, *M. tuberculosis* 02-171, also activated the TLR4 receptor in macrophages, leading to the production of IFN-β, and consequent increase of bacterial burden and lung pathology during early stages of mouse infection (Carmona et al. 2013). Therefore, the virulence capacity of lineage 2 strains may be explained by their ability to disrupt the host defences by inhibiting antimicrobial product, as well as inhibiting proinflammatory cytokines IL-1β, IL-18 and IL-12p40, and promoting anti-

inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (O'Garra et al. 2013; Mayer-Barber et al. 2011). Diversity in immune responses induced by MTBC is also observed outside lineage 2. Indeed, a lineage 3 strain was shown to induce less protective IL-12p40 and more anti-inflammatory IL-10 and IL-6 expression and secretion in infected human derived macrophages, compared to *M. tuberculosis* strains CDC1551 and H37Rv, both from lineage 4 (Newton et al. 2006).

III. The immune response to *M. tuberculosis* infection

III.I Overview

The infection with *M. tuberculosis* begins with the translocation of the bacteria to the lower respiratory tract following inhalation. *M. tuberculosis* will encounter AMs patrolling the airway, which are the dominant cell type that the bacteria is thought to infect early upon inhalation (Cohen et al. 2018). These cells internalize the bacteria through receptor-mediated phagocytosis, with numerous different receptors contributing to this process (Cadena, Flynn, and Fortune 2016). Uptake of *M. tuberculosis* activates an inflammatory response through the stimulation of multiple pattern recognition receptors (PRRs), that gets amplified when infected macrophages invade the subtending tissue of the lung (Killick et al. 2013). Once internalized, *M. tuberculosis* actively blocks phagosome fusion with the lysosome, thus ensuring its survival within the macrophage (Russell 2011). Then, through the activity of the ESX-1 secretion system, *M. tuberculosis* can disrupt the phagosomal membrane and a few bacteria might be found in the cytosol of the infected cells in the days following infection (Houben et al. 2012; van der Wel et al. 2007).

After infecting the AMs in the airways, *M. tuberculosis* gains access to the lung parenchyma, where the process of infection leads to the recruitment of an increasing number of cells into the site of infection, eventually generating a multicellular host response called a granuloma (Pagan and Ramakrishnan 2014). Neutrophils are one of the first and most abundant cells to infiltrate the lungs after *M. tuberculosis* infection, constituting a potent population of effector cells that can mediate both antimycobacterial activity and immunopathology during *M. tuberculosis* infection (Dallenga and Schaible 2016).

As the primary infection is established, either infected dendritic cells (DCs) or inflammatory monocytes transport *M. tuberculosis* to pulmonary lymph nodes for T cell priming (Wolf et al. 2008; Samstein et al. 2013). DCs play a central role in *M. tuberculosis* antigen presentation and are critical in bridging innate and adaptive immunity (Prendergast and Kirman 2013). In the lymph nodes, upon antigen presentation, the activated naïve T cells differentiate into effector Th responses, undergo clonal expansion and start responding to chemoattractant gradients that lead their migration from the lymph nodes to the site of the infection. Upon arrival to the lung, $CD4^+$ T cells produce IFN-γ and other cytokines, leading to further macrophage activation, cytokine production, induction of microbicidal mechanisms, and ultimately bacterial control (Cooper 2009) (Figure 3).

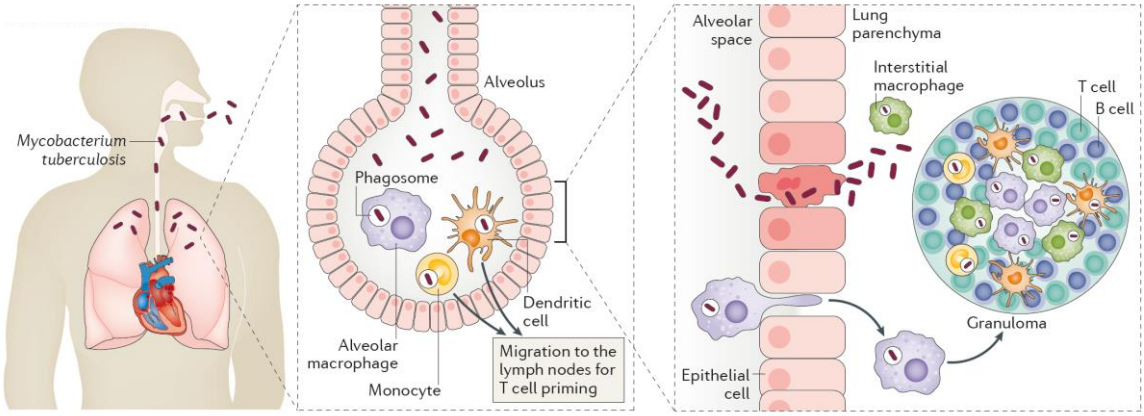

Figure 3 - The immune response to *M. tuberculosis*

Infection begins when *M. tuberculosis* enters the lungs via inhalation, reaches the alveolar space and encounters the resident AMs. If this first line of defence fails to eliminate the bacteria, *M. tuberculosis* invades the lung interstitial tissue, either by the bacteria directly infecting the alveolar epithelium or by the infected alveolar macrophages migrating to the lung parenchyma. Subsequently, either dendritic cells or inflammatory monocytes transport *M. tuberculosis* to pulmonary lymph nodes for T cell priming. This event leads to the recruitment of immune cells, including T cells and B cells, to the lung parenchyma to form a granuloma. Adapted from (Pai et al. 2016).

The concerted action of cellular and immune mediators during TB leads to the formation of granulomas, hallmark lesions of TB (Pagan and Ramakrishnan 2014). Granulomas are involved in both the control of the infection and, in some cases, the persistence of the pathogen (Lin et al. 2014). The granuloma illustrates the duality of *M. tuberculosis* infection. From the host perspective, the granuloma aims to avoid bacterial spread to the rest of the body. However, from the bacterial perspective, it is a growing collection of phagocytic cells to infect and replicate within (Antonelli et al. 2010). It has also been proposed that the granuloma might have a maximal bacterial burden, beyond which the infection will continue to progress (Lin et al. 2014). If the granuloma contains the infection without inducing substantial tissue pathology, then the person likely has LTBI and could be a candidate for preventive treatment.

In the context of my thesis, I will explore in more details specific aspects of the innate immune response and activation of macrophages during *M. tuberculosis* infection.

III.II Macrophages in tuberculosis

III.II.I The role of macrophages during *M. tuberculosis* infection

Macrophages play an important role in mycobacterial pathogenesis, since they are believed to be the primary cellular niche for *M. tuberculosis* during both early and chronic infection (McClean and Tobin 2016). They can eliminate *M. tuberculosis* through multiple mechanisms, such as reactive oxygen species and reactive nitrogen species, cytokines, phagosome acidification and autophagy of intracellular *M. tuberculosis*, among other processes (McClean and Tobin 2016). During early infection, *M. tuberculosis* is phagocytosed by AMs, which are the first cells to encounter the pathogen, and lead to the recruitment of different types of macrophages, such as monocyte-derived macrophages (Cadena, Flynn, and Fortune 2016). The recognition of pathogen-associated molecular patterns (PAMPs) from *M. tuberculosis*, such as glycolipids, lipoproteins, by macrophage PRRs, such as TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors, induces a network of coordinated signaling pathways that leads to distinct gene expression profiles in macrophages, at different stages of infection (Killick et al. 2013). The recognition of *M. tuberculosis* by PRRs and the subsequent cytokine network formed are discussed in more details below (sections III.III.I and III.IV).

Several populations of macrophages have been described in the context of *M. tuberculosis* infection (Flynn, Chan, and Lin 2011). AMs and more recently interstitial macrophages (IMs) have been described as the two major populations of macrophages in the lung (Guilliams et al. 2013; Tan and Krasnow 2016; Gibbings et al. 2017). Phenotypic markers defining IMs, through the expression of the integrins CD11c and CD11b, have indicated that these cells are recruited during *M. tuberculosis* infection (Srivastava, Ernst, and Desvignes 2014). It was observed that AMs exhibited lower stress and higher bacterial replication profiles than those in IMs. Accordingly, depletion of AMs reduced lung bacterial burdens, whereas depletion of IMs increased lung bacterial burdens. Thus, these different macrophage lineages respond divergently to *M. tuberculosis* infection, with IMs exhibiting nutritional restriction and controlling bacterial growth, while AMs represent a more nutritional permissive environment (Huang et al. 2018). Indeed, it has been revealed that early, productive *M. tuberculosis* infection occurs almost exclusively within AMs. Thereafter, *M. tuberculosis* infected AMs are localized into the lung interstitium through mechanism requiring *M. tuberculosis* ESX-1 secretion system. Relocalization of infected AMs precedes *M. tuberculosis* uptake by recruited monocyte-derived macrophages and neutrophils. This

dissemination process is driven by non-hematopoietic host myeloid differentiation primary response protein 88 (MyD88)/IL-1R inflammasome signaling (Cohen et al. 2018).

The development and function of macrophages are shaped by micro-environmental signals, which drive macrophage differentiation to M1 and M2 populations, the two extreme phenotypes of the macrophage polarization spectrum (Murray 2017; Ginhoux et al. 2016). Classically activated M1 macrophages, which are key effectors of the host response against intracellular bacteria and produce immune-stimulatory cytokines, are induced by microbial stimuli, such as lipopolysaccharide (LPS), or cytokines, such as IFN-γ, TNF-α, Granulocyte-macrophage colony-stimulating factor (Sica et al. 2015). During infection, *M. tuberculosis* is internalized and delivered to the acidic, hydrolytic environment of the phagolysosome. However, *M. tuberculosis* has evolved strategies to subvert the process of phagosome maturation (VanderVen et al. 2016). The phagosome in which *M. tuberculosis* resides is slightly acidified, remains interactive with the endosomal network, and shows limited acquisition of lysosomal hydrolase (Weiss and Schaible 2015). Classic activation of M1 macrophages with IFN-γ, produced by recruited T-cells, is required during infection to allow macrophages to overcome this process and to deliver the bacterium to an acidic lysosome (Schaible et al. 1998; Via et al. 1998). The killing of *M. tuberculosis* by activated macrophages is dependent on multiple factors, most significantly, the production of nitric oxide (NO), the low pH of the lysosome, and the delivery of antimicrobial peptides through the process of autophagy (Alonso et al. 2007; Gutierrez et al. 2004; MacMicking et al. 1997). Although these mechanisms are well organized, several studies demonstrated that *M. tuberculosis* can escape the phagosome and access the cytosol of its host cells (McDonough, Kress, and Bloom 1993; Myrvik, Leake, and Wright 1984; Simeone et al. 2012; van der Wel et al. 2007).

In contrast to protective M1 macrophages, alternatively activated M2 macrophages, which are poor antigen-presenting cells and suppressors of Th1 responses, are induced by IL-4, IL-13, as well as IL-10 and transforming growth factor (TGF) -β (Sica et al. 2015). These macrophage populations have been shown to play important roles in maintaining the balance between exacerbated pathology and control of mycobacterial growth. Indeed, mice deficient in arginase 1, a hallmark enzyme of M2 macrophages, showed a more proinflammatory macrophage response and demonstrated better protection against *M. tuberculosis* infection (Duque-Correa et al. 2014). During active TB, human monocytes are predisposed to differentiate toward M2-like macrophages, characterized by the $CD16^{+}CD163^{+}MerTK^{+}pSTAT3^{+}$ phenotype and increased protease-dependent mobility, pathogen permissivity and immunomodulation (Lastrucci et al. 2015). M2 macrophages can be induced via mycobacterial DaK (heat shock protein 70) in an IL-10-dependent manner (Lopes et al. 2016), which likely constitutes an immune evasion strategy. In addition to M2

macrophages, other myeloid populations appear to benefit *M. tuberculosis*. This is the case of myeloid-derived suppressor cells (MDSCs), which represent an innate immune cell population consisting of granulocyte $CD15^+$ and monocytic $CD14^+$ MDSCs (Dorhoi et al. 2019; Bruger et al. 2019). These cells have been described as lung-resident myeloid-derived suppressors induced during TB that can provide a niche for mycobacterial survival (Knaul et al. 2014). More recently, another population of macrophages, termed myeloid suppressor cells, which suppress T-cell responses via the secretion of IL-10 and TGF-β, has emerged as a novel class of immune cells exhibiting suppressive functions and regulating the infection and inflammation associated with TB (Khan, Hunter, and Jagannath 2016).

Other macrophage populations present in granulomas include multinucleated giant cells, epithelioid cells and foam cells (Feng et al. 2014; Peyron et al. 2008). Foamy macrophages, which exhibit an attenuated ability to mediate phagocytosis accompanied by reduced antigen processing capacity and increased secretion of TGF-β, can be induced by multiple *M. tuberculosis* triggers, such as mycolic acids, lipopeptides and early secretory antigen-6 (ESAT-6) (Peyron et al. 2008; Singh et al. 2015). More recently, it was reported that TLR2 signaling promotes macrophage polyploidy and suppresses genomic instability by regulating Myc and ATR expression, indicating that in the presence of persistent inflammatory stimuli, pathways involved in developing cancer cells surprisingly instruct a polyploid macrophage fate and regulate granulomatous tissue remodelling (Singh et al. 2015; Herrtwich et al. 2016). Interestingly, new evidence suggests that *M. tuberculosis* pathogenicity is intimately associated with its capacity to regulate host macrophage metabolism (Mehrotra et al. 2014). Upon *M. tuberculosis* infection, mononuclear phagocytes accumulate a stearic acid derivative, which promotes phagocyte differentiation into macrophages and enhances the effector function of phagocytes against *M. tuberculosis* (Mosquera-Restrepo et al. 2016). Furthermore, *M. tuberculosis* ESAT-6 was found to induce metabolic flux perturbations to drive foamy macrophages differentiation (Singh et al. 2015). During granuloma formation in TB, aggregating macrophages undergo a series of stereotyped morphological changes that lead them into a more epithelioid phenotype (Sutton and Weiss 1966). These epithelioid cells express at least one canonical epithelial marker, such as E-cadherin (Wanat et al. 2014). More recently, fundamental macrophage reprogramming events leading to macrophage-specific disruption of E-cadherin function were described. This macrophage reprogramming resulted in disordered granuloma formation, and enhanced immune access, decreased bacterial burden and increased host survival (Cronan et al. 2016). Thus, during mycobacterial infection, granuloma macrophages are broadly reprogrammed by epithelial modules, that can alter the trajectory of infection and the associated immune response.

Collectively, a deep understanding of macrophages functions during TB may lead the development of novel approaches to control TB via modulation of their phenotype, form, metabolism and function.

III.III Recognition of *M. tuberculosis* by pattern recognition receptors

III.III.I The recognition of *M. tuberculosis* by toll-like receptors

TLRs represent a family of type I transmembrane proteins that contain leucine-rich repeats and toll-interleukin 1 receptor (TIR) domains, and recognize PAMPs from pathogens. Signaling through TLRs requires the recruitment of the downstream adapters, including TIR domain-containing adapter protein, TIR domain-containing adapter protein inducing IFN-β (TRIF), TRIF-related adapter molecule, and MyD88 (Kawai and Akira 2010). Depending on their cellular localization and agonists, TLRs fall into two groups: plasma membrane-anchored TLR1, 2, 4, 5 and 6, which mainly recognize microbial membrane components, such as the Gram-negative bacterial endotoxin LPS; and endosomal TLR3, 7, 8 and 9, which predominantly detect microbial nucleic acids (Kawai and Akira 2010). TLR4 is an exception, as it is initially membrane anchored, but gets internalised upon activation, thus signaling from the endosomal compartment as well (Kagan et al. 2008). Upon pathogen infection, different TLRs recruit distinct adapter molecules to relay signals to downstream molecules, which results in the activation of multiple signaling pathways such as the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathways (Kawai and Akira 2010). These events culminate with the induction of proinflammatory cytokines and/or type I IFN. Changes in the expression and/ or activation status of TLRs can serve as useful markers of the immunological status in TB patients (Saraav, Singh, and Sharma 2014).

M. tuberculosis expresses many diverse lipoproteins and lipoglycans that can be recognized by different TLRs (Figure 4). This is the case of TLR2, that can recognize products encoded by lpqH (Pecora et al. 2006). TLR3 regulates mycobacterial RNA-induced IL-10 production through the TRIF signaling pathway (Bai et al. 2014). The *M. tuberculosis* recombinant leucine-responsive regulatory protein inhibits proinflammatory cytokine production and downregulates macrophage antigen presentation via TLR2-mediated activation of the PI3K/AKT pathway (Liu et al. 2016). Several *M. tuberculosis*-secreted proteins, such as PtpA and Mce3E, modulate TLR signaling by targeting the downstream molecules involved in the NF-κB and MAPK pathways (Wang et al. 2015; Li et al. 2015). Interestingly, a recent study demonstrated that G protein-coupled receptor 160 regulates mycobacteria entry into macrophages by activating MAPK/extracellular-signal-regulated kinase (ERK) signaling, which suggests a crosstalk between the G protein-coupled receptor and TLR signaling pathways during mycobacterial infection (Yang et al. 2016). Our group has shown that some *M. tuberculosis* strains activate TLR4 as a mechanism to induce IFN-β production in a TRIF-dependent pathway (Carmona et al. 2013).

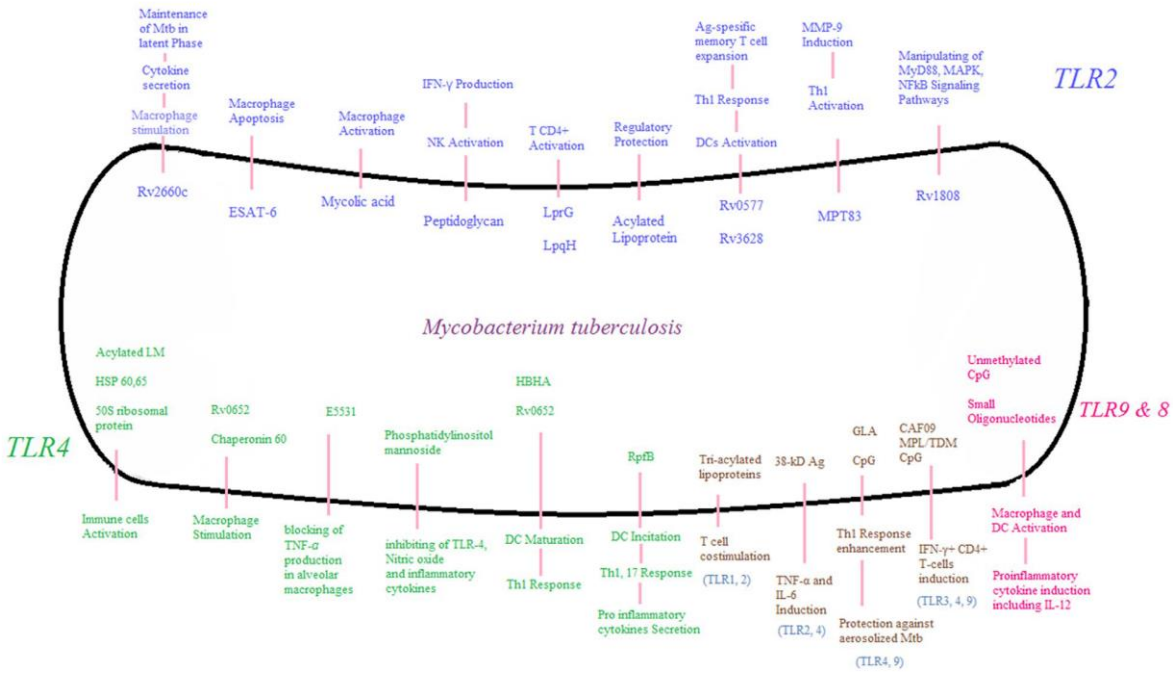

Figure 4 - Different *M. tuberculosis* components stimulate the immune system through TLRs.

The role of each TLR is depicted in this figure separately. TLRs interact with *M. tuberculosis* components and cause the activation of macrophages, natural (NK) cells, dendritic cells (DCs) and T cells and also induce cytokine secretion. Such roles of TLRs is crucial in primary identification of *M. tuberculosis* and development of appropriate immune responses to overcome the *M. tuberculosis* infection. LM: Lipomannan; Hsp: Heat Shock Protein; HBHA: Heparin-binding hemagglutinin; Rpf: Resuscitation-promoting factor; CAF: Cationic adjuvant formulation; MPL: Monophosphoryl lipid-A; TDM: Trehalose dimycolate; ESAT: Early secreted antigen; IFN-γ: Interferon-γ; Th: T helper; MMP: Matrix metalloproteinase; MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear factor κ-light-chain-enhancer of activated B cells; TNF: Tumor necrosis factor; IL: Interleukin. Adapted from (Faridgohar and Nikoueinejad 2017).

Mice deficient in the TLR adapter molecule MyD88 are highly susceptible to *M. tuberculosis* infection, suggesting a major role for this pathway in the innate defence against *M. tuberculosis* (Sugawara, Yamada, Mizuno, et al. 2003). TLR2-deficient mice exhibit defective granuloma formation following *M. tuberculosis* infection, although bacterial burden was similar to wild-type (WT) mice (Reiling et al. 2002; Bafica et al. 2005; Sugawara, Yamada, Li, et al. 2003; Drennan et al. 2004).

However, increasing the infection dose greatly enhanced the susceptibility of TLR2-deficient mice to infection, compared to WT mice (Drennan et al. 2004; Reiling et al. 2002). Consistently, TLR2 polymorphisms in humans are associated with enhanced susceptibility to pulmonary TB (Drennan et al. 2004). Our study has shown that TLR4-deficient mice could control growth of MTB strain from lineage 2. However, the bacterial burden remained higher in TLR4-deficient than WT mice, and higher than the ones found in mice infected with MTB H37Rv (Carmona et al. 2013). Genetic polymorphisms in TLR4 are linked to an increased susceptibility and severity of pulmonary TB in an Asian population in India (Najmi et al. 2010). TLR7 and TLR8 genetic polymorphisms are also associated with susceptibility to *M. tuberculosis* infection. Individuals with the TLR7 IVS2-151A/TLR8-129C genotype show increased phagocytosis and lower levels of immune activation due to a blockade of phagosome–lysosome fusion (Lai et al. 2016). Mice lacking TLR9 succumb to *M. tuberculosis* infection earlier than WT mice (Carvalho et al. 2011).

Additionally, polymorphisms in human TLR9 might have an important role in the susceptibility to *M. tuberculosis* infection (Bharti et al. 2014; Graustein et al. 2015). However, there is conflicting evidence concerning the protective effects of TLR signaling during *M. tuberculosis* infection. Indeed, a previous study demonstrated that unlike MyD88 deficiency, TLR2, TLR4 or TLR9 deficiency, even when combined, did not result in major susceptibility to *M. tuberculosis* infection in the mouse model (Holscher et al. 2008). Another study indicated that during *M. tuberculosis* infection, cytokines can be generated via a TLR- and caspase-1-independent mechanism (Mayer-Barber et al. 2010).

In conclusion, despite the presence of several TLR ligands in *M. tuberculosis* and the requirement of TLR signaling for macrophage function *in vitro* and data from animal and human studies, the final role of TLR signaling in the context of TB is not fully understood.

III.III.II The recognition of *M. tuberculosis* by Inflammasomes

The inflammasome is a high-molecular-weight complex formed in the cytosol of stimulated immune cells, that mediates the activation of inflammatory caspases (Martinon, Burns, and Tschopp 2002). Inflammasomes have been recognized for their crucial role in host defences against a multitude of pathogens (von Moltke et al. 2013). However, dysregulated activation of inflammasomes can lead to the development of cancer, autoimmune, metabolic and neurodegenerative diseases (Kayagaki et al. 2015; Shi et al. 2015). Therefore, the tight control of inflammasomes assembly and signaling is crucial to allow the immune system to initiate antimicrobial and inflammatory responses, while avoiding tissue damage. Five receptor proteins have been confirmed to assemble inflammasomes, which are the following: NLRP1 and NLRP3 (i.e. the NOD, leucine-rich repeat and pyrin domain (PYD)-containing protein family members); NLRC4 (which is the NOD, leucine-rich repeat and caspase activation and recruitment domain (CARD) domain-containing protein); AIM2 (the proteins absent in melanoma 2); pyrin. These canonical inflammasomes are complemented by non-canonical pathways, which target caspase-11 in mice and caspase-4/5 in human cells (von Moltke et al. 2013; Broz and Monack 2013). Two inflammasomes, containing NLRP3 and AIM2 molecules as sensor proteins, were found to play a crucial role in *M. tuberculosis*-induced immunity (Briken, Ahlbrand, and Shah 2013; Wassermann et al. 2015; Netea et al. 2010; Saiga et al. 2012).

The NLRP3 inflammasome-activated responses result in the release of significant amounts of caspase-1, leading to maturation and secretion of IL-1β and IL-18, and activation of pyroptosis (Liu and Lieberman 2017) (Figure 5). The process of NLRP3 activation requires at least two signals. The first consists of a priming signal eliciting the expression of NLRP3, pro-IL-1β and pro-IL-18 genes after TLR stimulation, and the second consists of activation signals leading to the autocatalytic activation of procaspase-1 and proteolytic cleavage of pro- IL-1β and pro-IL-18. In most of the cell types, NLRP3 priming is a prerequisite for deubiquitination and assembly of the NLRP3 inflammasome. Relocalization of NRLP3 to the mitochondria is followed by the secretion of mitochondrial factors into the cytosol, potassium efflux through membrane ion channels, and release of cathepsin, resulting in the destabilization of lysosomal membranes. Apoptosis-associated speck-like proteins (ASC) play an important role in the formation of an effective inflammasome platforms. ASC then recruits pro-caspase-1 through its C-terminal CARD and interacts with NLRP3 via its PYD, serving as a bridge between these two molecules. The autocatalysis of pro-caspase-1 results in its cleavage and transformation into active caspase-1, which in turn cleaves the precursors of IL-1β and IL-18, leading to their secretion

into the cytoplasm or induction (Broz and Dixit 2016; Guo, Callaway, and Ting 2015; dos Santos, Kutuzov, and Ridge 2012; Shaw, McDermott, and Kanneganti 2011).

The mechanisms behind the triggering of the NRLP3 inflammasome complex activation cascade are still elusive, with at least three possible models proposed. The first model suggests that the activation mechanism is associated with an efflux of potassium ions out of the cell and a reduction of their intracellular concentration. This model of activation occurs in monocytes/macrophages after stimulation with numerous stimuli, including ATP, nigericin, bacterial cells or components (Petrilli et al. 2007; Munoz-Planillo et al. 2009). The second model suggests damage of lysosomal membranes, and release of the phagosome content into cytosol as the inflammasome activation mechanism (Halle et al. 2008; Hornung et al. 2008). The third and most accepted model assumes that the induction of the NLRP3 inflammasome complex is caused by mitochondrial reactive oxygen species (Heid et al. 2013; Sorbara and Girardin 2011; Zhou et al. 2011; Nakahira et al. 2011). The common final step to all these models is the release of cathepsins into cytosol leading to the lysosomal destabilization and conversion of pro-caspase-1 into a biologically active form.

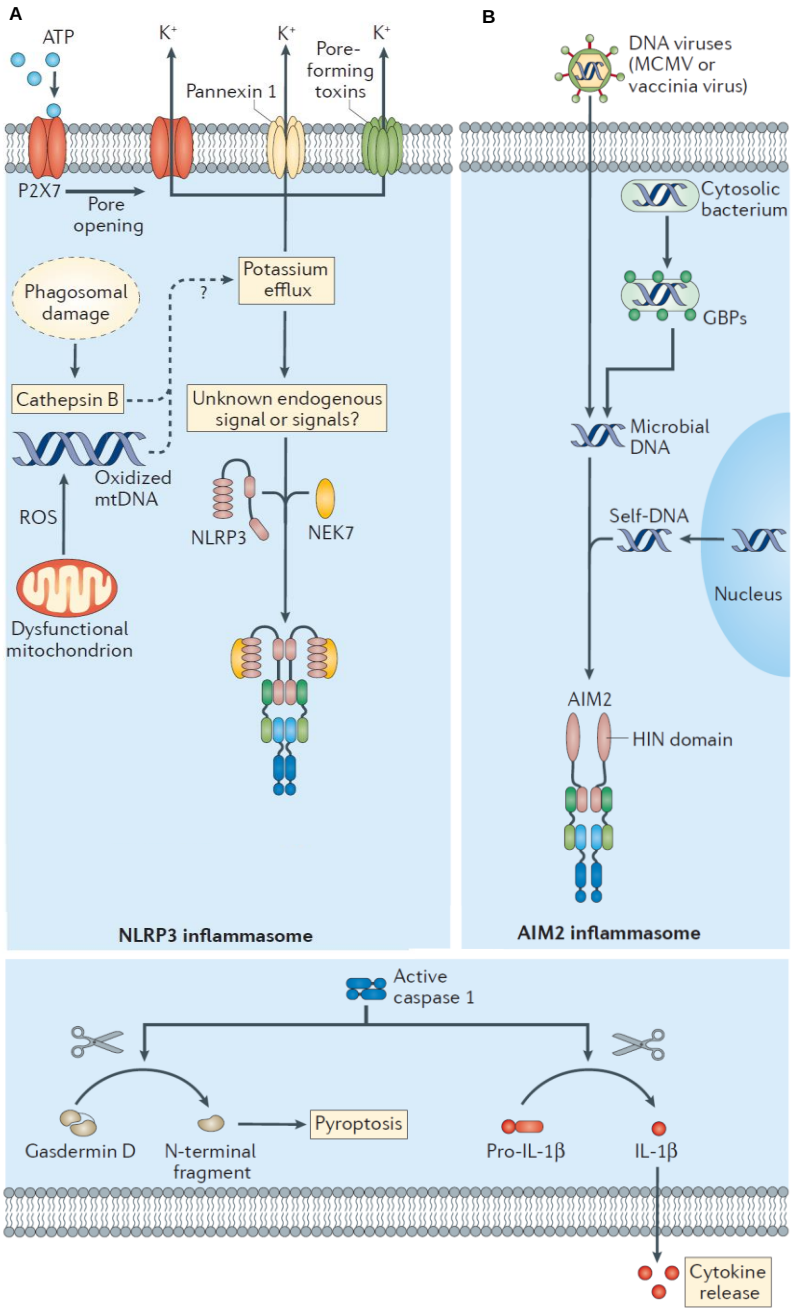

Figure 5 - The NLRP3 and AIM2 inflammasomes.

(A) Potassium efflux is a common event that is associated with a diverse array of NLRP3 stimuli. NLRP3 activation also requires NIMA-related kinase 7 (NEK7), which binds to the NLRP3 leucine-rich repeats (LRRs) and is required for its oligomerization. Activated receptors recruit the bipartite adaptor protein ASC, which consists of a pyrin domain (PYD) and a caspase recruitment domain (CARD), through homotypic PYD–PYD. The CARD of ASC is necessary to recruit pro-caspase 1 into the complex. **(B)** AIM2 detects the presence of double-stranded host or microbial DNA in the cytosol. Murine cytomegalovirus (MCMV), vaccinia virus and several intracellular bacteria activate AIM2. Recognition of microbial DNA requires bacteriolysis, which is facilitated by guanylate-binding proteins (GBPs). The recruitment of pro-caspase 1 to activated pyrin and AIM2 relies on the bipartite adaptor protein ASC. Following its proximity-induced autoproteolytic activation within the complex of NLRP3 and AIM2, caspase 1 processes pro-interleukin-1β (pro-IL-1β) and pro-IL-18 to their mature forms and cleaves gasdermin D. The N-terminal fragment of gasdermin D drives pyroptosis, which is a lytic type of cell death, and allows the release of mature IL-1β and IL-18 from the cell. mtDNA: mitochondrial DNA; P2RX7: P2X purinoceptor 7; ROS: reactive oxygen species. Adapted from (Broz and Dixit 2016).

The AIM2 receptor, possessing a C-terminal HIN-200 domain and an N-terminal PYD, triggers AIM2 inflammasome activation, pyroptosis, and release of IL-1β and IL-18 in response to cytosolic double-stranded (ds) DNA (Hornung et al. 2009; Fernandes-Alnemri et al. 2009) (Figure 5). The AIM2 inflammasome can be activated by DNA sequences having at least 80 base pairs in length in a sequence-independent manner (Roberts et al. 2009; Jin et al. 2012). The HIN-200 and PYD domains take part in forming a complex, which is maintained in an inactive state during homeostasis (Roberts et al. 2009; Jin et al. 2013). Binding dsDNA to HIN-200 facilitates oligomerization of AIM2, and results in the conformational change of PYD by exposing its N-terminal, allowing the recruitment of the adaptor protein ASC. The CARD of ASC binds the CARD of procapase-1, forming an active AIM2 platform. As described for NLRP3 above, upon autoactivation, caspase-1 directs the maturation and secretion of proinflammatory cytokines (Man, Karki, and Kanneganti 2016; Saiga et al. 2012; Hornung et al. 2009; Shaw, McDermott, and Kanneganti 2011; Wassermann et al. 2015). Interestingly, it was shown that during infection with *Legionella pneumophila*, AIM2 and caspase-11 induced membrane damage, which was sufficient and essential to activate NLRP3 inflammasome. These data reveal that different inflammasomes regulate on another activity to ensure an effective immune response to infection (Cunha et al. 2017).

Beside direct induction of proinflammatory cytokines secretion, the activated capase-1 triggers the pyroptotic death of infected cells. The cytosolic protein gasdermin D (GSDMD) is a key mediator of this process. The cleavage of GSDMD by active capase-1 results in the release of its N-terminal fragment, which forms pores in the plasma membrane of the infected cell, leading to the elimination of the pathogen (Liu and Lieberman 2017; Gaidt and Hornung 2016; Sborgi et al. 2016; Bergsbaken, Fink, and Cookson 2009). The pores disrupt cell membrane integrity allowing water influx, cell swelling, and osmotic lysis together with an efflux of small molecules, including proinflammatory cytokines. This process results in the death of both cell-free and intracellular microorganism, although it is not known whether GSDMD is able to permeabilize the membrane of the phagosome, and to kill the hidden bacteria within these organelles (Thurston et al. 2016).

As mentioned above, NLRP3 and AIM2 are the most studied inflammasomes in the context of TB (Briken, Ahlbrand, and Shah 2013). NLRP3- or ASC-deficient animals are characterized by impaired inflammasome formation and increased susceptibility to TB (van de Veerdonk et al. 2011; Eklund et al. 2014; Saiga et al. 2012; Lai et al. 2015; Chao et al. 2015). However, NLRP3- and ASC-deficient mice produced IL-1β and IL-18 levels comparable to those of WT mice, which suggests the involvement of inflammasome-independent pathways in the secretion of these cytokines (Netea et al. 2010; Mayer-Barber et al. 2010; Briken, Ahlbrand, and Shah 2013). Additionally, AIM2-deficent mice present

high infection burden and severe pathology to *M. tuberculosis* infection (Yan et al. 2018; Saiga et al. 2012). Infection of human macrophages with polymorphisms in NLPR3 and CARD8 presented an increased control of mycobacterial growth (Eklund et al. 2014). However, in the context of TB, polymorphisms in NLRP3 and CARD8 were associated with extrapulmonary TB and poor treatment outcome in the case of patients with pulmonary TB (Abate et al. 2019).

The mechanism underlying the activation of NLRP3 during *M. tuberculosis* infection is poorly understood. Nonetheless it was shown that the production of IL-1β required live intracellular bacteria expressing a functional ESX-1 secretion system. Through the production of ESAT-6 (an ESX-1 substrate with membrane-lytic capability), *M. tuberculosis* could induce caspase-1 activation and IL-1β secretion (Mishra et al. 2010). Additionally, AIM2 inflammasome has also been implicated in the intracellular recognition of *M. tuberculosis*. (Saiga et al. 2012; Wassermann et al. 2015). Indeed, co-localization of AIM2 with cytosolic DNA has been shown in macrophages infected with *M. tuberculosis* (Saiga et al. 2012). Some ESX-1-deficient *Mycobacterium smegmatis* mutants have shown limited capacity for AIM2 inflammasome activation. However, in contrast to non-tuberculous mycobacteria, *M. tuberculosis* mutant lacking ESX-1 secretion system failed to inhibit the inflammasome activation (Shah et al. 2013; Sugawara et al. 2001). Potassium efflux plays an important role in the activation of NLRP3 inflammasome during *M. tuberculosis* infection. However, disturbance of other ion fluxes through the action of ESX-1 can also contribute to NLRP3 activation (Dorhoi et al. 2012; Wassermann et al. 2015).

M. tuberculosis can inhibit the formation of NLRP3 and AIM2 inflammasomes both directly and indirectly, but the factors responsible for the inhibition have not been fully recognized up to now. Several studies have demonstrated that IFN-β can regulate inflammasome activity during infection (Jones et al. 2010; Tsuchiya et al. 2010; Mayer-Barber et al. 2011; Novikov et al. 2011; Guarda et al. 2011). This suggests that the mechanisms of inhibition during *M. tuberculosis* can involve the IFN-β-mediated induction of IL-10, which in turn suppresses IL-1β production (Mayer-Barber et al. 2011; Novikov et al. 2011; Guarda et al. 2011). Moreover, IFN-γ can also regulate inflammasome activity by inducing NO production in macrophages, consequently inhibiting NLRP3 assembling and IL-1β secretion (Mishra et al. 2013). In addition to the induction of inflammasome activation via PRRs, *M. tuberculosis* can secrete effector molecules that can restrict IL-1 production (Master et al. 2008). After a few days of invasion of the host organism, *M. tuberculosis* often escapes from the phagosome and creates difficulties in assessing the potential role of inflammasome during the initial stages of mycobacterial infection. Moreover, the initiation of phagocytosis causes a decrease in the levels of potassium ions in macrophages, which

have been found to be the most crucial inflammasome activators during infection with *M. tuberculosis* (Kanneganti et al. 2007).

III.III.III The recognition of *M. tuberculosis* by cGAS

Over the past two decades, the ability of host innate immune cells to sense microbial pathogens through recognition of their nucleic acids has emerged as a key feature of innate immunity in mammalian cells. Single-stranded RNAs or DNAs, RNA-DNA hybrids and cyclic dinucleotides are all recognized as foreign nucleic acids (Tan et al. 2018; Mankan et al. 2014; Janeway 1989).

In the case of DNA, three major receptors have been described in mammalian cells, which are TLR9, AIM2 and cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase (cGAS). The DNA sensor cGAS is activated through direct binding of DNA, which triggers conformational changes that induce enzymatic activity (Civril et al. 2013; Gao et al. 2013; Kranzusch et al. 2013; Zhang et al. 2013; Li et al. 2013).

Although any DNA can cause cGAS activation, the length of the DNA is important. Short DNAs of around 20 base pairs can bind to cGAS, but dsDNA superior to 45 base pairs can form more stable ladder-like networks of cGAS dimers, leading to stronger enzymatic activity (Li et al. 2013; Zhang et al. 2014). Active cGAS converts GTP and ATP into cyclic GMP (Wu et al. 2013). This endogenous second messenger is unique as it contains unusual mixed phosphodiester linkages between the 2´-hydroxyl group of GMP, forming a novel 2´3´-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) isomer (Zhang et al. 2013; Ablasser et al. 2013; Diner et al. 2013). The 2´3´-cGAMP product binds to the stimulator of interferon genes (STING), an endoplasmic reticulum (ER)-localized adaptor to form dimers, tetramers and higher-order oligomers (Ishikawa and Barber 2008; Ishikawa, Ma, and Barber 2009; Shang et al. 2019; Shu et al. 2012; Wu et al. 2013).

STING also binds to cyclic dinucleotides produced by bacteria directly, including cyclic diGMP, cyclic diAMP and bacterial cyclic GMP, all of which have conventional 3,5´phosphodiester linkages (Burdette et al. 2011). In general, the 2´3´-cGAMP is thought to bind with higher affinity to STING than to bacterial cyclic dinucleotides, which suggests that STING is more strongly activated when the cGAS receptor is engaged (Zhang et al. 2013). STING undergoes a conformational change upon cGAMP binding. This change of conformation leads to a 180º rotation of the ligand-binding domain, resulting in the formation of STING oligomers through side-by-side packing of dimeric STING molecules (Zhang et al. 2013; Gao et al. 2013; Zhou et al. 2018). Following cGAMP-driven conformational changes, STING traffics through the ER-Golgi intermediate compartment and the Golgi

apparatus in a process that is dependent on the cytoplasmic coat protein complex II and ADP-ribosylation factor GTPases (Dobbs et al. 2015; Gui et al. 2019). STING is also palmitoylated at the Golgi, and this post-translational modification is essential for its activation (Mukai et al. 2016). Following its translocation to the Golgi, STING interacts with TANK-binding kinase (TBK1), and IκB kinase-related kinases that control the activation of the transcription factor interferon regulatory factor (IRF) 3 (Hornung and Latz 2010; Ishikawa, Ma, and Barber 2009). Following its direct phosphorylation by TBK1, the C-terminal tail region of STING served as a docking site for IRF3, which is then phosphorylated and activated by TBK1 (Liu et al. 2015). Activated IRF3 dimerizes and translocates to the nucleus to regulate the transcription of IFN-β, a type I IFN (Agalioti et al. 2000) (Figure 6).

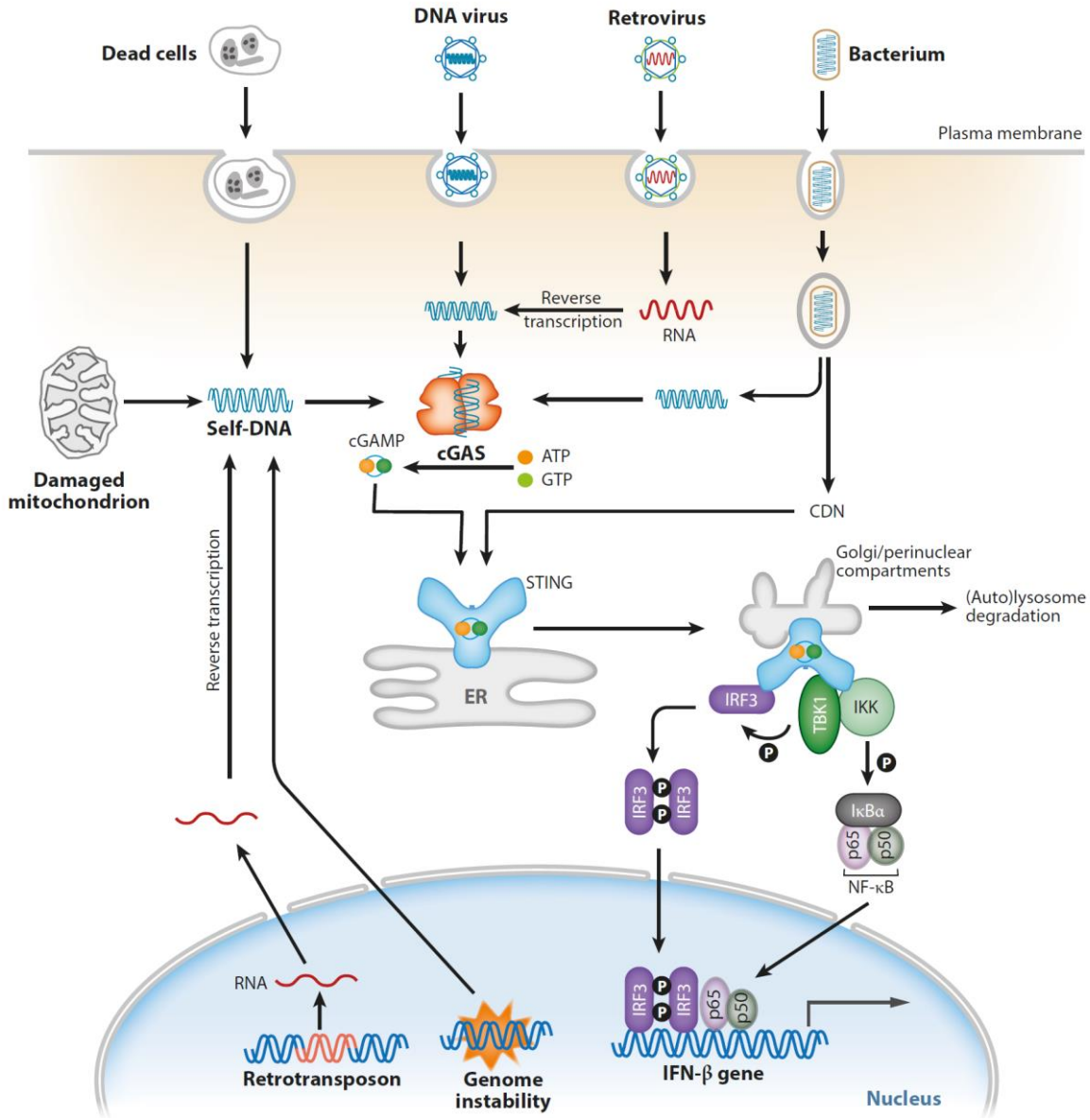

Figure 6 - the cGAS-STING DNA sensing pathway.

cGAS binds to DNA from invading microbes, including DNA viruses, retroviruses, and bacteria, or to self-DNA generated from DNA damage, reverse transcription of endogenous retroelements, damaged mitochondria, or phagocytosed dead cells. Such binding leads to activation of cGAS, which synthesizes the second messenger 2´3´- cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) from ATP and GTP. cGAMP is a high-affinity ligand for the endoplasmic reticulum (ER)-localized adaptor protein STING. After binding to cGAMP, STING undergoes conformational changes and translocates to perinuclear compartments, where it activates TBK1 and the IKK complex, leading to activation of transcription factors IRF3 and NF-κB, respectively. Nuclear translocation of these active transcription factors turns on type I interferons and proinflammatory cytokines. P: phosphoryl group. Adapted from (Tan et al. 2018).

The cGAS-STING pathway is important for the detection of several microbial infections, most importantly viruses (Tan et al. 2018). Recent years have witnessed significant progress in the study of mycobacterial recognition by cGAS. As already discussed, despite residing inside phagosomes after phagocytosis, *M. tuberculosis* can permeabilize the phagosomal membrane through its ESX-1 secretion system, which facilitates cytosolic exposure of bacterial DNA (Manzanillo et al. 2012; Watson et al. 2015). Accordingly, several studies have demonstrated an essential role for cGAS in recognition of *M. tuberculosis* and subsequently in IFN-β production (Collins et al. 2015; Wassermann et al. 2015; Watson et al. 2015). In the absence of cGAS, deficient mice succumbed significantly earlier than WT mice during chronic TB infection (Collins et al. 2015). Both AIM2 and cGAS have been shown to colocalize with and to be activated by *M. tuberculosis* -derived DNA in the cytosol, and the extent of which their pathways are activated is likely to be regulated by bacterial factors secreted through the ESX-1 secretion system (Wassermann et al. 2015).

III.IV Main molecular effectors of the immune response to *M. tuberculosis*

Tumour Necrosis Factor-α

TNF-α is a cytokine that is primarily produced by macrophages, but can be secreted by lymphocytes, mast cells, endothelial cells, and fibroblasts (Wajant, Pfizenmaier, and Scheurich 2003). Since most cells exhibit responsiveness to TNF-α, this cytokine is considered as a major proinflammatory mediator. The TNF-α mechanism of action depends on the activation of TNF receptors, mediated downstream by NF-κB, c-Jun N-terminal kinase (JNK), and p38 to promote proinflammatory responses, as well as apoptosis (Wajant, Pfizenmaier, and Scheurich 2003; Bazan 1993; Devin et al. 2001; Hsu et al. 1996; Hsu, Xiong, and Goeddel 1995; Jiang et al. 1999). Although it was originally described for its ability to promote necrosis of tumours, TNF-α has since been implicated in proliferation and differentiation of immune cells in multiple inflammatory processes and infections, including TB (Carswell et al. 1975; Faustman and Davis 2013; Keane et al. 1997; Keane, Remold, and Kornfeld 2000).

TNF-α is one of the first cytokines to be produced upon initial *M. tuberculosis* infection, playing an important role in coordinating the chemokine response within the lung, in facilitating the development of the granuloma, and in promoting the optimal macrophage activation when it is produced by both $CD4^+$ and $CD8^+$ T cells (Serbina and Flynn 1999;

Flynn and Chan 2001). In *M. tuberculosis* infection models, the importance of TNF-α is illustrated in mice deficient in the TNF receptor or following TNF-α neutralization (Flynn et al. 1995). In these models, TNF-α deficiency results in increased susceptibility to infection, with mice succumbing within 3 weeks, while harbouring a high bacterial burden (Flynn et al. 1995). Critically, although inflammatory cells accumulate at the site of *M. tuberculosis* infection in TNF-α-deficient mice, they do not conjoin to form granulomas (Flynn et al. 1995; Algood, Lin, and Flynn 2005; Roach et al. 2002; Bean et al. 1999). Neutralization of TNF-α leads to a decreased expression of important chemokines such as C-C chemokine ligand (CCL) 5, C-X-C chemokine ligand (CXCL) 9 and CXCL10 (Farber 1997; Cole et al. 1998; Griffith, Sokol, and Luster 2014). In the absence of TNF-α, T cells expressing C-X-C chemokine receptor 3 fail to encounter the ligands CXCL9 and CXCL10 required to recruit these cells into the granuloma. Therefore, the communication between infected phagocytes and the instructive T cells does not occur, resulting in loss of immunity (Farber 1997; Cole et al. 1998; Griffith, Sokol, and Luster 2014). Most importantly, the protective role of TNF-α during TB is also seen in humans. Immunotherapy to suppress TNF-α, commonly used for autoimmune and chronic inflammatory diseases, has been associated with higher incidences of TB reactivation (Shaikha, Mansour, and Riad 2012; Keane et al. 2001; Keane 2005; Raval et al. 2007; Gomez-Reino et al. 2003; Dixon et al. 2006; Askling et al. 2005; Tubach et al. 2009).

Interleukin-12

IL-12 also plays an important role as a link between innate and adaptive immune responses, as it is produced and influenced by multiple effector cells (Kobayashi et al. 1989; Gately et al. 1991). The main function of IL-12 in the context of TB is to promote Th1 cell differentiation. The bioactive IL-12 (IL-12p70), composed of IL-12p35 and IL-12/23p40 subunits, is primarily secreted by macrophages, DCs and B cells (Vignali and Kuchroo 2012; Ma and Trinchieri 2001; O'Shea and Paul 2002). Both TLR2 and TLR9 are necessary for optimal production of IL-12p40 in response to *M. tuberculosis* exposure, while the Mannose capped lipoarabinomannan of *M. tuberculosis* cell wall lipids have been shown to negatively regulate TLR-mediated IL-12 production by inducing an inhibitor of TLR signaling (Bafica et al. 2005; Pathak et al. 2005; Mishra et al. 2012). On the opposite, mycobacterial LprA, a TLR2 agonist, promotes IL-12p40 production, reflecting the need for *M. tuberculosis* to both induce and regulate IL-12p40 for its own ends (Pecora et al. 2006). The homodimers IL-12p40, IL-12(p40)2, antagonize IL-12-mediated immune responses through competitive binding to the IL-12Rβ1 (Gillessen et al. 1995; Gately et al. 1996; Mattner et al. 1993). However, in TB, it appears that IL-12(p40)2 can also function as an agonist of the IL-12R,

thus supporting dendritic cell migration to the draining lymph nodes, to promote T cell priming and differentiation (Holscher et al. 2001; Khader et al. 2006; Reinhardt et al. 2006). Specifically, following *M. tuberculosis* infection, DCs are thought to be the first immune cells to migrate into the draining lymph node, and this may occur in an IL-12p40 and IL-12Rβ1-dependent manner (Khader et al. 2006; Wolf et al. 2008; Robinson et al. 2010). Bone marrow derived DCs from mice deficient in IL-12p40 are unable to activate naïve T cells in the draining lymph node. Consequently, these T cells cannot migrate into the lung and thus protective adaptive responses fail (Khader et al. 2006). However, the treatment of the IL-12p40-deficient DCs with the homodimer IL-12(p40)2 is sufficient to restore the migration of these cells to the draining lymph nodes and to activate the naïve T cells (Khader et al. 2006). The expression of IL-12Rβ1 is also required to facilitate DCs migration to the draining lymph node, and indeed $CD11c^+$ cells in the *M. tuberculosis*-infected lung express an alternative splice variant of IL-12Rβ1 that augments IL-12Rβ1-mediated effects (Robinson et al. 2010). In particular, DCs expressing this splice variant exhibit enhanced migration from the infected lung to the draining lymph nodes and supported activation of *M. tuberculosis*-specific T cells (Robinson et al. 2010). Therefore, the activation of the IL-12Rβ1 is important for the effector function of IL-12p40 on DCs, hence the mediating recruitment and the function of $CD4^+$ T cells in response to TB.

IL-12 is expressed within the lung at the site of TB and its delivery to *M. tuberculosis*-infected mice decreases bacterial burdens, while the reduction of IL-12 by antibody administration increases bacterial burden (Zhang et al. 1994; Cooper et al. 1995). Interestingly, delivery of IL-12 also modestly improves the outcome of infection in mice lacking acquired cellular immunity, suggesting that IL-12 can mediate immunity via direct action on innate cells (Cooper et al. 1995). During *M. tuberculosis* infection with the Erdman strain, neither IL-12p35 nor p40-deficient mice could control bacterial growth as efficiently as WT mice. Mice genetically deficient in the IL-12p40 subunit are acutely susceptible to *M. tuberculosis* infection, whereas those lacking IL-12p35 exhibit prolonged survival relative to the IL-12p40-deficient mice (Cooper et al. 1997; Cooper et al. 2002). A stable and prolonged IL-12 production is required to maintain IFN-γ production and to limit bacterial growth in the long-term (Feng et al. 2005). The production of IL-12 in humans is critical in the context of TB. Patients with Mendelian susceptibility to mycobacterial disease (MSMD) present deficiencies in IL-12Rβ1, IFN-γR1, and IL-12p40, exhibit susceptibility to *M. tuberculosis* and develop BCGosis following the delivery of the BCG vaccine (Casanova and Abel 2002; Bogunovic et al. 2012; Bustamante, Arias, et al. 2011; Bustamante, Picard, et al. 2011; Filipe-Santos et al. 2006). Genetic causes for MSMD are associated with mutations in autosomal genes related to IL-12/IFN-γ-dependent signaling and IFN-γ-mediated activation of macrophages (Cooper et al. 1995). Indeed, an inactive IL-12R1 results in poor

accumulation of IFN-γ-producing memory T cells (Cleary et al. 2003). Humans with IL-12p40 deficiency present an inherent predisposition to *M. tuberculosis* infection (Ozbek et al. 2005; Dorman and Holland 2000; Picard et al. 2002; Altare et al. 2001; Caragol et al. 2003; Casanova and Abel 2002).

Interleukin-1

IL-1α, IL-1β and IL-18 are proinflammatory cytokines, belonging to the IL-1 family (Dinarello 1991). These cytokines are widely expressed by all nucleated cells of the body, including endothelial cells, monocytes, macrophages and neutrophils (Dinarello 1991). Expression of IL-1β and IL-18 is mediated in part by canonical pathways of inflammasome activation, as discussed above and widely reviewed (Gross et al. 2012; Sansonetti et al. 2000; Latz, Xiao, and Stutz 2013). These cytokines can also be induced by a non-canonical pathway, distinguished by the activation of caspase-8 and 11 on the precursor of the cytokine in the cytosol (Kayagaki et al. 2011; Bossaller et al. 2012).

IL-1α and IL-1β are interdependent proinflammatory cytokines critical for host protection during TB. Mice lacking either IL-1α or IL-1β or both are susceptible to acute and chronic *M. tuberculosis* infection (Mayer-Barber et al. 2011; Bourigault et al. 2013; Di Paolo et al. 2015; Guler et al. 2011; Gopal et al. 2014). IL-1α/β double-deficient mice share a similar susceptibility to infection as IL-1R1 and MyD88-deficient mice (Fremond et al. 2004; Fremond et al. 2007; Guler et al. 2011; Schneider et al. 2010). Anti-IL-1α and anti-IL-1α/β antibodies delivered subcutaneously to *M. tuberculosis*-infected mice have also been shown to result in loss of body weight and lethality. Further, lung sections from anti-IL-1α-treated mice exhibited lung parenchyma consumed by cellular infiltrates (Guler et al. 2011). During sterile mediated inflammation, IL-1α appears to be involved in the expression of proinflammatory cytokines such as IL-6 in primary fibroblasts, which may be associated with the mobilization of neutrophils (Suwara et al. 2014; Fielding et al. 2008). Recently, it has also been observed that upon activation of the inflammasome, IL-1β and IL-18 are capable of inducing the expression of the neutrophil recruiting cytokine IL-17 by Th17 cells (Lalor et al. 2011; Dunne et al. 2010; Chung et al. 2009). Consistent with this, IL-1R gene deficient mice infected with the *M. tuberculosis* strain HN878 produce decreased levels of IL-17 and decreased populations of IL-17-producing cells *in vitro* and *in vivo* (Gopal et al. 2014). IL-1β was also described to promote differentiation of monocytes into macrophages and to increase their phagocytic and antigen-presenting capacity (Selvaraj et al. 2008). Therefore it seems that one primary function of the IL-1/IL-1R pathway is to mediate the recruitment and the coordination of cellular responses by the induction of proinflammatory cytokines from the stroma (Mayer-Barber et al. 2011; Di Paolo et al. 2015). The protective role of IL-

1 in TB is now known to extend beyond the control of intracellular bacterial replication via enhancing the macrophage antimicrobial mechanisms. Recent studies have positioned IL-1β as one of the drivers of ILC3 responses required for early protection (Ardain et al. 2019). Moreover, the relocalization of AMs from the alveolar space to lung interstitium required both IL-1R and bacterial ESX-1 secretion system (Cohen et al. 2018). Finally, a role for IL-1R signaling to limit the proportion of infected cells, to disseminate and to afford protection has been recently revealed (Bohrer et al. 2018). The protective role of IL-1β during TB is also seen in humans. Polymorphism in IL-1 gene cluster was associated with the increased susceptibility to TB (Bellamy et al. 1998; Gomez et al. 2006).

Type I interferon

Type I IFN represent the largest group of cytokines, with at least thirteen gene products identified in humans and mice (Pestka, Krause, and Walter 2004). IFN-α/β is the most studied member of this family and is the focus of this section. In the context of *M. tuberculosis* infection, several studies have shown that distinct mycobacterial molecules and signaling pathways may be involved in the induction of type I IFN. Recognition of mycobacterial components by cytosolic sensors, such as NOD2, cGAS, STING, AIM2 have already been described to induce or regulate IFN-β secretion (Leber et al. 2008; Pandey et al. 2009; Collins et al. 2015; Wassermann et al. 2015; Watson et al. 2015; Shah et al. 2013). Additionally, our group has shown that *M. tuberculosis* strains that activate TLR4 induce IFN-β in a TRIF-dependent signaling (Carmona et al. 2013). Recognition by these different sensors activates complex cytosolic cascades of signal transduction toward TBK1, and IRF3/5/7-mediated transcription of IFN-α/β genes (McNab et al. 2015). Secreted IFN-α/β engages the IFN-α/β receptors 1 and 2 (IFNAR1/2) at the cell surface, which then activates dimers of the Janus kinase (JAK) and tyrosine kinase (TYK) (McNab et al. 2015; Honda, Takaoka, and Taniguchi 2006). The result is the activation of IFN-stimulated gene factor that interacts with IFN-stimulated response elements at the promoters of IFN-α/β regulated genes (McNab et al. 2015; Honda, Takaoka, and Taniguchi 2006).

Early reports on the infection of IFNAR-deficient mice with a low dose aerosol of *M. tuberculosis* strain Erdman did not indicate any major impact on the control of infection (Cooper et al. 2000). However, the use of strains with increased virulence, such as the *M. tuberculosis* strain HN878, has revealed an important strain-dependent outcome in relation to type I IFN. The pathogenesis of *M. tuberculosis* strain HN878 is associated with IFN-α/β dependent reduction in the activity of the proinflammatory cytokines IFN-γ, TNF-α, IL-6 and IL-12, accompanied by an increase of the anti-inflammatory IL-10 (Manca et al. 2001;

Ordway et al. 2007). Intranasal delivery of IFN-α/β to mice infected with *M. tuberculosis* strain HN878 also results in the increased bacterial burden and the reduced survival in contrast to IFN-γ treated mice (Manca et al. 2001). Additionally, the increased production of type I IFN associated with polyinosinic:polycytidylic acid (poly I:C) administration to *M. tuberculosis* infected mice, with deficiency on Tpl2 or with influenza co-infection, promoted susceptibility to *M. tuberculosis* infection (McNab et al. 2013; Redford et al. 2014; Antonelli et al. 2010).

Most importantly, recent study revealed a blood transcriptional signature discriminating active TB from LTBI and healthy individuals (Berry et al. 2010). The transcriptional analysis of peripheral blood from those exposed to TB showed that both IFN-γ and type I IFN signatures occur, but the type I IFN signature is predominantly associated with neutrophils (Berry et al. 2010). Using an advanced modular approach, a signature that discriminated active TB patients from LTBI individuals, or even those with acute viral and bacterial infections was further developed (Singhania, Verma, et al. 2018).

Given the aforementioned evidence for the role of type I IFN as a detrimental molecule in the context of TB, several studies have addressed the underlying mechanistic bases. Poly I:C treatment of infected mice resulted in elevated bacterial burdens through the increased recruitment of an apparently permissive $CD11b^{+}$ $GR1^{int}$ cell phenotype recruited via CCL2 and CCR2 (Antonelli et al. 2010). Further, in the context of *in vitro* macrophage infection, IFN-α/β signaling interferes with IFN-γ-mediated killing of *M. tuberculosis*, by promoting IL-10 production in an IL-27-independent manner (McNab et al. 2014). One hypothesis regarding the role of type I IFN during chronic *M. tuberculosis* infection is that the accumulation of plasmacytoid DCs in the lung provides a source of excess type I IFN which then inhibits the accumulation of $CD4^{+}$ and $CD8^{+}$ T cells in the lung (Ordway et al. 2007).

Despite all the evidence on the detrimental role of type I IFN in TB, this molecule is required for the initial steps of host immunity, to initiate the recruitment of phagocytes (Desvignes, Wolf, and Ernst 2012). Therefore, it is not surprising that the mechanisms regulating the expression and production of type I IFN in TB have been thoroughly addressed. One such mechanism relates to IL-1, which promotes prostaglandin E2 that mediates the inhibition of type I IFN-induced accumulation of permissive macrophages at the site of infection (Mayer-Barber et al. 2014). The underlying function of IL-1 in *M. tuberculosis* also appears to regulate type I IFN function. This process helps to maintain the balance between sufficient phagocytes in order to mediate control of the intracellular pathogen, while inhibiting the over recruitment of permissive macrophages mediated by type I IFN (Desvignes, Wolf, and Ernst 2012).

Type II Interferon

IFN-γ is a type II IFN that, although structurally related to the type I IFN, IFN-α and IFN-β, does not bind nor signal through IFNAR (Fallahi-Sichani et al. 2012). IFN-γ binds to the IFN-γ receptor (IFN-γR) composed by two ligand binding IFN-γR1 chains that associate with two signals transducing IFN-γR2 chains (Fallahi-Sichani et al. 2012). IFN-γ-IFN-γR binding induces signaling within the cell primarily through the JAK - signal transducers and activators of transcription (STAT) pathways and results in changes in both the migratory and functional capacity of multiple cell types such as macrophages, natural killer (NK) cells, T cells (Greenlund et al. 1994; Kovarik et al. 1998; Frucht et al. 2001). Classically, IFN-γ is a phagocyte activating cytokine, which instructs macrophages to exert microbicidal functions and other cells to change function. It enhances the antigen presentation through the expression of molecules from the major histocompatibility complex (MHC) class I and II. It also orchestrates the activation of innate immune system, regulates Th1 and Th2 cells balance, and controls cellular proliferation and apoptosis (Billiau 1996; Boehm et al. 1997). In the absence of IFN-γ, *M. tuberculosis* resides in an intracellular environment where there is little reactive radical production, the phagosome does not fuse with lysosomes and remains at neutral pH. There is also an ample supply of iron due to the location of the phagosome in the early endosomal pathway (Russell 2001).

In accordance with the fundamental role for IFN-γ for bacterial containment, mice that do not express IFN-γ due to targeted gene disruption are severely susceptible to both low dose aerosol and intravenous infection, and also exhibit poor macrophage activation and exacerbated granulocytic inflammation (Cooper et al. 1993; Flynn et al. 1993; Dalton et al. 1993). IFN-γ-deficient mice have extensive neutrophil-mediated pathology during *M. tuberculosis* infection (Nandi and Behar 2011). This is due to the role of IFN-γ in regulating Th17 cell responses, which in turn are important for neutrophil recruitment (Cruz et al. 2006; Cruz et al. 2010). Furthermore, and as mentioned above, genetic deficiencies in the IFN-γ pathway in humans belong to the MSMD (Zhang et al. 2008; Filipe-Santos et al. 2006). Autosomal complete recessive IFN-γR1-deficient patients exhibit a predisposition for mycobacterial infections manifesting early in life and with poor prognosis (Sologuren et al. 2011). IFN-γR2 deficiency has also been observed and results in a similar outcome to IFN-γR1-deficiency (Vogt et al. 2005; Dorman and Holland 1998).

Finally, it is worth mentioning that the production of IFN-γ is a very useful diagnostic tool which has been developed to be more selective than the older skin test assay. In this prominent test for *M. tuberculosis* exposure, *M. tuberculosis* antigens are used to stimulate IFN-γ release (Stefan et al. 2010; Abu-Taleb, El-Sokkary, and El Tarhouny 2011; Ferrara et al. 2009). While this test selects those who are exposed, it is not optimized to distinguish

those individuals who are infected but healthy from those in the process of developing active disease. Recently, studies have shown that patients that have more IFN-γ-producing T cells are actually more likely to progress to active disease, suggesting that this test may be optimised to identify those progressing toward disease (Diel et al. 2011).

Interleukin-10

IL-10 was initially considered a Th2 cell cytokine (Fiorentino, Bond, and Mosmann 1989). However, IL-10 can be produced by other T cell subsets including Th1 and Th17 cells, macrophages, some DC subsets, myeloid derived suppressor cells, B cells and neutrophils (Saraiva and O'Garra 2010). In addition, regulatory T cells (Tregs) are also a major source of IL-10 and serve to limit potentially pathogenic immune responses (Redford, Murray, and O'Garra 2011). Following mycobacterial stimulation, DCs and macrophages both produce IL-10 (Jang et al. 2004; Verreck et al. 2004). In macrophages, IL-10 can block phagosome maturation and macrophage activation, allowing a niche for *M. tuberculosis* to replicate and survive within the phagosome (O'Leary, O'Sullivan, and Keane 2011). In addition, IL-10 can inhibit aspects of IFN-γ-mediated macrophage activation (Oswald et al. 1992). In DCs, mycobacterial-induced IL-10 production inhibits antigen presentation through the down regulation of MHC class II molecules, the decreased IL-12 production and the inhibition of DC trafficking to the lymph nodes for T cell priming (Moore et al. 2001; Richardson et al. 2015).

In keeping with this regulatory role for IL-10, IL-10 gene deficient mice infected with *M. tuberculosis* exhibit increased Th1 and Th17 responses, which coincided with improved *M. tuberculosis* control during chronic infection (Redford et al. 2010). However, this effect is not dramatic and indeed some challenge models fail to show an impact of IL-10 gene deficiency (North 1998; Jung et al. 2003; Higgins et al. 2009). In vaccine models, blocking IL-10 at the time of BCG vaccination, or using IL-10 gene deficient mice in BCG vaccination and *M. tuberculosis* challenge experiments, demonstrates that IL-10 limits IFN-γ and IL-17 responses during priming, and decreases vaccine-induced protection against *M. tuberculosis* infection (Redford, Murray, and O'Garra 2011; Gopal et al. 2012). Importantly, differences related to the role of IL-10 were reported depending on the nature of the *M. tuberculosis* strains being examined. Indeed, infection of mice with *M. tuberculosis* strain HN878 induces the rapid emergence of IL-10-producing Tregs that inhibit Th1 cell responses (Ordway et al. 2007). However, a more recent study has shown that despite the multiple immune sources of IL-10 during *M. tuberculosis* infection, activated effector T cells are the major cellular source associated with IL-10-induced TB susceptibility (Moreira-Teixeira et al. 2017).

In the context of human TB, meta-analyses studies suggest that polymorphisms in the IL-10 gene, specifically in European and Asian populations, are significantly associated with TB risk (Ke et al. 2015). Further, antigen-specific IL-10 production is found in pulmonary TB patients, and along with TNF-α production can be used to reliably distinguish between latent TB and pulmonary TB (Jeong et al. 2015; Tebruegge et al. 2015). In addition, the increased accumulation of Tregs expressing IL-10 correlated with the increased bacterial burden and more severe TB in an Indian population. Furthermore, a high level of IL-10 at the end of the treatment in pulmonary TB patients was associated with TB recurrence (Kumar et al. 2015; Eum et al. 2008; Lago et al. 2012). Moreover, stimulation of T cells from anergic patient with PPD resulted in IL-10 production but not IFN-γ, and T cells failed to proliferate upon stimulation. That event might help *M. tuberculosis* to escape immune surveillance (Boussiotis et al. 2000). Finally, TB patients co-infected with helminths had decreased antigen-specific IFN-γ and IL-17 responses, which was dependent on IL-10, since its blockade significantly increased the frequencies of IFN-γ-producing cells (George et al. 2015; George et al. 2014).

Nitric oxide

NO is an endogenous molecular gas produced at different sites of the body as a result of the conversion of L-arginine and molecular oxygen to *N*-hydroxy-L-arginine (Mikaili, Moloudizargari, and Aghajanshakeri 2014). This reaction is catalysed by the enzyme NO synthase (NOS). There are three isoforms NOS: neuronal NOS1, inducible NOS2, and endothelial NOS3. These isoforms of NOS differ with respect to their regulation, their cellular and tissue distribution, and duration of NO production (MacMicking, Xie, and Nathan 1997; Stuehr 1999). The NO production in the context of the immune response has been mainly associated with NOS2 (Nathan 1992).

NO generation is a feature of genuine immune-system cells, such as DCs, NK cells, mast cells and phagocytes (monocytes, macrophages, microglia, Kupffer cells, eosinophils, and neutrophils), as well as other cells involved in immune reactions, for instance endothelial cells, epithelial cells, vascular smooth muscle cells, fibroblast and hepatocytes (Bogdan, Rollinghoff, and Diefenbach 2000). The expression of NOS2 in these cells is regulated by cytokines and determined primarily by *de novo* synthesis and stability of NOS2 messenger RNA (mRNA) and proteins (MacMicking, Xie, and Nathan 1997; Rodriguez-Pascual et al. 2000; Carpenter, Cordery, and Biden 2001). Activation of the NOS2 gene promoter is an important mode of NOS2 regulation by cytokines, which has been analysed mostly in mouse macrophages, human hepatocyte, and epithelial cell lines. The

participating transcription factors include NF-κB, activator protein 1, STAT-1α, IRF1, nuclear factor-IL6, and high-mobility group-I(Y) proteins (MacMicking et al. 1997; Kleinert et al. 1998; Dlaska and Weiss 1999; Pellacani et al. 2001; Ganster et al. 2001). Depending on the cytokine or microbial stimulus and cell type, different upstream signaling pathways involved can promote (e.g. JAK1, JAK2 and TYK2; MAPK p38, ERK1/2 and JNK; protein kinase C, protein phosphatase 1 and 2S) or inhibit (e.g. PI3K, protein tyrosine phosphatase) NOS2 expression (Bogdan, Rollinghoff, and Diefenbach 2000; Karaghiosoff et al. 2000; Chakravortty et al. 2001; Chan et al. 2001; Kristof, Marks-Konczalik, and Moss 2001).

Another factor that determines NOS activity is the availability of arginine. High-output production of NO, such in macrophages, depends on extracellular L-arginine even when an adequate level of intracellular arginine is present (Chang, Liao, and Kuo 1998). Extracellular arginine concentration is strongly modulated by arginase (Wu and Morris 1998). This enzyme, which can also be released into the extracellular space, degrades arginine to urea and ornithine, and exists in at least two isoforms, arginases 1 and 2. In macrophages and bone marrow-derived DCs, Th2 cytokines (IL-4 with or without IL-10, IL-13), TGF-β, LPS or dexamethasone plus cyclic AMP have been found to strongly increase arginase 1 or 2 (Munder et al. 1999; Gotoh and Mori 1999). The upregulation of arginase prior to the induction of NOS2 by IFN-γ plus TNF or LPS prevents the NO production by substrate depletion (Bogdan, Rollinghoff, and Diefenbach 2000; Coccia et al. 2000).

NO plays an important role in bacteriostatic and bactericidal processes as part of the host defence mechanism against pulmonary infections (Flesch and Kaufmann 1991; Appelberg and Orme 1993). In the context of *M. tuberculosis* infection, the production of TNF-α, IL-1β, along with IFN-γ produced by T cells, can induce macrophages to produce NO via the action of NOS2 (Kuo et al. 2000; Lee et al. 1993; Wang et al. 2001). The capacity of NO to modify DNA, proteins and lipids can result in the death of both microbes and host (Chan et al. 1995). NO can also interact with accessory protein targets such as iron-sulfur groups, heme groups or amines, resulting in the inactivation of enzymes or protein modification (Gow, Thom, and Ischiropoulos 1998). Additionally, NO can play a regulatory function in TB. NO production during *M. tuberculosis* infection can regulate IL-1β production by inhibiting the assembly of the NLRP3 inflammasome, consequently promoting immunopathology control (Mishra et al. 2013).

The importance of NO production in the context of *M. tuberculosis* infection has also been shown *in vivo* and *ex vivo*. Mice treated with NOS2 inhibitors or deficient for NOS2 gene presented an increase severity of infection and of exacerbation, compared to WT mice (Boom 1996; Flynn et al. 1998; Sciorati et al. 1999; Cooper et al. 2000; Kuo et al. 2000). In contrast to murine model of TB, there is controversy regarding the role of NO to kill and inhibit *M. tuberculosis* in human (Nathan and Shiloh 2000). Study has shown that inhibition

of mycobacterial growth by human AMs was independent on NO production (Aston et al. 1998). However, several studies suggested that NO production by *M. tuberculosis*-infected human macrophages, macrophages cell lines and epithelial cells induced mycobacteriostatic activity against *M. tuberculosis* (Nicholson et al. 1996; Jagannath, Actor, and Hunter 1998; Kwon 1997; Rich et al. 1997; Rockett et al. 1998). Additionally, polymorphism in NOS2 gene influences the susceptibility to TB, suggesting a role for NOS2 in pathogenesis in mycobacterial infection (Gomez et al. 2007; Jamieson et al. 2004).

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Chapter II – Objectives

The regulation of the immune response during TB is crucial to ensure protection whilst preventing pathology. This regulation depends on the balance between cellular and molecular interactions at the site of infection. While some cytokines, such as TNF-α and IFN-γ, are known to promote protection, the specific role of other cytokines during TB remains to be fully defined. Such is the case for type I IFN, a cytokine known to be protective during viral infections, but that has been mainly associated with increased disease severity in TB. However, its full role during TB remains controversial, and its mechanism of action has not been completely understood. Another challenging field in TB relates to the genetic diversity of the pathogen. Indeed, recent studies position the diversity of *M. tuberculosis* as an important modulator of the immune response. However, how *M. tuberculosis* diversity orchestrates the immune response to drive certain disease severities remains unknown. In this context, the overarching goal of my thesis was to answer two specific questions:

1. What is the role of type I IFN during infection with a virulent TLR4-activating *M. tuberculosis* clinical isolate?

2. Could the diversity of *M. tuberculosis* strains influence the host immune response towards a specific TB severity?

Chapter III – Results

I. Type I IFN Inhibits Alternative Macrophage Activation during *Mycobacterium tuberculosis* Infection and Leads to Enhanced Protection in the Absence of IFN-γ Signaling

This chapter has been published in *The Journal of Immunology*.

Type I IFN Inhibits Alternative Macrophage Activation during *Mycobacterium tuberculosis* Infection and Leads to Enhanced Protection in the Absence of IFN-γ Signaling

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Tuberculosis causes ∼1.5 million deaths every year, thus remaining a leading cause of death from infectious diseases in the world. A growing body of evidence demonstrates that type I IFN plays a detrimental role in tuberculosis pathogenesis, likely by interfering with IFN-γ–dependent immunity. In this article, we reveal a novel mechanism by which type I IFN may confer protection against *Mycobacterium tuberculosis* infection in the absence of IFN-γ signaling. We show that production of type I IFN by *M. tuberculosis*–infected macrophages induced NO synthase 2 and inhibited arginase 1 gene expression. In vivo, absence of both type I and type II IFN receptors led to strikingly increased levels of arginase 1 gene expression and protein activity in infected lungs, characteristic of alternatively activated macrophages. This correlated with increased lung bacterial burden and pathology and decreased survival compared with mice deficient in either receptor. Increased expression of other genes associated with alternatively activated macrophages, as well as increased expression of Th2-associated cytokines and decreased TNF expression, were also observed. Thus, in the absence of IFN-γ signaling, type I IFN suppressed the switching of macrophages from a more protective classically activated phenotype to a more permissive alternatively activated phenotype. Together, our data support a model in which suppression of alternative macrophage activation by type I IFN during *M. tuberculosis* infection, in the absence of IFN-γ signaling, contributes to host protection. ***The Journal of Immunology*****, 2016, 197: 4714–4726.**

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* infection, is a leading cause of mortality and morbidity, causing ∼1.5 million deaths every year (1). Despite the efforts devoted to the understanding of this disease, mechanisms determining whether protection or pathogenesis results from *M. tuberculosis* infection remain poorly understood. An in-depth understanding of these mechanisms is critical for the design of novel preventive and therapeutic strategies based on the modulation of the immune response.

The initiation of the immune response to *M. tuberculosis* relies on bacterial recognition by pattern recognition receptors, such as TLR, by host sentinel cells (2). Recognition of *M. tuberculosis* triggers the production of key cytokines, chemokines, and antimicrobial molecules that are crucial to activate microbicidal

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Abbreviations used in this article: *Arg1*, arginase 1; dKO, double KO; ICVS, Life and Health Sciences Research Institute; IFNAR, type I IFN receptor; KO, knockout; MOI, multiplicity of infection; NOS2, inducible NO synthase 2; TB, tuberculosis; WT, wild-type.

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mechanisms in innate immune cells and for the establishment of the adaptive immune response, restriction of bacterial growth, and, ultimately, host resistance (3–6). Among key cytokines produced by innate immune cells, IL-12 is critical for the induction of protective Th1 responses and IFN-γ production (5, 7–9). In turn, IFN-γ is crucial for full activation of macrophages, enhancing the production of cytokines and expression of microbicidal mediators, such as the inducible isoform of the enzyme NO synthase (NO synthase 2 [NOS2]) that is critical for controlling bacterial growth (9–13). Indeed, mice deficient in IFN-γ (*Ifng*$^{-/-}$ mice) or NOS2 (*Nos2*$^{-/-}$ mice) are extremely susceptible to *M. tuberculosis* infection, supporting the essential role of IFN-γ and NO in immunity against *M. tuberculosis* infection (10–14).

Different strains of *M. tuberculosis* interact with host TLRs in a distinct way, which is likely to shape the downstream immune response and disease outcome. We recently showed that, although most of the *M. tuberculosis* strains tested primarily activate TLR2, some activate TLR4 (15). TLR4 activation by *M. tuberculosis* was found to result in the expression of host-protective factors (e.g., TNF, IFN-γ, and NOS2) and to limit bacterial growth during in vivo infection (15). Despite the protective role of TLR4, a hypervirulent strain of *M. tuberculosis* recognized predominantly by this receptor was also found to induce high levels of type I IFN during infection (15), a cytokine that was associated with exacerbated disease (16, 17). Infection with other hypervirulent strains of *M. tuberculosis* showed a correlation between increased levels of type I IFN and increased virulence in mouse models of *M. tuberculosis* infection (18–20). *M. tuberculosis* infection of mice deficient in the type I IFN receptor (IFNAR) (*Ifnar*$^{-/-}$ mice) largely results in reduced bacterial load and/or increased survival compared with wild-type (WT) mice (19–22). Additionally, overexpression of type I IFN during *M. tuberculosis* infection provided robust evidence for the detrimental effects of type I IFN during TB (18, 23–26). Induction of high levels of type I IFN by direct instillation of type I IFN (18) or a type I IFN inducer (23, 25) into the lungs of *M. tuberculosis*–infected mice promoted disease severity. Abrogation of a negative regulator of type I IFN signaling increased host susceptibility to *M. tuberculosis* infection (24). Coinfection of mice with influenza A virus and *M. tuberculosis* resulted in increased bacterial loads in a type I IFN–dependent manner (26). Furthermore, a potential negative role for type I IFN was also revealed in human TB, because patients with active TB showed a prominent type I IFN–inducible blood signature (27–30) that correlated with the extent of radiographic disease (27) and that diminished upon successful treatment (30, 31). Thus, studies in mouse and humans highlight a potentially detrimental, rather than protective, role for type I IFN during TB (16–30).

The mechanisms that mediate type I IFN–dependent TB exacerbation are a major topic of investigation in the field (16, 17). Recent studies described that type I IFN suppresses the expression of protective proinflammatory cytokines (e.g., IL-1, TNF, and IL-12) while inducing the immune-suppressive cytokine IL-10 during *M. tuberculosis* infection (18–21, 32–34). Generation and trafficking to the lung of *M. tuberculosis*–permissive myeloid cells in response to induced type I IFN may also contribute to exacerbated disease (22, 23, 35). Type I IFN also was shown to impair Th1 cell responses in in vivo mouse models (18, 20, 32, 36) and to inhibit IFN-γ–induced antimicrobial responses in murine macrophages (33) and human monocytes (37, 38). The interplay between type I and type II IFN is further supported by a recent report by Desvignes et al. (39) describing an unanticipated protective role for type I IFN during *M. tuberculosis* infection in the absence of IFN-γ signaling. Mice deficient in both type I and type II IFNRs (*Ifngr*$^{-/-}$ × *Ifnar*$^{-/-}$ mice) showed increased pulmonary pathology and early mortality following *M. tuberculosis* infection compared with single type II IFNR–deficient (*Ifngr*$^{-/-}$) mice (39). The investigators unmasked the involvement of type I IFN in the recruitment and/or survival of myeloid cells into the lungs and restriction of *M. tuberculosis* infection of these cells when IFN-γ signaling was absent (39). A putative protective role for type I IFN in the absence of IFN-γ signaling also was suggested in human TB based on the observation that administration of type I IFN, together with multidrug antimycobacterial treatment, had beneficial effects against disseminated *Mycobacterium avium* infection in a patient with IFN-γR deficiency (40).

In this article, we describe a novel mechanism for type I IFN in regulating macrophage activation during infection with a virulent strain of *M. tuberculosis* in the absence of IFN-γ signaling. Using a TLR4-activating virulent strain of *M. tuberculosis* that induces high levels of type I IFN, we detected increased levels of arginase 1 (*Arg1*) gene expression in the lungs, along with other genes associated with alternatively activated macrophages, when IFNAR signaling was abrogated in IFN-γR–deficient mice following in vivo infection. This correlated with increased lung bacterial loads and pathology, as well as increased Th2-associated cytokines and decreased TNF levels. Thus, our data indicate that suppression of alternative macrophage activation during *M. tuberculosis* infection by type I IFN confers protection against *M. tuberculosis* infection in the absence of IFN-γ signaling.

Materials and Methods

Ethics statement

All animal experiments were performed in strict accordance with the recommendations of the European Union Directive 2010/63/EU and were previously approved by the Portuguese National Authority for Animal Health (Direção Geral de Alimentação e Veterinária).

Mice

C57BL/6 WT mice and knockout (KO) mice (all backcrossed ≥10 generations to the C57BL/6 background) were bred and housed at the Life and Health Sciences Research Institute (ICVS) (WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice), Instituto de Investigação e Inovação em Saúde (WT, *Tnf*$^{-/-}$, *Nos2*$^{-/-}$ mice), and at The Francis Crick Institute, Mill Hill Laboratory (WT, *Ifnar*$^{-/-}$ and *Ifngr*$^{-/-}$ mice). *Ifngr*$^{-/-}$ and *Ifnar*$^{-/-}$ mice were crossed to obtain double-KO (dKO) mutant mice (*Ifngr*$^{-/-}$ × *Ifnar*$^{-/-}$). Bones from *Il1r*$^{-/-}$ and *Ticam*$^{-/-}$ mice (and WT controls) were generously provided by Dr. Teresa Pais (Instituto de Medicina Molecular, Lisbon, Portugal) and by Dr. Luigina Romani (University of Perugia, Perugia, Italy), respectively. Mice were bred and maintained for experiments in accordance with the European Union Directive 2010/63/EU or the United Kingdom Home Office regulation and the Animal Scientific Procedures Act, 1986. For infections with *M. tuberculosis*, animals were housed under barrier conditions in the Animal Biosafety Level 3 facility at ICVS. Mice were matched for sex and age for use in experiments.

Bacteria

M. tuberculosis strains H37Rv Pasteur and BTB 02-171 were kindly provided by Dr. Pere-Joan Cardona (Experimental Tuberculosis Unit, Barcelona, Spain) and Dr. Gunilla Källenius (Karolinska Institutet, Stockholm, Sweden), respectively. *M. tuberculosis* strains were grown in Middlebrook 7H9 liquid media for 7–10 d, diluted into Proskauer Beck medium with 0.05% Tween 80 for further expansion to mid-log phase, and frozen in 1-ml aliquots at −80°C, as previously described (15). All stocks were checked for endotoxin contamination using the *Limulus* assay (Sigma) and found to be negative.

Bone marrow–derived macrophages

Bone marrow–derived macrophages (macrophages) were differentiated from bone marrow precursors cultured in complete DMEM (containing 10% FBS, 1% sodium pyruvate, 1% HEPES and 1% L-glutamine; all from Life Technologies) supplemented with 20% L929 cell-conditioned media, as previously described (15). Briefly, total bone marrow cells were cultured in microbiological petri dishes (Sterilin) and kept at 37°C and 5% CO_2.

Cells were fed on day 4 with 4 ml of complete DMEM containing 20% L929 cell-conditioned media. On day 7, macrophages were harvested, counted, and seeded into 24-well tissue culture plates (Nunc) at 0.5×10^6 cells per well in culture medium. Cells were infected with *M. tuberculosis* strains at a multiplicity of infection (MOI) of 2. In some experiments, macrophages were stimulated with LPS (0.5 μg/ml; Sigma). When indicated, polymyxin B (5 μ/ml; Sigma), cycloheximide (10 μg/ml; Sigma), rIFN-β (2 ng/ml; PBL), or rIFN-γ (5 ng/ml; R&D Systems) was added at the time of infection. At specific time points postinfection, total RNA was isolated and/or supernatants were collected.

Enumeration of intracellular M. tuberculosis *in macrophages*

To determine the number of intracellular *M. tuberculosis* CFU present in macrophages following in vitro infection, supernatants were removed, and macrophages were washed with PBS and lysed with saponin (Sigma). This suspension was serially diluted and plated onto Middlebrook 7H11 agar (BD Biosciences) plates containing OADC, and colonies were counted after 3 wk of incubation at 37°C. Differences in bacterial uptake among WT, *Tlr4*$^{-/-}$, *Ifnar*$^{-/-}$, and *Nos2*$^{-/-}$ macrophages were monitored by enumerating CFU at 4 h postinfection, with no significant differences observed (data not shown).

Experimental infection

Mice were infected with *M. tuberculosis* strains H37Rv or BTB 02-171 via the aerosol route using an inhalation exposure system (Glas-Col) calibrated to deliver ~100–200 CFU to the lungs. The infection dose was confirmed by determining the number of viable bacteria in the lungs of three to five mice 3 d after the aerosol infection. For bacterial load determination, mice were euthanized by CO_2 inhalation, and the lungs were aseptically excised and individually homogenized, followed by plating of serial dilutions of the organ homogenate on Middlebrook 7H11 agar (BD Biosciences) supplemented with OADC and Panta. CFU were counted after 3 wk of incubation at 37°C.

Quantitative real-time PCR analysis

Total RNA from infected lungs or cultured macrophages was extracted with TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Thermo Scientific). Target gene mRNA expression was quantified by real-time PCR (Bio-Rad CFX96 Real-Time System with C1000 Thermal Cycler) and normalized to *Hprt1* or *Ubiquitin* mRNA levels. Target gene mRNA expression was quantified using SYBR Green (Thermo Scientific) and specific oligonucleotides (Invitrogen) for *Tnf* ([F] 5′-GCC ACC ACG TCT TCT GTC T-3′, [R] 5′-TGA GGG TCT GGG CCA TAG AAC-3′) and *Ubiquitin* ([F] 5′-TGG CTA TTA ATT ATT CGG TCT GCAT-3′, [R] 5′-GCA AGT GGC TAG AGT GCA GAG TAA-3′) or TaqMan primer probes (Applied Biosystems) for *Nos2* (Mm00440502_m1), *Arg1* (Mm00475988_m1), *Ym1* (Mm00657889_mH), *Fizz1* (Mm00445109_m1), *Il4* (Mm00445260_m1), *Il5* (Mm00439646_m1), *Il13* (Mm00434204_m1), *Il10* (Mm00439614_m1) and *Hprt1* (Mm00446968_m1).

Histology

Whole lungs were perfused in situ with PBS. The right upper lobe of infected lungs was excised and fixed in 3.7% phosphate-buffered formalin for 1 wk. Then tissue was embedded in paraffin and cut into 3-μm-thick sections. Lung specimens were stained with H&E and subjected to microscopic morphometric analysis. Lung surface area of inflammation was measured using ImageJ software (version 1.50e; National Institutes of Health). Briefly, entire lung sections were analyzed, and the areas corresponding to the whole section and individual lesions were manually selected and measured. The percentage of total lung area involved with inflammation was calculated by dividing the cumulative area of inflammation by the total lung surface area for each sample.

NOS2 detection by immunofluorescence

NOS2 was detected by immunofluorescence with a rabbit anti-mouse NOS2 primary Ab IgG (M-19; Santa Cruz Biotechnology) and an Alexa Fluor 488 goat anti-rabbit secondary Ab IgG (Invitrogen), as previously described (15). DAPI was used to detect nuclei. Images were acquired on an Olympus BX61 fluorescence microscope using Cell^P software.

Measurement of NO production

NO production was quantified in cell culture supernatants by the Griess reaction. Absorbance was measured at 550 nm using a microplate reader (Bio-Rad), and NO concentration was assessed using a sodium nitrite standard curve (0–100 μM analyzed in duplicate).

Arginase activity assay

ARG1 activity in lung homogenates was determined using the Arginase Activity Assay Kit (Sigma), according to the manufacturer's instructions. Briefly, 1×10^6 lung cells were lysed for 10 min in 10 mM Tris-HCl (Calbiochem) (pH 7.4) containing 1 μM pepstatin A, 1 μM leupeptin, and 0.4% (w/v) Triton X-100 (all from Sigma). Each sample was incubated for 2 h at 37°C in the presence of assay reagent and then incubated with urea reagent for 1 h at room temperature. Urea levels were subsequently detected and calculated according to the manufacturer's instructions. ARG1 activity is expressed as U/l, and data are shown as fold induction relative to uninfected control mice.

Cytokine determination by ELISA

TNF concentration in the supernatants of infected macrophages was determined by ELISA using a commercially available kit (eBioscience), according to the manufacturer's instructions. Cytokine levels from uninfected cells were below the assay level of detection (20 pg/ml; data not shown).

Flow cytometry

Myeloid cell populations from infected lungs were characterized using flow cytometry. For cell surface staining, Abs against CD11b (M1/70; eBioscience), CD11c (N418), Ly6G (1A8), and Ly6C (HK1.4; all from BioLegend) were used. For intracellular staining of NOS2, lung cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% saponin (Sigma) before staining with anti-NOS2 Ab (CXNFT; eBioscience). Samples were acquired on a LSR II flow cytometer with FACSDiva software (BD Bioscience). Data were analyzed using FlowJo software (TreeStar).

Statistics

Data are shown as the mean ± SEM. Statistical tests, as described in the figure legends, were used to compare experimental groups, with $p < 0.05$ considered significant. GraphPad Prism 6 (GraphPad) was used for data analysis and preparation of all graphs.

Results

Early and elevated Nos2 *expression induced by infection with a virulent TLR4-activating strain of* M. tuberculosis *results in protection and not pathology*

Although it is generally recognized that NOS2 plays an essential role in the host defense against some strains of *M. tuberculosis* (12, 14, 41, 42), other studies assign a detrimental role to NOS2 in the context of *M. avium* infection (43) and other bacterial infections (44, 45). Previous data from our group showed that TLR4 activation by *M. tuberculosis* BTB 02-171 induced an early and elevated expression of *Nos2* in the lungs of infected mice that was accompanied by enhanced lung pathology; this was not seen when the TLR2-activating laboratory reference strain H37Rv was used (15). To investigate whether early and elevated levels of *Nos2* expression could explain the enhanced pathology, WT and *Nos2*$^{-/-}$ mice were aerosol infected with *M. tuberculosis* strain H37Rv or BTB 02-171. As expected, bacterial loads were significantly increased in the lungs of H37Rv-infected *Nos2*$^{-/-}$ mice by day 30 postinfection, but no major differences in lung bacterial loads were observed at earlier times post-H37Rv infection (days 18 and 24 postinfection) compared with WT mice (Fig. 1A). However, higher bacterial loads were detected in the lungs at these early times, in addition to day 30, postinfection with BTB 02-171 in *Nos2*$^{-/-}$ mice compared with WT mice (Fig. 1B), in keeping with an earlier and elevated induction of *Nos2* transcription and NOS2 expression (15) (Supplemental Fig. 1). Increased bacterial loads observed in the absence of NOS2 were accompanied by enhanced lung pathology, which was quantified by the extent of lung area with inflammatory lesions at day 24 postinfection (Fig. 1C, 1D), confirming the protective, rather than detrimental, role of early and elevated *Nos2* levels observed during BTB 02-171 *M. tuberculosis* infection.

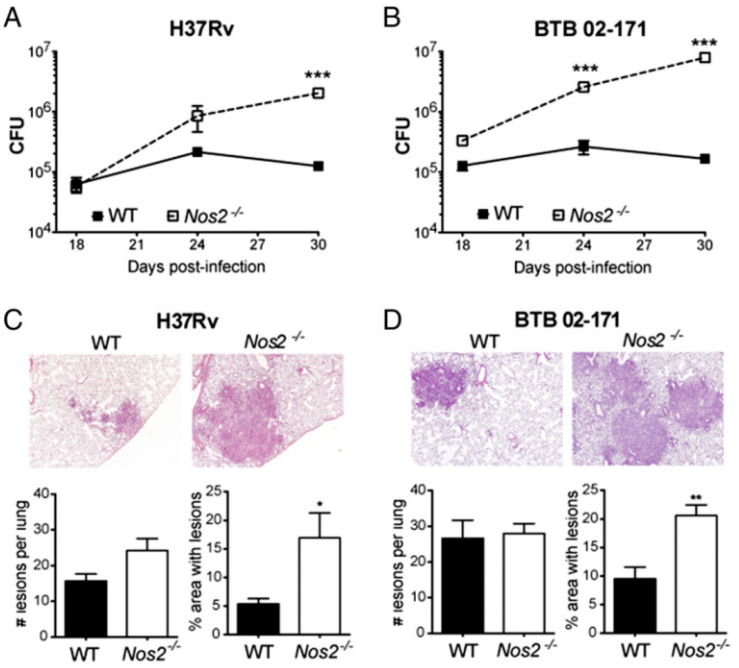

FIGURE 1. *Nos2* expression is essential for early control of virulent *M. tuberculosis* growth and immunopathology. WT and *Nos2*$^{-/-}$ mice were infected with *M. tuberculosis* strains H37Rv (**A** and **C**) or BTB 02-171 (**B** and **D**). (A and B) At the indicated days postinfection, lung cell suspensions were prepared, diluted, and plated onto 7H11 agar to determine the number of mycobacterial CFU in the lungs. Data points show the mean ± SEM for five mice per group. ***$p < 0.001$, two-way ANOVA corrected for multiple comparisons with a Bonferroni test. (C and D) H&E-stained lung tissue from WT and *Nos2*$^{-/-}$ infected mice was analyzed blindly at day 24 postinfection. Representatives of one of five animals are shown (original magnification ×4; upper panels). Morphometric analysis of the number and size of inflammatory lesions are also shown (lower panels). Each bar represents mean ± SEM for five mice per group. Data are representative of two independent experiments. *$p < 0.05$, **$p < 0.01$, unpaired *t* test.

TLR4 recognition of a virulent M. tuberculosis *strain induces high* Nos2 *transcription and NO production by infected macrophages*

To investigate the mechanisms underlying high *Nos2* induction, we infected mouse bone marrow–derived macrophages (macrophages) with *M. tuberculosis* strain H37Rv or BTB 02-171. *Nos2* mRNA, NOS2 protein, and NO secretion were strongly upregulated upon macrophage infection with *M. tuberculosis* strain BTB 02-171, but not H37Rv (Fig. 2A–C), despite similar infection levels (Supplemental Fig. 2A), thus replicating the in vivo results. These data suggest that the higher induction of *Nos2* by BTB 02-171 may be a direct response of the macrophage to bacterial ligands, which is consistent with the fact that these phagocytes are among the first cells to recognize *M. tuberculosis* (4) and are well known for their capacity to upregulate *Nos2* and produce NO as a bactericidal mechanism (46). Because TLRs are capable of differential recognition of *M. tuberculosis* strains (15), we infected WT and *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ macrophages with the BTB 02-171 strain and measured NO secretion by quantifying the amount of nitrites in the supernatant 24 h later. TLR2 deficiency led to a minor reduction in nitrite concentrations; however, the lack of TLR4 nearly completely abrogated NO production (Fig. 2D). In line with this, *Nos2* transcription was also greatly reduced at 6 h postinfection in *Tlr4*$^{-/-}$ and *Ticam*$^{-/-}$ macrophages, but not in *Tlr2*$^{-/-}$ macrophages, compared with WT controls (Supplemental Fig. 2B, 2C). The upregulation of *Nos2* expression and NO production was a direct effect of the mycobacteria, because production of NO by BTB 02-171–infected macrophages was not affected by a concentration of polymyxin B (an endotoxin inhibitor) that completely abrogated NO production by macrophages stimulated with 500 ng/ml of LPS (Supplemental Fig. 2D). TLR4-dependent *Nos2* induction and NO production by infected macrophages were also observed postinfection with *M. tuberculosis* strain Harlingen (Supplemental Fig. 2E, 2F), which also was described as a TLR4-activating strain (15). These data suggest that TLR4 activation and the downstream TRIF pathway are required to initiate the signaling cascade involved in the upregulation of *Nos2* and NO production by macrophages infected with certain strains of *M. tuberculosis* that signal through TLR4 and are known to induce type I IFN production by infected macrophages.

Type I IFN induces NO production by M. tuberculosis*–infected macrophages*

Our data showed that some *M. tuberculosis* strains induce high levels of *Nos2* transcription and NO production by macrophages by activating TLR4, whereas other strains only activate macrophages via TLR2. To investigate whether de novo protein synthesis was required for maximal *Nos2* induction by the TLR4-activating strains, WT macrophages were infected with *M. tuberculosis* BTB 02-171 strain in the absence or presence of cycloheximide (an inhibitor of protein biosynthesis), and *Nos2* transcription was measured 6 h postinfection. Blockade of de novo protein synthesis strongly inhibited *Nos2* induction following macrophage infection with the BTB 02-171 strain (Supplemental Fig. 2G), indicating that specific mediators produced upon TLR4 stimulation were required to induce *Nos2* transcription in infected macrophages. Thus, we investigated whether macrophage-derived type I IFN might induce the production of NO in response to *M. tuberculosis* by infecting WT and *Ifnar*$^{-/-}$ macrophages with the TLR4-activating BTB 02-171 strain. We found that NO production was completely abrogated and *Nos2* transcription was greatly inhibited in the absence of type I IFN signaling (Fig. 2E, Supplemental Fig. 2H). Other candidate mediators produced downstream of TLR4 activation and with a described role in the induction of NOS2 were also tested. Absence of IL-1R signaling did not affect *Nos2* transcription and only slightly reduced NO production by macrophages upon BTB 02-171 infection (Supplemental Fig. 2I, 2J). Partial decreases in the expression of *Nos2* and NO production were detected in the absence of TNF (Supplemental Fig. 2K, 2L), suggesting that TNF, although able to modulate levels of these mediators, was not absolutely required to induce NO production by BTB 02-171–infected macrophages.

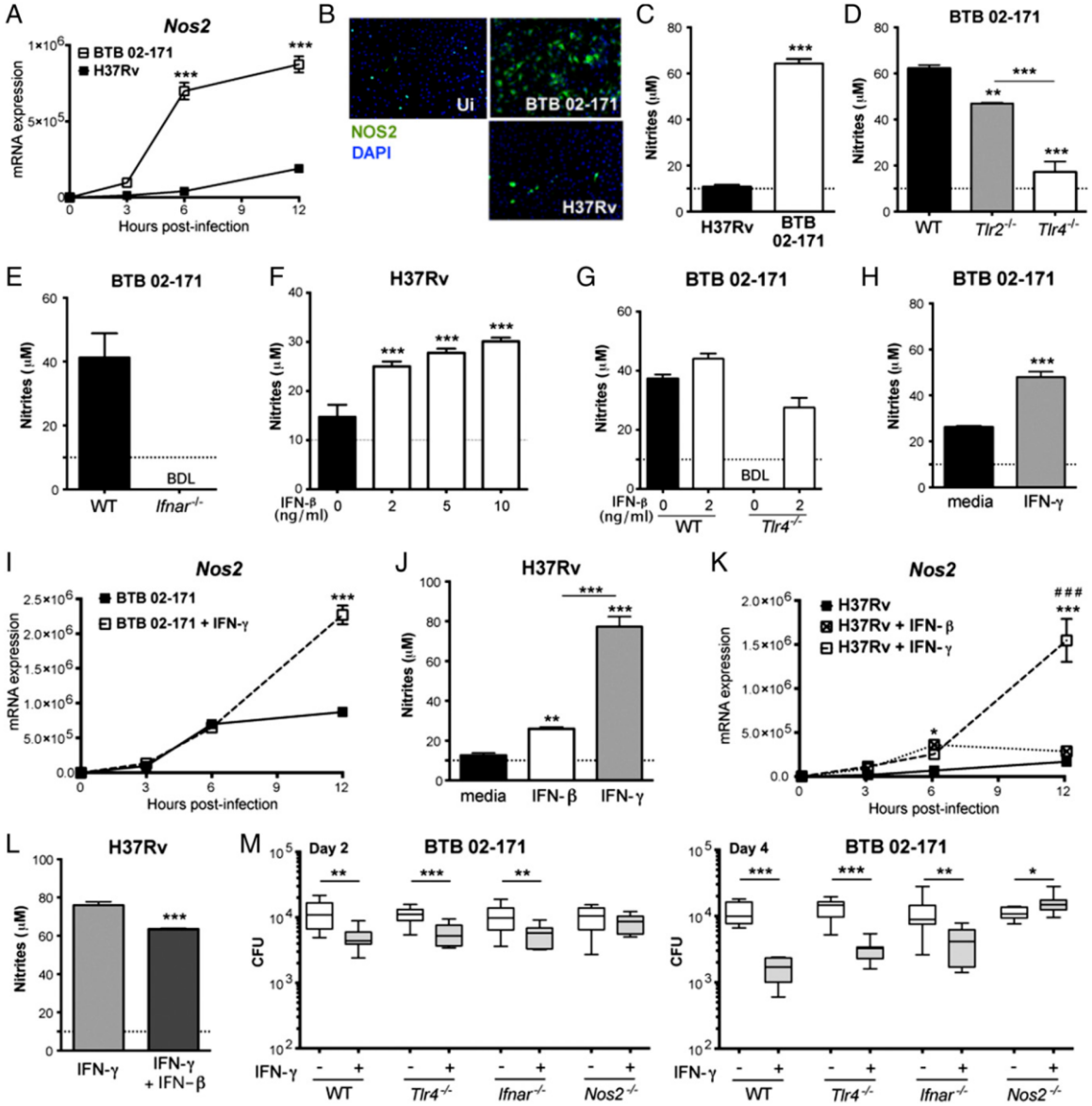

FIGURE 2. Type I IFN induces *Nos2* expression and NO production by *M. tuberculosis*–infected macrophages. (**A**–**C**) WT macrophages were infected with *M. tuberculosis* strains H37Rv or BTB 02-171 (MOI = 2). (A) At the indicated hours postinfection, *Nos2* mRNA levels were determined by quantitative real-time PCR and normalized to the expression of *Hprt1*. (B) Expression of NOS2 by uninfected (Ui) or infected macrophages was determined by immunofluorescence at 8 h (green signal indicates NOS2 stain, and blue signal indicates cell nuclei). (C) NO production was determined by Griess reagent assay of nitrites in culture supernatants at 24 h. WT, $Tlr2^{-/-}$, and $Tlr4^{-/-}$ (**D**) or WT and $Ifnar^{-/-}$ (**E**) macrophages were infected with BTB 02-171, and NO levels in culture supernatants were determined by Griess reagent assay at 24 h. (**F**) WT macrophages were infected with H37Rv in the presence of increasing concentrations of rIFN-β, and NO levels in culture supernatants were determined by Griess reagent assay at 24 h. (**G**) WT and $Tlr4^{-/-}$ macrophages were infected with BTB 02-171 in the presence or absence of rIFN-β, and NO levels in culture supernatants were determined by Griess reagent assay at 24 h. WT macrophages were infected with BTB 02-171 (**H** and **I**) or H37Rv (**J** and **K**) in the presence or absence of rIFN-β or rIFN-γ. (H and J) NO levels in culture supernatants were determined by Griess reagent assay at 24 h. (I and K) *Nos2* mRNA levels were determined at the indicated hours postinfection. (**L**) WT macrophages were infected with H37Rv in the presence of rIFN-γ alone or rIFN-γ plus rIFN-β, and NO levels in culture supernatants were determined by Griess reagent assay at 24 h. Graphs show mean ± SEM of triplicate samples. (**M**) WT, $Tlr4^{-/-}$, $Ifnar^{-/-}$, and $Nos2^{-/-}$ macrophages were infected with BTB 02-171 (MOI = 0.5) in the presence or absence of rIFN-γ. Media were removed at 4 h postinfection, cells were washed in PBS, and fresh media was replaced. At day 2 (left panel) and day 4 (right panel) postinfection, cells were washed in PBS and lysed in 0.2% saponin, and bacterial loads were enumerated. Graphs show minimum to maximum CFU per well (five wells per experiment). Data are representative of at least two independent experiments. Significance was determined using two-way ANOVA corrected for multiple comparisons with a Bonferroni test (A, I, and K), an unpaired *t* test (C, H, L, and M), or one-way ANOVA with Bonferroni correction test (D, F, and J). Significance is relative to control group except in (K), where significance is shown relative to H37Rv (*) or to H37Rv + IFN-β ($^{\#}$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#\#\#}p < 0.001$. BDL, below detection level.

Absence of IL-10 slightly reduced *Nos2* transcription, but it did not affect NO production by macrophages upon BTB 02-171 infection (Supplemental Fig. 2M, 2N).

In further support of a role for type I IFN, addition of increasing concentrations of rIFN-β to macrophages infected with the TLR2-activating H37Rv laboratory strain resulted in significantly increased NO production (Fig. 2F). Although NO production by BTB 02-171–infected WT macrophages was not further enhanced by IFN-β, NO production by *Tlr4*$^{-/-}$ macrophages was rescued by the addition of IFN-β (Fig. 2G). Altogether, our findings indicate that TLR4-dependent induction of type I IFN resulted in the induction of NO production by macrophages in response to BTB 02-171 infection.

Type I and II IFNs differentially regulate Nos2 induction in M. tuberculosis*–infected macrophages*

IFN-γ is known as the major *Nos2* regulator in *M. tuberculosis* infection (11, 13, 47). We have now shown that the induction of *Nos2* transcription in *M. tuberculosis*–infected macrophages may occur independently of IFN-γ, via type I IFN signaling (Fig. 2, Supplemental Fig. 2). Because both IFNs can induce *Nos2* transcription, we next investigated their relative contribution to NO production in response to *M. tuberculosis* infection. We infected WT macrophages with the BTB 02-171 strain in the presence of rIFN-γ at the time of infection. Enhanced NO production was observed in infected macrophages treated with IFN-γ (Fig. 2H). *Nos2* mRNA levels were also higher in infected macrophages treated with IFN-γ compared with untreated macrophages at 12 h (Fig. 2I), although similar levels were detected at earlier times postinfection (3 and 6 h). These findings suggest that both IFNs, macrophage-derived type I IFN and exogenous IFN-γ, may cooperate for maximal *Nos2* induction or that IFN-γ may induce higher levels of *Nos2* than type I IFN upon *M. tuberculosis* infection. To distinguish between these two possibilities, we infected WT macrophages with the H37Rv strain and added IFN-β or IFN-γ at the time of infection. Although IFN-β and IFN-γ induced NO production by H37Rv-infected macrophages, IFN-γ was significantly more potent than maximal doses of IFN-β (Fig. 2J). Similar levels of *Nos2* mRNA were induced by IFN-β and IFN-γ early postinfection (up to 6 h); however, although we observed a plateau in *Nos2* levels between 6 and 12 h postinfection in the presence of IFN-β, IFN-γ continued to increase the levels of *Nos2* transcription after 6 h postinfection (Fig. 2K). We showed previously that type I IFN impairs IFN-γ's effects on cytokine production by *M. tuberculosis*–infected macrophages (33). Therefore, we examined whether type I IFN might affect IFN-γ's activation of NO production by infected macrophages. WT macrophages were infected with the H37Rv strain and concomitantly treated with IFN-γ alone or IFN-γ plus IFN-β. NO production was reduced slightly when both IFN-γ and IFN-β were added to macrophages compared with IFN-γ alone (Fig. 2L), suggesting that, although type I IFN itself can stimulate NO production by *M. tuberculosis*–infected macrophages, it can also impair IFN-γ–dependent induction of NO production.

We next investigated whether type I IFN–induced *Nos2* could mediate restriction of *M. tuberculosis* in infected macrophages. WT, *Tlr4*$^{-/-}$, *Ifnar*$^{-/-}$, and *Nos2*$^{-/-}$ macrophages were infected with the BTB 02-171 strain, in the presence or absence of IFN-γ, and bacterial loads were assessed 2 and 4 d postinfection (Fig. 2M). Similar bacterial loads were detected at days 2 and 4 postinfection among WT, *Tlr4*$^{-/-}$, *Ifnar*$^{-/-}$, and *Nos2*$^{-/-}$ macrophages in the absence of IFN-γ (Fig. 2M). This suggests that TLR4 or type I IFN signaling each did not induce bacterial clearance by infected macrophages in vitro. IFN-γ treatment induced a significant reduction in bacterial load in WT, *Tlr4*$^{-/-}$, and *Ifnar*$^{-/-}$ macrophages but not in *Nos2*$^{-/-}$ macrophages (Fig. 2M). Thus, *Nos2*-dependent bacterial clearance by infected macrophages required the presence of IFN-γ. Altogether, our data highlight a differential regulation of *Nos2* transcription in *M. tuberculosis*–infected macrophages by type I and type II IFNs; IFN-γ is a more potent inducer of NO production, by sustaining increased *Nos2* transcription for longer times postinfection, and this seems to be crucial for efficient bacterial clearance by macrophages.

Type I IFN controls bacterial growth and immunopathology in the lungs of mice infected with a virulent M. tuberculosis *strain in the absence of IFN-γ signaling*

The recent discovery of a protective role for type I IFN in *M. tuberculosis* infection in the absence of IFN-γ signaling (39), together with our observation that induction of *Nos2* by type I IFN is masked by the dominant effect of IFN-γ, led us to investigate whether type I IFN might contribute to host protection by inducing macrophage microbicidal mechanisms during *M. tuberculosis* infection in vivo. To address this, we started by assessing the role of type I and type II IFN signaling during infection with *M. tuberculosis* strain BTB 02-171. To this end, *Ifngr*$^{-/-}$ and *Ifnar*$^{-/-}$ mice were intercrossed to obtain *Ifngr*$^{-/-}$ × *Ifnar*$^{-/-}$ (dKO) mice. WT, *Ifnar*$^{-/-}$, *Ifngr*$^{-/-}$, and dKO mice were aerosol infected with a low-dose of virulent *M. tuberculosis* strain BTB 02-171. Similar to the previous study with *M. tuberculosis* strain H37Rv (39), dKO mice succumbed to BTB 02-171 infection significantly earlier than did *Ifngr*$^{-/-}$ mice (median survival, 32 versus 35 d, respectively, $p < 0.0025$; Fig. 3A), whereas none of the WT or *Ifnar*$^{-/-}$ mice succumbed to disease, even at late stages of infection (surviving >200 d; data not shown). In contrast to what was reported with H37Rv infection, where bacterial loads were no different in dKO mice compared with *Ifngr*$^{-/-}$ mice (39), bacterial loads were significantly higher in the lungs of dKO mice compared with *Ifngr*$^{-/-}$ mice at days 24 and 27 post–BTB 02-171 infection (Fig. 3B). This suggests that type I IFN also plays a role in controlling bacterial growth in the absence of IFN-γR during in vivo infection with the TLR4-activating *M. tuberculosis* strain BTB 02-171. WT and *Ifnar*$^{-/-}$ mice had lower lung bacterial loads than did *Ifngr*$^{-/-}$ and dKO mice, and no difference in bacterial loads between WT and *Ifnar*$^{-/-}$ mice was observed during the time of the experiment (Fig. 3B) or at later time points (data not shown). Tissue sections of infected lungs were examined to assess pulmonary histology. Increased bacterial loads observed in the absence of both type I and type II IFN receptors were accompanied by enhanced lung pathology at day 27 post–BTB 02-171 infection compared with *Ifngr*$^{-/-}$ mice (Fig. 3C, 3D). The extent of the inflammatory infiltrates was significantly greater in dKO mice than in single-KO and WT mice, showing extensive areas of granulomatous inflammation (Fig. 3C, 3D). Enhanced lung pathology correlated with increased numbers of neutrophils in the lungs of dKO and *Ifngr*$^{-/-}$ mice, whereas the number of other myeloid cell populations was similar or reduced in the absence of IFN-γR but not in dKO compared with *Ifnar*$^{-/-}$ or WT mice (Fig. 4). This is in keeping with the previous report by Desvignes et al. (39) using *M. tuberculosis* strain H37Rv for infection.

Type I IFN suppresses expression of genes associated with alternatively activated macrophages in M. tuberculosis*–infected lungs in the absence of IFN-γ signaling*

We next examined the contribution of each IFN pathway to the induction of *Nos2* transcription upon in vivo infection with *M. tuberculosis* strain BTB 02-171. As expected, *Nos2* expression was significantly lower in infected lungs from *Ifngr*$^{-/-}$ mice

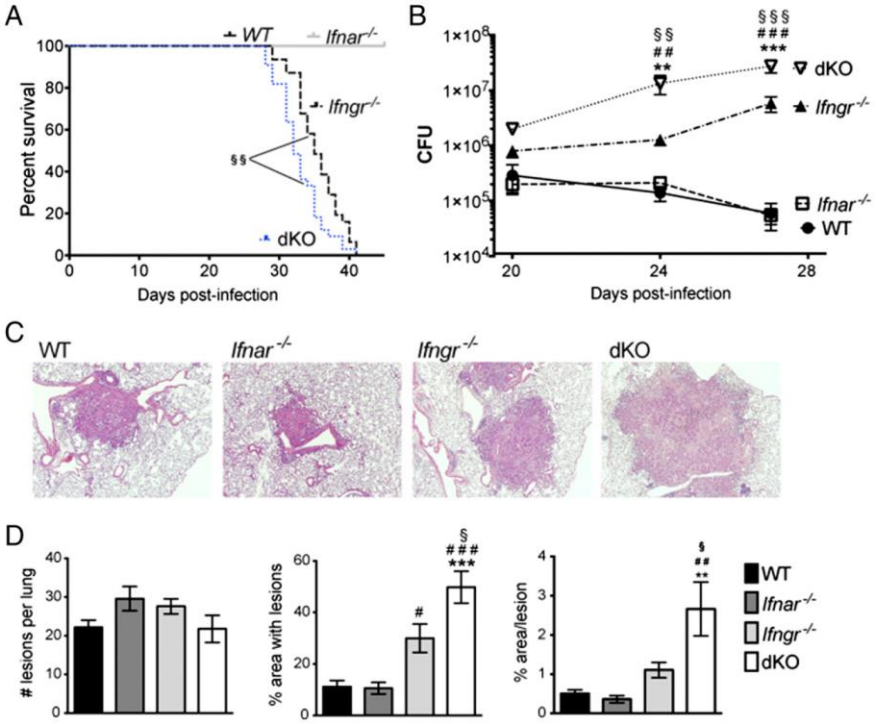

FIGURE 3. Type I IFN contributes to host protection during infection with virulent *M. tuberculosis* strain in the absence of IFN-γR. WT, *Ifnar*$^{-/-}$, *Ifngr*$^{-/-}$, and *Ifngr*$^{-/-}$ × *Ifnar*$^{-/-}$ (dKO) mice were infected with *M. tuberculosis* strain BTB 02-171. (**A**) Percentage of survival for three independent experiments with 10 mice per group. $^{\S\S}p < 0.01$, dKO versus *Ifngr*$^{-/-}$ mice, log-rank test. (**B**) At the indicated days postinfection, lung cell suspensions were prepared, diluted, and plated onto 7H11 agar to determine the number of mycobacterial CFU in the lungs. (**C** and **D**) H&E-stained tissue of infected lungs at day 27 postinfection was analyzed blindly. (C) Representative images from one of five animals per group (original magnification ×4). (D) Morphometric analysis of the number and size of inflammatory lesions. Each bar represents mean ± SEM for five mice per group. Data are representative of two independent experiments. Significance was determined using two-way ANOVA (B) or one-way ANOVA (D), corrected for multiple comparisons with a Bonferroni test. Significance is shown relative to WT (*), *Ifnar*$^{-/-}$ ($^{\#}$), or *Ifngr*$^{-/-}$ (§). $^{*,\#,\S}p < 0.05$, $^{**,\#\#,\S\S}p < 0.01$, $^{***,\#\#\#,\S\S\S}p < 0.001$.

compared with WT mice at day 20 postinfection (Fig. 5A). However, IFNAR signaling did not appear to contribute to *Nos2* induction, because similar or increased levels of *Nos2* were observed between dKO and *Ifngr*$^{-/-}$ mice or between *Ifnar*$^{-/-}$ and WT mice, respectively (Fig. 5A). Similarly, the number of NOS2-expressing cells in infected lungs was not affected by the loss of IFNAR signaling, but it was greatly reduced in the absence of IFN-γR signaling (Supplemental Fig. 3A). These findings indicate that the protective role of type I IFN during *M. tuberculosis* infection, in the absence of IFN-γ responses, did not correlate with *Nos2* expression in whole infected lungs.

It was shown that *M. tuberculosis* infection induces *Arg1* expression in macrophages, which suppresses NOS2 activity and *M. tuberculosis* killing by these cells (48). Indeed, mice lacking ARG1 exhibit reduced bacterial burden compared with ARG1-competent control mice (48, 49). Because NOS2 and ARG1 are hallmarks of two extremes of macrophage polarization (classically and alternatively activated macrophages, respectively) (50), we hypothesized that the decrease in *Nos2* expression in *Ifngr*$^{-/-}$ and dKO mice may be accompanied by an increased expression of *Arg1*. Therefore, we measured *Arg1* gene expression and ARG1 activity in the lungs of WT, *Ifnar*$^{-/-}$, *Ifngr*$^{-/-}$, and dKO mice infected with *M. tuberculosis* strain BTB 02-171. Low levels of *Arg1* expression were detected in the lungs of WT and *Ifnar*$^{-/-}$ mice at day 20 post–BTB 02-171 infection (Fig. 5B). Loss of IFN-γR led to an increased expression of *Arg1* in infected lungs (Fig. 5B). Strikingly, loss of both type I and type II IFNRs resulted in significantly higher *Arg1* expression than that observed in the absence of IFN-γR alone (Fig. 5B). Similarly, ARG1 activity was significantly enhanced in the lungs of infected dKO mice compared with *Ifngr*$^{-/-}$ mice (Supplemental Fig. 3B), indicating that type I IFN suppresses *Arg1* gene expression and ARG1 activity in the absence of IFN-γR. In addition, increased pulmonary expression of other markers associated with alternatively activated macrophages, *Ym1* and *Fizz1*, was detected in dKO mice compared with *Ifngr*$^{-/-}$, *Ifnar*$^{-/-}$, and WT mice (Fig. 5C, 5D).

To further understand the molecular basis for the switch between classically activated and alternatively activated macrophages observed during *M. tuberculosis* infection in the absence of IFNAR and IFN-γR, we measured the expression of several cytokines previously associated with the control of macrophage polarization (50, 51). Coincident with the increased expression of genes associated with alternatively activated macrophages (Fig. 5B–D), we found decreased expression of *Tnf*, a cytokine associated with classically activated macrophages (50, 51), in the lungs of infected dKO mice compared with *Ifngr*$^{-/-}$, *Ifnar*$^{-/-}$, and WT mice (Fig. 5E). Furthermore, the presence of markers associated with alternatively activated macrophages also coincided with an increase in mRNA expression of Th2 cytokines, such as IL-4, IL-5, and IL-13, but not IL-10, in infected lungs in the absence of IFN-γR that was enhanced significantly by the loss of IFNAR in the absence of IFN-γR (Fig. 5F–I).

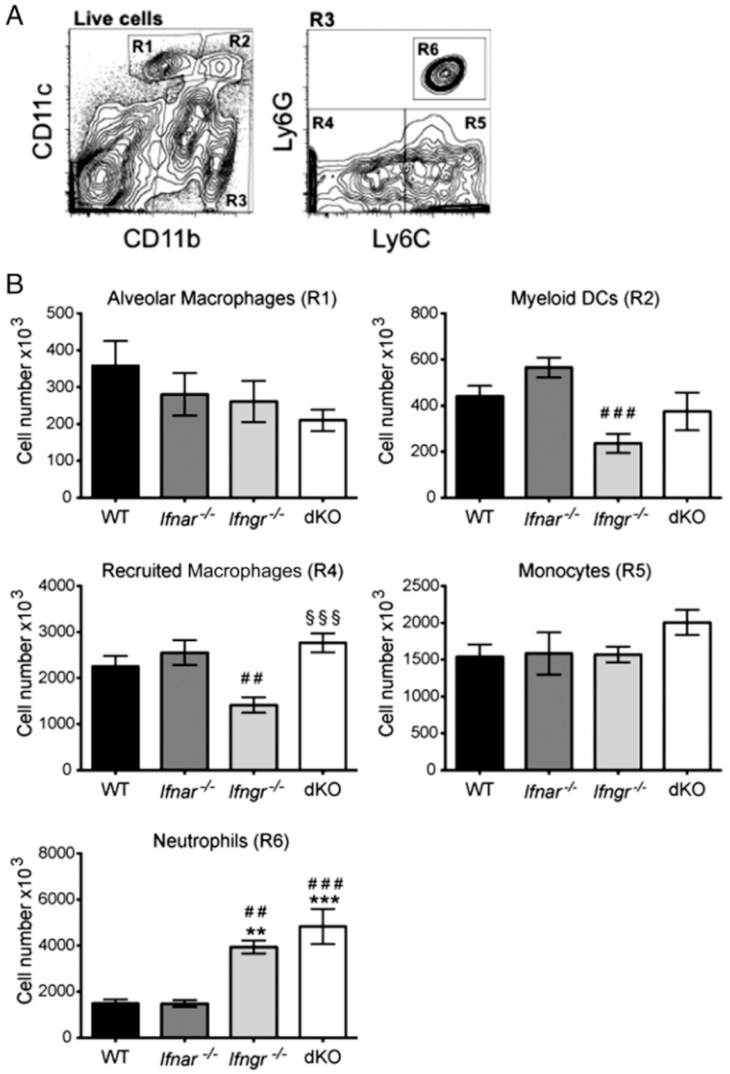

FIGURE 4. Type I and II IFNs regulate lung myeloid cell recruitment during *M. tuberculosis* infection. WT, $Ifnar^{-/-}$, $Ifngr^{-/-}$, and $Ifngr^{-/-} \times Ifnar^{-/-}$ (dKO) mice were infected with the *M. tuberculosis* strain BTB 02-17. Myeloid cell populations in infected lungs were characterized by flow cytometry 24 d postinfection. (**A**) Lung cells were gated on single live cells using forward and side scatter parameters. Gating strategy for quantification of myeloid cell populations is shown. R1 ($CD11b^{lo}$ $CD11c^{+}$): alveolar macrophages; R2 ($CD11b^{hi}$ $CD11c^{+}$): myeloid dendritic cells (DCs); gating on R3 ($CD11b^{hi}$ $CD11c^{neg}$), R4 ($CD11b^{hi}$ $CD11c^{neg}$ $Ly6C^{lo/neg}$): recruited macrophages; R5 ($CD11b^{hi}$ $CD11c^{neg}$ $Ly6C^{int/hi}$ $Ly6G^{neg}$): monocytes; and R6 ($CD11b^{hi}$ $CD11c^{neg}$ $Ly6C^{int}$ $Ly6G^{hi}$): neutrophils. (**B**) Cell number of myeloid cell populations. Each bar represents mean ± SEM for five mice per group. Data are a pool of two independent experiments. Significance is shown relative to WT (*), $Ifnar^{-/-}$ ($^{\#}$), or $Ifngr^{-/-}$ (§). $^{**,\#\#}p < 0.01$, $^{***,\#\#\#}p < 0.001$, one-way ANOVA corrected for multiple comparisons with a Bonferroni test.

Our data demonstrate that, in the absence of IFN-γ signaling, additional loss of type I IFN signaling results in very high levels of expression of markers associated with alternatively activated macrophages in infected lungs, which likely suppress bacterial killing.

Type I IFN suppresses Arg1 expression in M. tuberculosis*–infected macrophages*

To evaluate whether macrophage-derived type I IFN could have a direct effect on suppressing *Arg1* induction in response to *M. tuberculosis* infection, we infected WT and $Ifnar^{-/-}$ macrophages with *M. tuberculosis* strain BTB 02-171 and examined the subsequent effects on *Arg1* mRNA expression. This revealed that macrophage-derived type I IFN suppressed *Arg1* induction in infected macrophages, because *Arg1* levels were significantly higher in $Ifnar^{-/-}$ macrophages than in WT macrophages at 6 h post–BTB 02-171 infection (Fig. 6A). Macrophage-derived TNF also suppressed *Arg1* induction in infected macrophages, because *Arg1* levels were significantly higher in $Tnf^{-/-}$ macrophages compared with WT macrophages (Fig. 6B). Although loss of type I IFN signaling decreased *Tnf* expression during in vivo infection in the absence of IFN-γR (Fig. 5E), TNF production in response to *M. tuberculosis* infection in vitro was not significantly different between WT and $Ifnar^{-/-}$ macrophages (Supplemental Fig. 4A). Therefore, both type I IFN and TNF suppressed *Arg1* expression in infected macrophages, although the inhibition of *Arg1* expression by type I IFN was not accompanied by increased levels of TNF production in vitro. In contrast, macrophage-derived IL-10 induced *Arg1* expression in infected macrophages, because *Arg1* levels were significantly reduced in $Il10^{-/-}$ macrophages compared with WT macrophages (Fig. 6C). Because type I IFN and TNF induced *Nos2* gene expression and NOS2 activity in *M. tuberculosis*–infected macrophages (Fig. 2E, Supplemental Fig. 2H, 2K, 2L), we assessed whether suppression of *Arg1* expression by type I IFN and TNF was dependent on NOS2. Similar levels of *Arg1* expression were detected in $Nos2^{-/-}$ and WT macrophages infected with the BTB 02-171 strain (Supplemental Fig. 4B), indicating that suppression of *Arg1* transcription by type I IFN and TNF appeared to be independent of *Nos2* induction or NOS2 activity. Taken together, our results show that type I IFN might

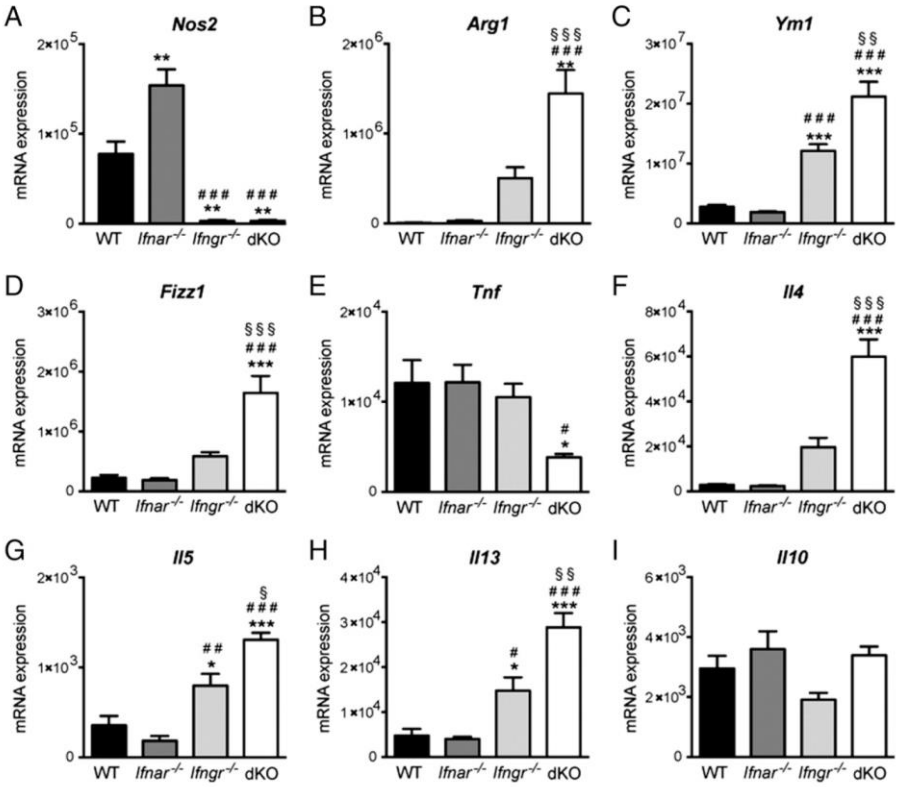

FIGURE 5. Concomitant loss of type I and type II IFN signaling increases the expression of genes associated with alternatively activated macrophages in *M. tuberculosis*–infected lungs. WT, $Ifnar^{-/-}$, $Ifngr^{-/-}$, and $Ifngr^{-/-} \times Ifnar^{-/-}$ (dKO) mice were infected with the *M. tuberculosis* strain BTB 02-171. At day 20 postinfection, RNA was extracted from infected lungs and *Nos2* (**A**), *Arg1* (**B**), *Ym1* (**C**), *Fizz1* (**D**), *Tnf* (**E**), *Il4* (**F**), *Il5* (**G**), *Il13* (**H**), and *Il10* (**I**) mRNA expression was analyzed by quantitative real-time PCR and normalized to the expression of *Hprt1*, with the exception of *Tnf*, which was normalized to *Ubiquitin* expression. Each bar represents mean ± SEM for five mice per group. Data are representative of two independent experiments. Significance is shown relative to WT (*), $Ifnar^{-/-}$ (#), or $Ifngr^{-/-}$ (§). $^{*,\#,\S}p < 0.05$, $^{**,\#\#,\S\S}p < 0.01$, $^{***,\#\#\#,\S\S\S}p < 0.001$, one-way ANOVA corrected for multiple comparisons with a Bonferroni test.

inhibit the differentiation of alternatively activated macrophages by direct downregulation of *Arg1* transcription in *M. tuberculosis*–infected macrophages, although other factors further potentiate this mechanism in vivo (Fig. 7).

Discussion

Type I IFN plays a role in *M. tuberculosis* persistence and TB pathogenesis (16–30, 32, 33). However, in particular cases, such as in multidrug resistant TB patients treated with antimycobacterial drugs (52, 53) or in patients with mutations in their IFN-γR suffering from mycobacterial infections (40, 54), type I IFN could provide some level of protection. Thus, unraveling the molecular mechanisms underlying this protective role may offer new targets for host-direct therapies in these individuals. In the mouse model of infection, a protective role was attributed to type I IFN, in the absence of IFN-γ–dependent immunity, by influencing the recruitment and/or survival of potential target cells in infected lungs (39). In this article, we describe a previously unappreciated role for type I IFN in regulating macrophage activation during *M. tuberculosis* infection, revealing a novel mechanism by which type I IFN may confer protection against *M. tuberculosis* infection in the absence of IFN-γ signaling (Fig. 7). Our findings demonstrate that type I IFN induces *Nos2* and inhibits *Arg1* expression following infection of macrophages with *M. tuberculosis*, resulting in high NO production by infected macrophages. The suppression of *Arg1* expression by type I IFN, and indeed of other

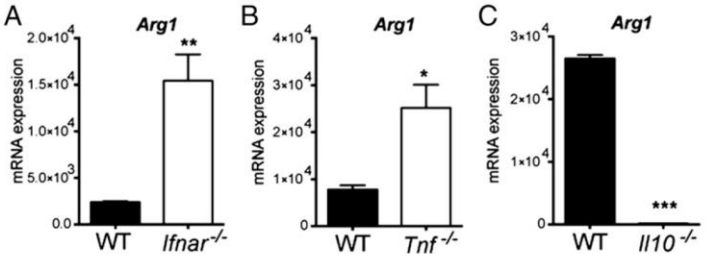

FIGURE 6. Type I IFN and TNF suppress *Arg1* transcription in macrophages infected with *M. tuberculosis*. WT and $Ifnar^{-/-}$ (**A**), WT and $Tnf^{-/-}$ (**B**), or WT and $Il10^{-/-}$ (**C**) macrophages were infected with BTB 02-171 (MOI = 2), and *Arg1* mRNA levels were determined by quantitative real-time PCR at 6 h postinfection and normalized to the expression of *Hprt1*. Graphs show mean ± SEM of triplicate samples. Data are representative of at least two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, unpaired *t* test.

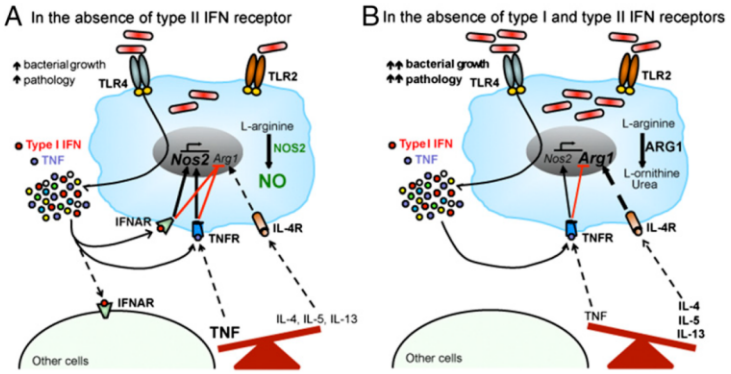

FIGURE 7. Schematic summary of the regulation of macrophage activation by type I IFN during *M. tuberculosis* infection. (**A** and **B**) Activation of TLR4 in macrophages by specific *M. tuberculosis* strains leads to the production of type I IFN, in addition to other cytokines (e.g., TNF) (15). Type I IFN induces *Nos2* and inhibits *Arg1* gene expression and protein activity in infected macrophages, thus regulating macrophage activation toward a more protective phenotype. Induction of *Nos2* and suppression of *Arg1* transcription in infected macrophages are further potentiated by macrophage-derived TNF. In vivo, in the absence of IFN-γ signaling, type I IFN suppresses the expression of markers associated with alternatively activated macrophages following *M. tuberculosis* infection, likely by direct regulation of macrophage activation, as well as by modulating TNF and Th2-associated cytokine expression, contributing to host protection.

genes associated with alternatively activated macrophages, was also observed during in vivo infection in the absence of IFN-γ signaling. These alterations correlated with control of bacterial growth and pathology. Taken together, our data show that, although masked by the dominant effect of IFN-γ, type I IFN has a regulatory function in macrophage activation during *M. tuberculosis* infection; it contributes to host protection by suppressing the switching of macrophages from a more protective, classically activated phenotype to a more permissive, alternatively activated phenotype.

Mutations in both IFN-γR chains, *IFNGR1* and *IFNGR2*, were identified in humans and have been associated with increased susceptibility to mycobacterial infections (9). It was reported that adjunctive treatment with IFN-α confers clinical benefits in patients with mutations in the IFN-γR who are suffering from mycobacterial diseases (40, 54). Our results provide important information that may help to explain the mechanism underlying the beneficial effect of IFN-α treatment and provide novel targets for host-directed therapy to improve future treatment of patients with IFN-γR mutations or with compromised IFN-γ responses.

NO synthesis by NOS2 is critical for effective immunity and host protection against virulent strains of *M. tuberculosis* (12–14, 41, 55). Although *M. tuberculosis* strain BTB 02-171 induces strong *Nos2* expression early after in vivo infection, this strain still causes severe disease, as shown by increased bacterial loads and enhanced lung pathology (15). In some bacterial infections, an exacerbated expression of NOS2 was associated with a more severe disease outcome, likely due to NO-mediated cytotoxicity and tissue damage and suppression of the immune response to the pathogen (43–45). Although we cannot completely exclude the hypothesis that elevated *Nos2* expression can contribute to disease severity during infection with *M. tuberculosis* strain BTB 02-171, mice deficient for NOS2 were extremely susceptible to infection with this strain. Additionally, compared with the less virulent laboratory strain H37Rv, *Nos2*$^{-/-}$ mice showed earlier susceptibility to BTB 02-171 infection that correlates with earlier induction of elevated levels of *Nos2* expression following infection with this strain. Temporal differences in *Nos2* induction following infection with different strains of *M. tuberculosis* may explain previous reports showing a protective role for NOS2 during the late, but not the early, phase of infection (41).

Surprisingly, we found that the expression of *Nos2* in macrophages infected with *M. tuberculosis* strain BTB 02-171 was independent of the presence of IFN-γ, a key cytokine for activation of macrophages during *M. tuberculosis* infection to produce large amounts of NO (11, 13, 47, 56). We then investigated the molecular mechanisms underlying this IFN-γ–independent *Nos2* induction and NO production and found a requirement for TLR4/TRIF signaling. Type I IFN was reported to contribute to the induction of NO production following stimulation of macrophages with the TLR4 ligand LPS (57). We found that type I IFN signaling was required for transcriptional induction of *Nos2* and consequent NO production in macrophages infected with BTB 02-171. Furthermore, addition of IFN-β significantly enhanced NO production following infection with the H37Rv strain, which, on its own, induced little production of NO by infected macrophages.

Nevertheless, our results show that IFN-γ is a stronger inducer of NO production by *M. tuberculosis*–infected macrophages than type I IFN, likely due to prolonged transcriptional induction of *Nos2*. Furthermore, IFN-γ–induced NOS2, but not type I IFN–induced NOS2, appears to be required for efficient bacterial control by macrophages. We reported previously that type I IFN impairs IFN-γ's effects on cytokine production by macrophages infected with *M. tuberculosis* (33). Type I IFN completely abrogated the ability of IFN-γ to enhance IL-12 and TNF production and to inhibit IL-10 production by macrophages in response to H37Rv infection, although it did not impair IFN-γ inhibition of IL-1β production (33). We now show that type I IFN can also downregulate IFN-γ–dependent induction of NO production by infected macrophages. Because IFN-γ–dependent inhibition of IL-1β production during *M. tuberculosis* infection seems to be mediated by *Nos2* (33, 58), our recent finding may explain why the ability of IFN-γ to suppress IL-1β production by infected macrophages was indeed increased by the presence of type I IFN (33). Although IL-10 is an important mediator of the suppressive effect of type I IFN on IL-12 and TNF macrophage production induced by IFN-γ, IL-10 did not play a role in suppressing the IFN-γ–dependent induction of NO production by *M. tuberculosis*–infected macrophages (data not shown).

We report in this article that, in vivo, *Nos2* expression required IFN-γ signaling because *Nos2* mRNA levels were significantly lower in infected lungs from mice deficient for IFN-γR compared

with WT mice, in agreement with previous reports (11, 13, 32). Type I IFN inhibited IFN-γ–induced *Nos2* transcription slightly, in line with our in vitro data and with another study reporting an IFNAR-mediated inhibition of IFN-γ–induced NOS2 expression in lung myeloid cells (32). However, type I IFN–dependent *Nos2* induction was not detected in infected lungs at the time points analyzed postinfection. One explanation is that type I IFN–induced *Nos2* transcription is transient and not sustained over time, in contrast to IFN-γ–dependent *Nos2* induction. This hypothesis is supported by our in vitro data showing that, although both type I and type II IFNs induced similar levels of *Nos2* early following macrophage infection, increased levels of *Nos2* were only sustained over time in the presence of IFN-γ. *Nos2* induction was reported to be critical to control neutrophil-mediated pulmonary pathology following *M. tuberculosis* infection (58). Therefore, induction of *Nos2* by type I IFN (even if transient or in low levels) could explain the increased numbers of neutrophils and enhanced lung pathology observed in the absence of both IFN-γR and IFNAR. In addition to enhanced pathology, we observed increased lung bacterial loads in the absence of both IFN-γR and IFNAR, compared with the absence of IFN-γR alone, which contrasts with a previous study of *M. tuberculosis* strain H37Rv (39). The strain of *M. tuberculosis* used in this study, BTB 02-171, is more virulent and induces higher levels of type I IFN in the lungs of infected mice than does the H37Rv strain (15), which may account for some of the differences observed between the previous study and ours (39).

The increased susceptibility to *M. tuberculosis* infection that we observed in the absence of both IFN-γR and IFNAR signaling correlated with increased expression levels of genes associated with alternatively activated macrophages, such as *Arg1*, *Ym1*, and *Fizz1*, in the lungs. A positive correlation between ARG1 and human TB was suggested recently based on the increased expression of *ARG1* in monocytes isolated from peripheral blood of patients with active TB compared with those with latent TB (49). ARG1 was also reported to be expressed in granuloma-associated macrophages of lung tissues from patients with TB (59). Likewise, *M. tuberculosis* infection can induce *Arg1* expression in murine macrophages (48, 60), and specific elimination of *Arg1* in macrophages decreased lung bacterial loads during in vivo infection (48, 49). How macrophage expression of *Arg1* during *M. tuberculosis* infection increases susceptibility to *M. tuberculosis* infection remains unclear. It was suggested that ARG1 impairs bacterial growth restriction by infected macrophages by suppressing NOS2 activity and preventing NO production (48), which could be part of the mechanism observed in our study of *M. tuberculosis* infection in the absence of IFN-γR and IFNAR signaling. As another mechanism, we consider the possibility that ARG1 activity may supply substrates for *M. tuberculosis* growth and survival, as was suggested for *Leishmania* species (61).

Alternative macrophage activation is typically induced by IL-4Rα activation (50, 51). Differentiation of alternatively activated macrophages with IL-4 in vitro was reported to inhibit macrophage antimicrobial responses to *M. tuberculosis* (62). Moreover, Th2 cell responses were associated with TB pathogenesis by mediating the alternative activation of macrophages during *M. tuberculosis* infection (49, 63, 64). We detected increased levels of Th2-associated cytokines in infected lungs in the absence of IFN-γR, which were further enhanced by the concomitant absence of IFNAR, suggesting that increased IL-4Rα signaling may drive alternative activation of lung macrophages in *Ifngr*$^{-/-}$ and dKO mice. In addition, decreased levels of TNF expression were detected in the absence of both IFN-γR and IFNAR. TNF was recently shown to hamper alternative activation of macrophages in murine models of cancer by suppressing Th2-associated cytokine expression (65). Although the effect of TNF on the expression of Th2-associated cytokines remains to be clarified in our in vivo model, our data show that TNF directly inhibits *Arg1* expression in *M. tuberculosis*–infected macrophages. In contrast, IL-10 is required for maximal induction of *Arg1* expression in *M. tuberculosis*–infected macrophages in vitro. Overexpression of IL-10 by macrophages and monocytes (under control of the CD68 promoter) was shown to induce the expression of *Arg1* and other markers associated with alternative macrophage activation during *M. tuberculosis* infection, increasing host susceptibility to TB (66). However, similar levels of IL-10 expression were detected in infected lungs in the presence or absence of IFN-γR and IFNAR, suggesting that IL-10 does not play a major role in the regulation of *Arg1* expression in our in vivo model. Type I IFN inhibits *Arg1* expression by infected macrophages in our in vitro model, in which Th2-associated cytokines are absent, and TNF production was not affected by the absence of IFNAR. These findings point to a direct suppressor function of type I IFN on the transcriptional induction of *Arg1* in macrophages following *M. tuberculosis* infection. Therefore, during in vivo infection, *Arg1* expression may be directly inhibited by type I IFN signaling and indirectly inhibited by type I IFN–dependent regulation of TNF and Th2-associated cytokine expression.

In summary, our findings demonstrate that inhibition of alternative macrophage activation by type I IFN correlates with control of bacterial growth and pathology during infection with a virulent strain of *M. tuberculosis* in the absence of IFN-γR. Type I IFN inhibits transcriptional induction of *Arg1* in infected macrophages. Moreover, in the absence of IFN-γR, IFNAR signaling inhibits the expression of Th2-associated cytokines and enhances TNF expression in infected lungs, which likely further contributes to the suppression of alternative macrophage activation in vivo. In addition to furthering our understanding of the modulation of macrophage activation during *M. tuberculosis* infection, these data provide evidence for a novel mechanism by which type I IFN, in the absence of IFN-γR, may confer protection against *M. tuberculosis* infection; this offers new avenues to develop host-direct therapies for patients with compromised IFN-γ responses.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Information

Type I IFN inhibits alternative macrophage activation during *Mycobacterium tuberculosis* infection and leads to enhanced protection in the absence of IFN-γ signaling

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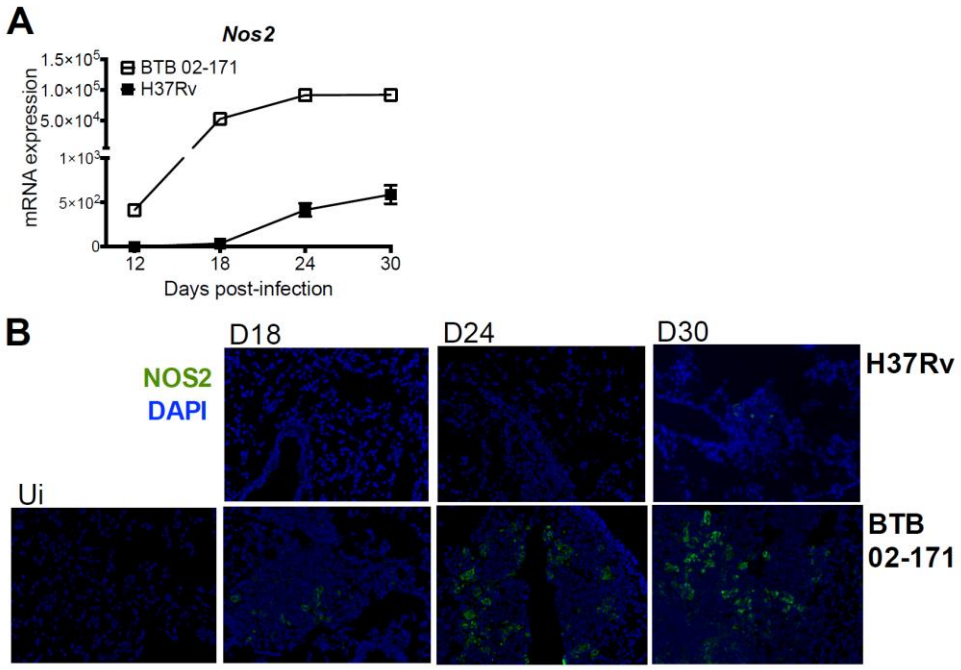
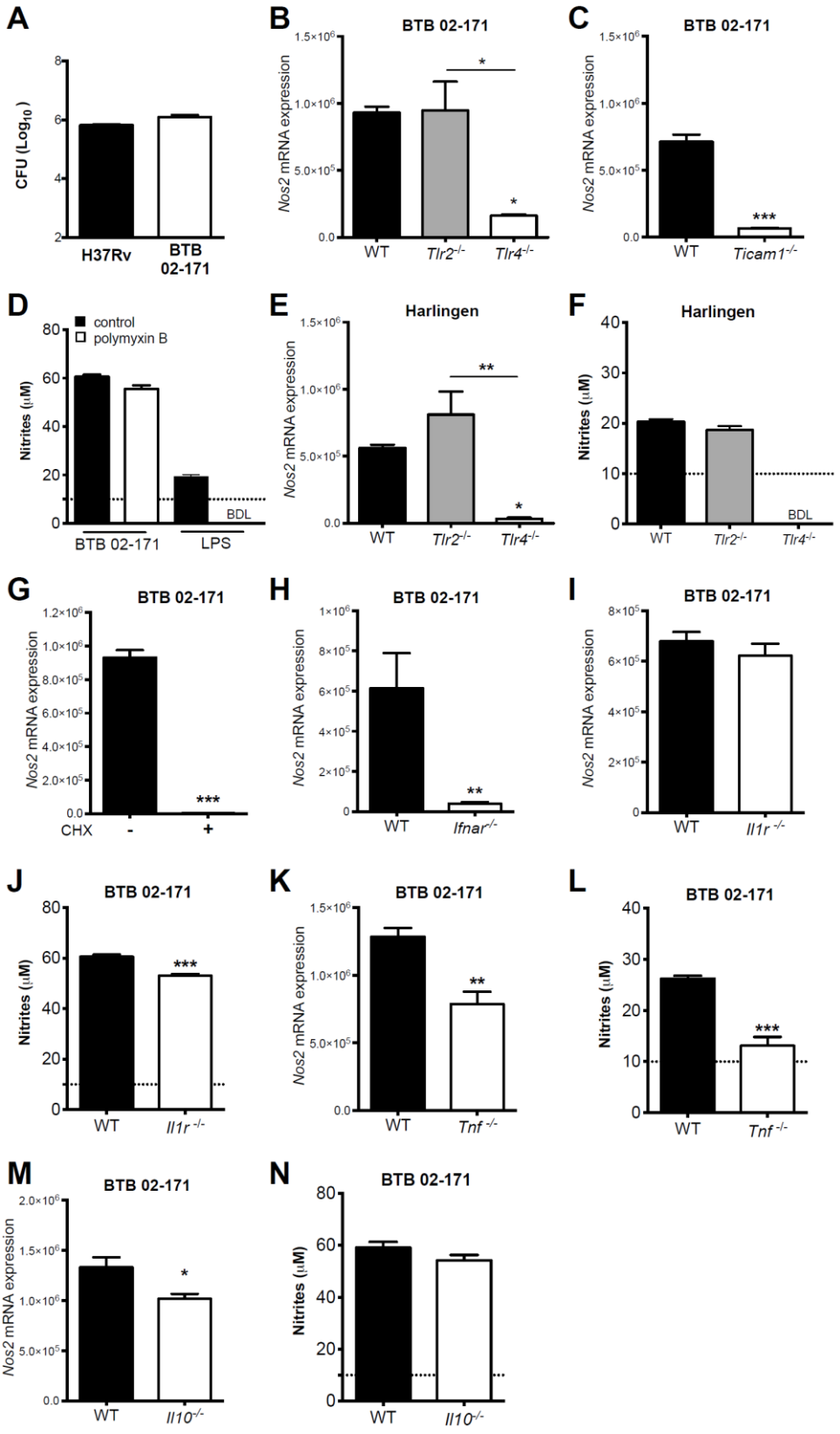
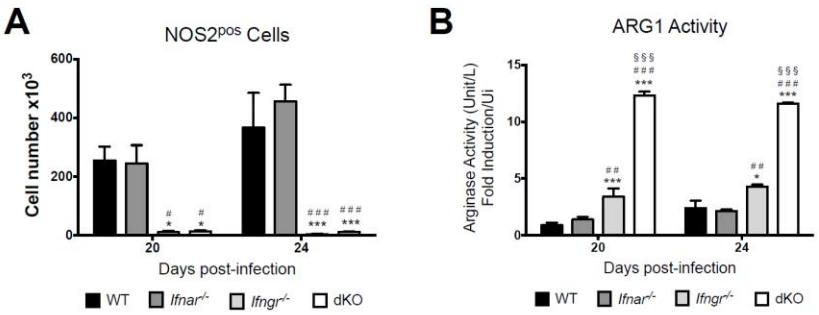


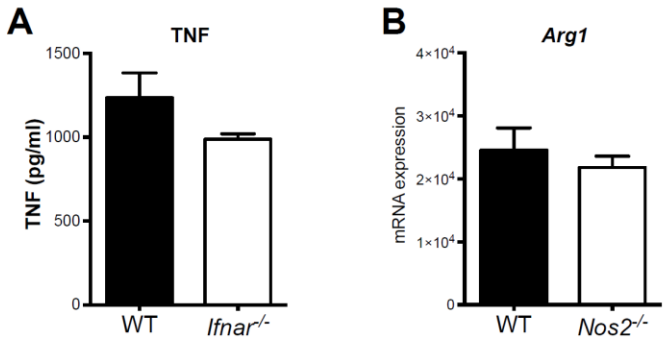
Supplemental Figure 1. Virulent *M. tuberculosis* strain BTB 02-171 induces high levels of NOS2 in infected lungs early after infection. WT mice were infected with *M. tuberculosis* strains H37Rv (closed squares) or BTB 02-171 (open squares). (**A**) At indicated days post-infection, RNA was extracted from infected lungs and *Nos2* expression analyzed by qRT-PCR and normalized to the expression of *Hprt1*. Data points show the mean±SEM for 5 mice per group. (**B**) Expression of NOS2 in the lung tissue of uninfected (Ui) or infected mice was determined by immunofluorescence (green signal indicates NOS2 stain and blue signal indicates cell nuclei). Represented are 4x magnifications of one animal out of 3 per group. Data are from one independent experiment for each *M. tuberculosis* strain.


A
CFU (Log10)
H37Rv
BTB 02-171
B
BTB 02-171
Nos2 mRNA expression
WT
Tlr2-/-
Tlr4-/-
C
BTB 02-171
Nos2 mRNA expression
WT
Ticam1-/-
D
control
polymyxin B
Nitrites (μM)
BDL
BTB 02-171
LPS
E
Harlingen
Nos2 mRNA expression
WT
Tlr2-/-
Tlr4-/-
F
Harlingen
Nitrites (μM)
BDL
WT
Tlr2-/-
Tlr4-/-
G
BTB 02-171
Nos2 mRNA expression
CHX
H
BTB 02-171
Nos2 mRNA expression
WT
Ifnar-/-
I
BTB 02-171
Nos2 mRNA expression
WT
Il1r-/-
J
BTB 02-171
Nitrites (μM)
WT
Il1r-/-
K
BTB 02-171
Nos2 mRNA expression
WT
Tnf-/-
L
BTB 02-171
Nitrites (μM)
WT
Tnf-/-
M
BTB 02-171
Nos2 mRNA expression
WT
Il10-/-
N
BTB 02-171
Nitrites (μM)
WT
Il10-/-

Supplemental Figure 2. Macrophage-derived type I IFN induces *Nos2* expression in macrophages infected with TLR4-activating *M. tuberculosis* strains. (**A**) WT macrophages were infected with *M. tuberculosis* strains H37Rv or BTB 02-171 for 4h (MOI=2). Cells were then extensively washed and lysed and the bacterial burden determined by CFU counting in 7H11 agar plates. (**B** and **C**) WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ (**B**) or WT and *Ticam*$^{-/-}$ (**C**) macrophages were infected with BTB 02-171 and *Nos2* mRNA levels were determined by qRT-PCR at 6h post-infection and normalized to the expression of *Hprt1*. (**D**) WT macrophages were infected with BTB 02-171 or stimulated with 0.5μg/ml LPS in the presence or absence of 5μg/ml polymyxin B. NO production was determined by Griess reagent assay of nitrites in culture supernatants at 24h post-infection. (**E** and **F**) WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ macrophages were infected with *M. tuberculosis* strain Harlingen. (**E**) *Nos2* mRNA levels were determined by qRT-PCR at 6h. (**F**) NO levels in culture supernatants were determined by Griess reagent assay at 24h. (**G**) WT macrophages were infected with BTB 02-171 in the presence or absence of 10μg/ml cycloheximide (CHX) and *Nos2* mRNA levels were determined by qRT-PCR at 6h. (**H**) WT and *Ifnar*$^{-/-}$ macrophages were infected with BTB 02-171 and *Nos2* mRNA levels were determined by qRT-PCR at 6h. (**I-N**) WT and *Il1r*$^{-/-}$ (**I** and **J**) or WT and *Tnf*$^{-/-}$ (**K** and **L**) or WT and *Il10*$^{-/-}$ (**M** and **N**) macrophages were infected with BTB 02-171. (**I**, **K** and **M**) *Nos2* mRNA levels were determined by qRT-PCR at 6h. (**J, L** and **N**) NO levels in culture supernatants were determined by Griess reagent assay at 24h. Graphs show mean±SEM of triplicate samples, except for (**A**) which shows data from 6 wells. Data are from one (**A-D**, **F**, **G**, **I**), two (**E**, **J-N**) or three (**H**) independent experiments. Significance was determined using one-way ANOVA with Bonferroni correction test (**B**, **E**) or unpaired *t* test (**C**, **G-N**). Significance relative to control group, unless otherwise indicated in the figure. *p<0.05, **p<0.01, ***p<0.001.



Supplemental Figure 3. Protective role of type I IFN during *M. tuberculosis* infection, in the absence of IFN-γR, correlates with lower ARG1 activity in infected lungs. WT, *Ifnar*$^{-/-}$, *Ifngr*$^{-/-}$ and *Ifngr*$^{-/-}$ x *Ifnar*$^{-/-}$ (dKO) mice were infected with the *M. tuberculosis* strain BTB 02-17. (**A**) At the indicated days post-infection, lung cell suspensions were prepared and total NOS2 expressing cells were determined by flow cytometry (gated on single live cells using forward and side scatter parameters). (**B**) ARG1 activity was measured as urea production by lysed lung cells after addition of L-arginine. Each bar represents mean±SEM for 5 mice per group. Data are from one experiment. Significance was determined using two-way ANOVA corrected for multiple comparisons with a Bonferroni test. Significance is shown relative to WT (*), *Ifnar*$^{-/-}$ (#) or *Ifngr*$^{-/-}$ (§). *p<0.05, **p<0.01, ***p<0.001.



Supplemental Figure 4. Absence of NOS2 has no impact on *Arg1* expression in macrophages infected with virulent *M. tuberculosis* strain BTB 02-171. (**A**) WT and *Ifnar*$^{-/-}$ macrophages were infected with BTB 02-171 (MOI=2) and levels of TNF in culture supernatants were determined by ELISA at 24h post-infection. (**B**) WT and *Nos2*$^{-/-}$ macrophages were infected with BTB 02-171 (MOI=2) and *Arg1* mRNA levels were determined by qRT-PCR at 6h post-infection and normalized to the expression of *Hprt1*. Graphs show mean±SEM of triplicate samples. Data are representative of two (**A**) or one (**B**) experiments.

II. *Mycobacterium tuberculosis* Associated with Severe Tuberculosis Evades Cytosolic Surveillance Systems and Modulates IL-1β Production

The results presented in this Chapter are submitted for publication.

I performed all of the experiments leading to the preparation of the M. tuberculosis clinical isolates used and their functional analysis. I contributed to the analysis and interpretation of data and design of new experiments.

Mycobacterium tuberculosis associated with severe tuberculosis evades cytosolic surveillance systems and modulates IL-1β production

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S

Summary

The genetic diversity of *M. tuberculosis* was neglected in the past, but is increasingly recognized as a determinant of immune responses and clinical outcomes of TB. However, how this bacterial diversity orchestrates immune responses to direct distinct TB severities remains unknown. We studied 681 patients with pulmonary TB and found that phylogenetically related *M. tuberculosis* isolates from cases with mild disease induced robust cytokine responses in macrophages. In contrast, bacteria isolated from severe TB patients failed to do so. Using representative isolates, we show that *M. tuberculosis* inducing a low cytokine response in macrophages also diminished activation of cytosolic surveillance systems, including cGAS and the inflammasome, suggesting a novel mechanism of immune escape. Isolates exhibiting this evasion strategy carried mutations generating *sigA* recognition boxes or affecting components of the ESX-1 secretion system. We conclude that host interactions with different *M. tuberculosis* strains may result in variable TB severities.

Keywords: tuberculosis, genetic diversity, clinical severity, macrophages, interleukin-1β, inflammasome, type I IFN, ESX-1 secretion

Introduction

The establishment of disease upon infection depends on the balance between the virulence of the pathogen and the susceptibility of the host, but is often also orchestrated by the immune system. Pathogens have thus evolved several strategies to thwart the host immune response. *M. tuberculosis*, estimated to infect a quarter of the human population (Houben and Dodd 2016) and to kill over 1.6 million people every year (WHO 2018), is no exception. During its parallel evolution with the human host (Comas et al. 2013), *M. tuberculosis* developed important immune evasion mechanisms, including virulence factors aimed at preventing elimination by macrophages (Liu, Liu, and Ge 2017), and strategies to modulate T cell responses to favor transmission (Comas et al. 2010). Unlike many other pathogens, *M. tuberculosis* is an obligate human pathogen with no environmental reservoir, and for which transmission relies on disease establishment (Gagneux 2018). Thus, *M. tuberculosis* must create a balance between damaging the host (virulence) and finding the opportunity to spread - a balance which is ultimately achieved by modulating the host immune response. Interestingly, some strains of *M. tuberculosis* are more transmissible than others, and this transmission potential varies in different human genetic backgrounds (Coscolla and Gagneux 2014). Therefore, one can expect a relevant role for host and pathogen diversity in transmission, through the modulation of host immune responses and disease establishment. However, *M. tuberculosis* diversity is seldom considered in TB research.

The human adapted TB-causing bacteria are part of the MTBC, and can be divided into 7 distinct lineages that exhibit a strong phylogeographical structure (Gagneux 2018). Despite harboring little DNA sequence variation as compared to other bacteria (Achtman 2008), strains of the MTBC differ in their capacity to modulate the immune response (Bastos et al. 2017). Pathogen diversity within the MTBC also impacts the clinical manifestation of TB (Coscolla and Gagneux 2014; Bastos et al. 2017). What remains unknown, however, is the interaction between pathogen-induced immune-modulation and disease severity. In other words, how does the natural diversity in *M. tuberculosis* isolates direct the host immune response towards a certain TB presentation. Our study represents the first endeavor towards understanding this question. We studied well-defined patient and pathogen populations to disclose the relevant immune responses leading the various disease outcomes. Our results reveal that phylogenetically related *M. tuberculosis* isolated from severe TB cases developed mechanisms to escape cytosolic recognition and consequently lower cytokine production by host cells. This study contributes with new perspectives on the modulation of host immunity to TB, with the potential to inform the design of host- and pathogen-directed therapies to combat this devastating disease.

Methods

Experimental Models and Subject Details

Ethics statement for Human sample collection

The study protocol was approved by the Health Ethics Committees of the CHSJ (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. Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Recovery of *M. tuberculosis* clinical isolates

Bacterial samples (n=133) of the subjects in the TB group were recovered from stored primary cultures of *M. tuberculosis* clinical isolates at the Clinical Microbiology department of CHSJ. Two hundred µL of inoculum were plated and smeared uniformly on solid Mycobacteria 7H11 agar supplemented with 10% Oleic Albumin Dextrose Catalase (OADC) growth supplement, 0,5% glycerol and PANTA antibiotic mixture. The plates were incubated at 37°C for 4 to 8 weeks. Grown colonies were gently rubbed and transferred to 20 mL of Middlebrook 7H9 liquid medium (BD Biosciences, San Jose, USA) supplemented with 10% OADC, 0,2% glycerol and 0.05% Tween® 80 (Sigma-Aldrich, St. Louis, USA). Alternatively, the stored primary cultures were re-grown in MGIT tubes using a BACTEC instrument (BD) and once a positive signal was obtained, transferred to 20 mL of Middlebrook 7H9 liquid medium, as stated above. All Middlebrook 7H9 liquid cultures were incubated at 37°C with constant 120 rpm shaking for an additional 7-10 days, to increase the bacterial biomass.

Preparation of bacterial stocks

Twenty-six selected *M. tuberculosis* clinical isolates (Supplemental Table 1) were grown in 200 mL of Middlebrook 7H9 liquid medium supplemented with 10% OADC and 0,2% glycerol. Bacterial suspensions were aliquoted in cryovials, frozen and stored at -80ºC. Bacterial quantification was performed by thawing 3 to 5 vials and plating several serial dilutions in 7H11 agar medium supplemented with 10% OADC and 0.5% glycerol. The plates were incubated 21 to 28 days at 37ºC before colony count.

PBMC isolation and CD14$^+$ cell purification

Blood from donors was collected and processed within 2 hours. PBMCs were separated using Histopaque 1077 at room temperature (Sigma-Aldrich) and following the protocol for mononuclear cell separation of SepMate-50 tubes (StemCells). Cells were resuspended in complete RPMI-1640 medium (10% Fetal bovine serum, 1% Sodium Pyruvate, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfic acid (HEPES) and 1% L-glutamine) (cRPMI) (GIBCO), counted and plated in 24 well plates at a concentration of $0.5x10^6$ cell/mL in 500 µl of cRPMI.

CD14$^+$ cells were isolated from cryopreserved PBMCs. PBMCs were magnetic labelled with CD14 MicroBeads (Miltenyi Biotec) and isolated through positive selection using MACS Separation Columns MS (Miltenyi Biotec), according to the manufacturer's protocol. Purified cells (~95% pure) were counted and plated in 96 well plates at a concentration of $0.5x10^6$ cell/mL in 200 µl of cRPMI (GIBCO).

THP-1-ASC-GFP culture

The THP1-ASC-Green Fluorescent Protein (GFP) (InvivoGen) cell line was maintained in cRPMI (GIBCO) supplemented with 100µg/mL Zeocin (InvivoGen), at 37ºC and 5% CO_2. Cells were used before reaching 20 passages.

Animals

Eight-to-12-week-old male or female mice used to generate bone marrow-derived macrophages (BMDMs) were of the following origins: C57BL/6 WT, *Tlr2*-deficient (-/-) and *Tlr4*$^{-/-}$ mice were maintained and provided by the animal facility of i3S; *Ifnar*$^{-/-}$ and matched C57BL/6 WT were kindly provided by Dr. Jocelyne Demangeau, Intituto Gulbenkian de Ciência, Oeiras, Portugal; *Aim2*$^{-/-}$ and matched C57BL/6 WT femurs were kindly provided by Dr. Thomas Henry, CIRI-Centre International de Recherche en Infectiologie (Institut National de la Santé et de la Recherche Médicale), Lyon, France, under the MTA OM-217356. Animals were kept under specific-pathogen free conditions, with controlled temperature (20-24ºC), humidity (45-65%), light cycle (12h light/dark) and *ad libitum* food and water. All experiments were performed in strict accordance with recommendation of European Union Directive 2010/63/EU and previously approved by Portuguese National Authority for Animal Health – *Direção Geral de Alimentação e Veterinária*.

BMDMs generation

Protocols were as previously published (Moreira-Teixeira et al. 2016). Briefly, mice tibias and femurs were collected and bone marrow flushed with complete DMEM medium (10% Fetal bovine serum, 1% Sodium Pyruvate, 1% HEPES and 1% L-glutamine) (cDMEM) (GIBCO). Cells were counted and plated in Petri dishes at the concentration of $0,5x10^6$ cells/mL in 8mL with 20% L-cell conditioned medium (V/V). On day 4, cDMEM supplemented with 20% L-929-cell conditioned medium was added to the cells. On day 7, cells were harvested, counted, plated in 24 well plates at a concentration of $1x10^6$ cell/mL in 500 µl of cDMEM.

Method Details

Study population

A cohort of 813 culture-confirmed TB cases diagnosed at a University-affiliated hospital (CHSJ, Porto) during a 7-year (2007-2013) period was reviewed to derive a study group of pulmonary TB cases (Supplemental Figure 1-A). The overall demographic and clinical features of this population have been described (Bastos et al. 2016). Patients in the study group were divided into four main groups: no known comorbidities (TB group); HIV-coinfected (HIV-TB group); diabetics (DM-TB group); other comorbidities or immunological suppression, including hepatitis C virus chronic infection, alcohol abuse, end-stage chronic kidney failure, malignancy, cirrhosis or chronic liver failure, heart failure, chronic obstructive pulmonary disease, and patients with structural lung disease, such as silicosis, fibrosis, or bronchiectasis (Other).

Clinical severity classification

The clinical records of the patients in the TB group were retrospectively reviewed to stratify the severity of TB at presentation. A classification flowchart was developed (Figure 1-C) taking into consideration the site of TB involvement, baseline levels of hemoglobin and C-reactive protein, the severity of symptoms and the chest radiography findings for intrathoracic TB. Digital images of plain chest radiographs were blind-graded by two independent clinicians, using a previously published decision tree (Supplemental Figure 1-C; (Berry et al. 2010)). Disagreements between the two clinicians were resolved through a consensus assessment by a third reader.

Human ancestry

Individual ancestry estimates were computed using Structure (Pritchard et al. 2000) considering four ancestral contributors (African, European, East Asian and Native American biogeographical origins; K=4); known genotypes from the HGDP-CEPH panel (Pereira et al. 2012) and the 1000 Genomes (Consortium 2015), complemented with Colombian (Ossa et al. 2016) and Peruvian Amerindians (unpublished data), were used as learning reference samples to perform unsupervised clustering analyses of Portuguese controls (Porto TB-contacts) and TB patients (Porto TB-cohort) (100000 burnin followed by 100000 MCMC repetitions; 3 replicate runs). Aligned averaged clustering was then obtained with CLUMPP (Jakobsson and Rosenberg 2007) and plotted with DISTRUCT (Rosenberg 2004).

Genomic DNA extraction from bacterial suspensions

Bacterial suspensions were pelleted, resuspended in water to a final volume of 1 mL and inactivated with 0.5 mL of a 1:2 phenol-water solution for DNA extraction and homogenized with zirconia beads in TEN buffer, in the Fast-Prep24 bead beater (MP Biomedicals) at 4 M/s for 30 seconds, twice. DNA was then extracted with chlorophorm, precipitated with absolute ethanol and sodium acetate, resuspended in TE buffer, quantified by spectrophotometry (NanoDrop® 1000, Thermo Scientific, Wilmington, USA) and normalized to a standard concentration of 200 ng/µL.

MTBC lineage and sublineage genotyping

MTBC lineage and sublineage genotyping was performed by a custom TaqMan® real-time PCR assay (Applied Biosystems, Carlsbad, USA), using single-nucleotide polymorphisms (SNPs) as stable genetic markers, as previously described (Stucki et al. 2012). Results were analyzed with the Bio-Rad CFX Manager™ 3.1 and genotypes determined using the Allele Discrimination software.

Whole-genome sequencing, variant filtering and phylogenetic analysis

DNA sequencing of bacterial isolates was performed with Illumina MiSeq and HiSeq 2000/2500 paired-end technology. Raw reads were subjected to quality trimming with Trimmomatic (Bolger, Lohse, and Usadel 2014) v0.38 (minimum read length 20 and average base quality 20 in 4-base sliding windows). Visual inspection after trimming was performed with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) v0.11.7 and MultiQC (Ewels et al. 2016) v1.0. Filtered reads were mapped to the

reconstructed MTBC ancestral strain (Comas et al. 2010) with bwa-mem (Li 2013) and converted to BAM files with SAMtools (Li et al. 2009) v.1.3.1. Duplicated reads were removed with Picard (http://broadinstitute.github.io/picard) Mark Duplicates v2.18.14. Single nucleotide polymorphisms and insertions/deletions were called with Pilon (Walker et al. 2014) v1.22 (minimum depth of 5 valid read pairs, minimum mapping quality 20 and minimum base quality 20) and filtered with bcftools (http://samtools.github.io/bcftools) v1.3.1 (keeping only biallelic sites with variant frequency >75% and with a minimum distance from insertion and deletion of bases (InDels) of 4 nucleotides). Genomic variants were annotated with SnpEff (Cingolani et al. 2012) v4.3t according to the *M. tuberculosis* H37Rv reference genome annotation (NC_000962.3; GCF_000195955.2). To minimize false positives, previously identified repetitive genomic regions, mobile elements and genes containing ≥50 bp nucleotide chunks identical to other parts of the genome (Coscolla et al. 2015; Koch et al. 2017) were not considered to further analysis. Variants exclusive from either low or high IL-1β-inducing strains were assigned only on sites that had the reference allele successfully called on all the isolates belonging to the opposite phenotype.

Phylogenetic reconstruction was derived from a multiple sequence alignment containing only single nucleotide polymorphisms (n=10748) and allowing less than 5% of strains per site with missing information. Maximum likelihood (ML) tree was obtained with RaxML (Stamatakis 2015) v7.2.8 using GTR GAMMA model with estimate of proportion of invariable sites, 1000 rapid bootstrap replicates and searching for the best-scoring ML tree. Tree was rooted on the MTBC ancestor and annotated using iTOL (Letunic and Bork 2019) v4.3.3.

The global reference dataset was constructed with samples from (Ates et al. 2018; Bos et al. 2014; Brites et al. 2018; Chiner-Oms, Berney, et al. 2019; Coll et al. 2014; Comas et al. 2013; Comas et al. 2015; Guerra-Assuncao et al. 2015; Stucki et al. 2016; Zignol et al. 2018). We applied the pipeline explained above to derive an alignment and a phylogeny with all the downloaded samples (n = 9240). After that, we run Treemer (Menardo et al. 2018) to reduce the number of strains with a minimum loss of genetic diversity (0.05 of the initial diversity). The final alignment consisted in 4528 MTBC strains. Variants identified in the Porto dataset were mapped to the phylogeny and the number of occurrences (homoplasies) across the phylogeny was recorded.

Cell wall analysis

M. tuberculosis isolates 4I2 and 6C4 were grown in Middlebrook 7H9 liquid medium complemented with 10% OADC and 0,2% glycerol until a dense bacteria suspension was obtained. The bacteria were pelleted by centrifugation and inactivated by autoclave sterilization before lipid extraction. Polar and apolar lipids were extracted using described methods (Dobson et al. 1985). Extracted lipids were resuspended in chloroform:methanol (2:1). Free and apolar lipid extracts using a solvent system consisting of chloroform/methanol/water (60:16:2). A 2D thin-layer chromatography (TLC) system B (direction 1: Petroleum ether 60-80/ acetone (92:8) – 3 runs; direction 2: Toluene/acetone (95:5) – 1 run) and system C (direction 1: Chloroform/ methanol (96:4) – 1 run; direction 2: Toluene/acetone (80:20) – 1 run) was also used. 10% phosphomolybdic acid staining followed by charring was used to reveal lipids in TLCs.

Cell infection

Before infection, mycobacterial clumps were disaggregated by gentle passaging through a 25G needle. For PBMC infection, a multiplicity of infection (MOI) of 1 bacteria: 1 cell (MOI of 1) was used for infection. THP-1-ASC-GFP cells were primed with 100nM PMA for 24h, and allowed to rest for 4 days in fresh medium without PMA, before being infected. MOIs of 1 or 0.1 were used for infection of THP-1-ASC-GFP cells. BMDMs were infected with a MOI of 2. For specific experiments, MOIs of 0.5, 1, 1.5, 5 and 10 were used to infect BMDMs. At different time points, cells or supernatants were recovered for RNA or protein analysis. Culture supernatants were filter sterilized prior to be used for protein detection. For immunofluorescent detection of ASC specks, infected cells were washed with PBS and fixed with formalin 2%. For rifampicin treatment condition, bacteria were treated with 500µg/mL of antibiotic for 18-24h before been pelleted. Loss of viability was confirmed by plating treated bacteria on 7H11 agar plates.

THP-1 ASC specks quantification

Fixed cells were stained with 4´,6-diamidino-2-phenylindole (DAPI) and an IN CELL analyzer 2000 was used for plate image acquisition. Images were analyzed using Fiji (Schindelin et al. 2012).

Cell treatment with chemical inhibitors or agonists for specific signaling pathways

For specific experiments, cells were treated with 0.1, 1 or 10µM of the NLRP3 inhibitor, MCC950 (InvivoGen), or the caspase 1 inhibitor, VX-760 (InvivoGen); 10 or 20µM of the cGAS inhibitor, RU.521 (InvivoGen); 25µg/mL of the K^+ (potassium) channel inhibitor, Glybenclamide (InvivoGen); 10µM of the cathepsin B inhibitor, ZRLR (kindly provided by Eva Wieczerzak, (Reich et al. 2009)); 5µM of the phagocytosis inhibitor, cytochalasin D (Sigma-Aldrich); or with 5mM of ATP (InvivoGen); 1µg/mL of the retinoic acid-inducible gene I (RIG-I) agonist, 5'ppp-dsRNA (InvivoGen).

mRNA analysis by real-time PCR

Total RNA from infected cells was extracted with TRIzol Reagent (Invitrogen, California, USA), according to the manufacturer's instructions. cDNA was synthesized using the ProtoScript First Strand cDNA Synthesis kit (New England Biolabs, Massachusetts, USA) and gene expression was analyzed by RT-PCR using the Bio-Rad CFX Manager™ 3.1, as describe before (Bhatt et al. 2018). Targeted *Il1β*, *Ifnβ*, *Tnf* and *Il10* mRNA expression was quantified using SYBR Green (Thermo Fisher) and specific oligonucleotides, and normalized to ubiquitin mRNA levels. *Ccl2* and *Cxcl10* mRNA expression was quantified using TaqMan gene expression master mix (Applied Biosystems), and normalized to hypoxanthine phosphoribosyltransferase (HPRT).

Cytokine determination

Cytokines were detected in supernatants of *in vitro* infected cultures by immunoassay. This included Multiplex (Invitrogen) for human IL-1β, IL-12p40 and IL-10, or Enzyme-linked immunosorbent assay (ELISA) for mouse IL-1β (Invitrogen), IFN-β (BioLegend) and TNF (Invitrogen) as indicated in the Figure legends.

Global transcriptomic and pathway analyses

Total RNA from infected or non-infected cells was extracted as above and treated with Turbo DNA-free kit (Invitrogen) before sequencing. Targeted RNA sequencing was performed by GenCore, i3S (Institute of Health Innovation and Research) using Ion AmpliSeq™ Transcriptome Mouse Gene Expression Kit. All subsequent analyses were performed in R [v3.5.1]. RNA expression levels in the count matrix were normalized using the trimmed mean of M-values and computed as counts per million using edgeR package [v3.24.1] (McCarthy, Chen, and Smyth 2012; Robinson, McCarthy, and Smyth 2010).

Differential expression analysis was performed using edgeR and limma [v3.38.3] (Ritchie et al. 2015) packages. Genes with less than 15 raw counts in all samples were excluded. Differentially expressed genes were determined through linear model fitting. Empirical Bayes moderated t-statistics test was performed and genes with and genes with adjusted p-value ≤ 0.05 and log2 fold-change ≤-2 or ≥2 were considered significant. Pathway analysis was performed using ReactomePA package [v1.26.0] (Yu and He 2016). Adjustment of p-values for multiple testing was performed using the Benjamini-Hochberg (BH) procedure (https://www.jstor.org/citation/ris/10.2307/2346101) in both differential expression analysis and pathway analysis.

Quantification and Statistical Analysis

Data were analysed using GraphPad Prism software, version 8.1.0. Student's t test was used to determine differences between two different groups and one-way ANOVA for more than two groups. Post-tests were applied to multiple comparisons as referred in Figure legends. Data was checked for normality and log normality. Other statistical tests included Fisher's exact test and Pearson correlation, as indicated in the Figure legends. Differences were considered significant for p≤0.05 and represented as follows: *p≤0.05; **p≤0.01; ***p≤0.001 and ****p≤0.0001. Bean plots were generated with the R package beanplot (Kampstra 2008).

Data and Code Availability

Generated data from RNA-Seq are deposited on NCBI GEO database accession code GSE138580 (please use the following token to access data: qnozigcqlrczrkn).

Results

Characterization of a TB cohort to investigate *M. tuberculosis* determinants of host immune responses

To investigate whether *M. tuberculosis*-associated determinants may contribute to the pathogenesis of TB through the manipulation of host immune responses, we started by stratifying TB severity in a fully characterized population of TB patients (Bastos et al. 2016). From a cohort of 813 culture-positive TB cases (Bastos et al. 2016), 681 adult pulmonary TB patients were selected (Supplemental Figure 1-A) and classified according to the presence of risk factors for TB in: no known risk factors (TB), HIV co-infection (HIV-TB), diabetes mellitus (DM-TB), and various comorbidities or pharmacologically immunosuppression (Other) (Figure 1-A). To exclude the effect of host-related comorbidities on TB presentation, we next focused on TB only patients. The genetic makeup of 60 TB patients (Supplemental Figure 1-A) was investigated through a validated panel of autosomal ancestry-informative markers (AIMs). This analysis was performed alongside a group of Portuguese reference genomes and a group of TB contacts from Porto (Figure 1-B). The ancestry of both the Porto-TB cohort and the Porto-TB contacts seemed homogeneous, rooted on Portuguese (Figure 1-B) and European (Supplemental Figure 1-B; (Pereira et al. 2012; Bycroft et al. 2019)) makeups. Next, a clinical decision system to assign the severity of TB at presentation was developed based on chest X-ray classification (Supplemental Figure 1-C; (Berry et al. 2010)) combined with systemic clinical parameters (Figure 1-C). This classification system was applied to the 133 TB cases, for which all required data was available (Supplemental Figure 1-A). The most common TB presentation was moderate, followed by mild and severe (Figure 1-D). Although a higher proportion of men was observed in the severe TB group, both the age of the patient and reported time of symptoms were similarly distributed within the three TB severity groups (Supplemental Figure 1-D).

In parallel, we investigated the genetic structure of the infecting *M. tuberculosis* population. Bacterial isolates corresponding to 117 TB cases were recovered (Supplemental Figure 1-A). A large predominance of the MTBC L4 and of sublineage L4.3/Latin-American-Mediterranean (LAM) was revealed within the pathogen population (Figure 1-E), in line with other reports focused in Europe (Lopes et al. 2013; Stucki et al. 2016). This pathogen populational structure was replicated across the different TB severity groups (Figure 1-F).

M. tuberculosis isolated from patients with severe TB induce lower cytokine responses in infected human peripheral blood mononuclear cells than *M. tuberculosis* isolated from mild TB cases

Our findings so far suggest that host-pathogen sympatry breaks are not likely to explain the severity of TB in the cohort under study. We next questioned whether *M. tuberculosis* isolated from patients presenting different TB severities interacted differently with human immune cells. For this, we selected L4 isolates from the mild (n=10), moderate (n=9) or severe (n=7) TB groups (Supplemental Table 1). Each of these isolates was used to infect PBMC cultures isolated from 8-10 donors who had been IGRA+ for more than 2 years, did not undergo preventive antibiotherapy and had no past TB episode. We decided against cells from active TB patients, as their cytokine responses are altered by the disease status. We also decided against IGRA- donors, as within this population certain individuals might present innate resistance to TB and altered myeloid cell responses to infection (Verrall et al. 2019). The PBMC composition of the recruited individuals was similar and followed the expected values for the different cell populations (Supplemental Figure 2-A). The supernatants from the infected cultures were collected 24h post-infection and the amounts of IL-12p40, IL-1β, IFN-β and IL-10 quantified. Deficiencies in IL-12p40 and IL-1β are both linked to TB susceptibility (O'Garra et al. 2013), and altered levels of IL-1β have been associated with different TB severities in patients (Mayer-Barber et al. 2014). IFN-β and IL-10 play a detrimental role in TB, and have been implicated in the cross-regulation of IL-12p40 and IL-1β (Moreira-Teixeira et al. 2018; Redford, Murray, and O'Garra 2011; Mayer-Barber et al. 2014). Interestingly, as compared to *M. tuberculosis* isolated from mild TB cases, we found that *M. tuberculosis* isolates associated with severe or moderate forms of TB induced lower cytokine responses by PBMCs (Figure 1-G). IFN-β secretion was below detection level for all cases. Although PBMCs from different donors varied in their absolute amount of cytokine production, *M. tuberculosis* inducing high cytokine production did so for all donors and the same was observed for low cytokine inducers (Supplemental Figure 2-B). These data suggest that the pattern of cytokine responses are primarily controlled by bacterial factors and that an association between the levels of cytokines triggered and TB severity might be in place.

Whole-genome analysis reveals common polymorphisms in groups of low-intermediate cytokine-inducing *M. tuberculosis* clinical isolates

To search for bacterial genomic determinants associated with the differential potential to induce cytokine responses in PBMCs, the whole-genome sequence of the 26 *M. tuberculosis* clinical isolates was generated. A total of 3,112 unique polymorphisms (SNPs and InDels) were found in comparison with the inferred sequence of the MTBC ancestor (Comas et al. 2010). The maximum pairwise distance between any two clinical isolates was 456 mutations (Figure 2-A), a value that falls within the expected for *M. tuberculosis* strains from the same lineage (Bastos et al. 2017). Five groups of clinical isolates (6C4, 5F9 and 3A7; 6C1 and 6D3; 5C8 and 1B2; 5I5 and 5A9; 1E1 and 2G8), possibly being part of transmission chains (<12 SNPs; Supplemental Figure 3-A) were identified. Taking IL-1β as a proxy for low versus high cytokine responses, the phylogenetic analysis using 3,077 genome-wide SNP positions revealed no clear associations between phylogeny and IL-1β induction (Figure 2-B). Unbiased comparative analysis between the selected *M. tuberculosis* isolates revealed 21 genes harboring polymorphisms shared exclusively by more than 5 low IL-1β-inducing isolates and 3 genes harboring polymorphisms shared exclusively by 3 high inducing isolates (Figure 2-C).

If some of the identified polymorphisms indeed lead to a modulation of cytokine induction, we would expect signatures of selection acting upon them. Homoplastic positions, those appearing multiple times and independently in the phylogeny, are generally good surrogates of the action of positive selection (Chiner-Oms, Sanchez-Buso, et al. 2019; Mortimer, Weber, and Pepperell 2018). By comparing the identified polymorphisms to a collection of 4528 strain genomes representative of the global diversity of the MTBC, we found that 5 SNP positions from our Porto dataset also emerged in unrelated strains in the phylogeny more than one time. Notably, we found a synonymous position that occurred five times in strains from different lineages (data not shown). Although unlikely to alter the structure of the underlying protein, this synonymous mutation generates a new internal TANNT *sigA* recognition box. We have shown before that MTBC uses newly generated TANNT boxes as a way to adapt to changing environments (Chiner-Oms, Berney, et al. 2019). In fact, among the synonymous SNPs observed, three generate new regulatory boxes (data not shown). Taken together, the evolutionary analysis suggests that at least part of the polymorphisms and genes identified are under selection in epidemiological settings.

Given the previous association of the ESX-1 secretion system with cytokine responses, particularly IL-1β (Orgeur and Brosch 2018), a directed analysis to identify

polymorphisms in this pathway was performed. We found common genetic variants shared by 5 clinical isolates (6C4, 3A7, 1C7, 5F9 and 5D6) inducing low IL-1β levels, whilst absent in high IL-1β inducers (Figure 2-D). In particular, *M. tuberculosis* isolate 5D6 harbored two SNPs in genes encoding ESX-1 secretion system components (*EccD1* and *EspA*) and isolates 6C4, 3A7 and 5F9 shared two SNPs in the intergenic regions *espR-Rv191* and *PPR35-Rv1919c* (Figure 2-D). No SNPs exclusive of either group of isolates were identified in the other ESX-1 components or regulators encoding genes. Thus, our analyses support that low IL-1β induction results from different evolutionary events and highlight the diversity of bacterial genetic pathways possibly underlying the induction of low cytokine responses.

In clinico-selected *M. tuberculosis* isolates induce distinct IL-1β in monocytes and macrophages independently of the host TB status

M. tuberculosis isolates 6C4, 3A7 and 5F9 belong to a probable transmission cluster (Supplemental Figure 3-A) and consistently induce the secretion of low IL-1β by infecting cells, thus suggesting a “stable phenotype”. Isolate 6C4 was selected as a representative of this transmission cluster and of the low-cytokine/severe TB group and *M. tuberculosis* 4I2 as a representative of the high-cytokine/mild TB. The clinical differences between these two isolates are listed in Supplemental Table 1. Both isolates belong to the LAM sublineage of L4 and showed similar growth curves in axenic media (Supplemental Figure 3-B). We next measured the production of IL-1β induced upon infection of cells from different origins with *M. tuberculosis* isolate 4I2 or 6C4. Differential IL-1β induction was observed upon infection of PBMCs from IGRA+ (Figure 3-A) or IGRA- (Figure 3-B) donors. Similar findings were observed upon infection of purified monocytes from IGRA- individuals (Figure 3-C), macrophages differentiated from the human monocytic cell line THP-1 (Figure 3-D) or mouse bone marrow-derived macrophages (BMDMs, Figure 3-E). The differential pattern of IL-1β expression is thus independent of the host and its TB status, being instead strongly dictated by the infecting bacteria. Of note, the differential response observed was also independent of the batch of *M. tuberculosis*, as stocks grown independently yielded the same profile of IL-1β induction in infected cells.

Distinct global transcriptional changes are induced in macrophages by *M. tuberculosis* isolates associated with different TB severities

To gain insights on the mechanisms underlying the differential cellular response to the selected *M. tuberculosis* isolates, a targeted RNA-Sequencing for gene expression analysis (AmpliSeq; (Papp et al. 2018; Li et al. 2015)) was obtained for BMDMs before and after infection with either isolate. An overall similarity between replicates and groups was detected for genes with a >2-log fold change in their expression upon infection and a statistical difference in expression (adjusted p-values of $\leq$0.05) (Supplemental Figure 4-A). Principal component analysis (PCA) showed that over 75% of the differences observed between samples, described by PC1, are mainly between BMDMs left uninfected and BMDMs infected with the 4I2 *M. tuberculosis* isolate (Figure 4-A). In response to infection with *M. tuberculosis* isolate 4I2, 1.271 transcripts were differentially expressed (Figure 4-B and -C). Strikingly, only 389 transcripts were differentially regulated in BMDMs infected with isolate 6C4 (Figure 4-B and -C).The full list of differentially expressed genes (>2-log fold change; adjusted p-values of $\leq$0.05; Supplemental Figure 4-B) is not provided in this thesis. Canonical pathway analysis (ReactomePA; Figure 4-D and -E) revealed that whereas infection with isolate 4I2 activated PRR-related pathways, such as TLRs, NLRs and MyD88 or TRIF cascades, infection with 6C4 did not (Figure 4-D and -E). An overview of the interactions between the activated pathways and the significantly altered genes in each pathway upon infection is shown in Supplemental Figure 4-C and Figure 4-D. Thus, infection with *M. tuberculosis* isolate 4I2 resulted in a more robust and complex alteration of the gene expression profile of host BMDMs.

Enhanced activation of the inflammasome in macrophages infected with *M. tuberculosis* 4I2 isolate underlies increased IL-1β secretion

Next, a heatmap of differentially regulated genes within the cytokine/interleukin–related pathways was generated. Although an overall higher expression of the genes in these pathways was noted in BMDMs infected with *M. tuberculosis* isolate 4I2, the upregulation of the *Il1b* mRNA was detected at the same level in BMDMs infected with either isolate (Figure 5-A). This was validated by real-time PCR in both BMDMs (Figure 5-B) and human CD14+ monocytes (Supplemental Figure 5-A). These observations suggest that the recognition of either *M. tuberculosis* isolate by BMDMs may be similar, thus

leading to an equivalent transcription of the *Il1b* gene. Indeed, for both *M. tuberculosis* isolates the production of IL-1β by infected BMDMs was fully dependent on TLR2 activation (Figure 5-C), with no contribution of TLR4 triggering (Supplemental Figure 5-B). Therefore, the differential regulation of IL-1β by the two *M. tuberculosis* isolates likely occurs at the post-transcriptional level, perhaps through differential inflammasome activation. To test this hypothesis, we used THP-1-ASC-GFP reporter cells (Stutz et al. 2013). The percentage of ASC-speck positive cells in PMA-differentiated reporter cells was higher in the case of *M. tuberculosis* 4I2 infections (Figure 5-D and Supplemental Figure 5 -C). Furthermore, chemical inhibition of NLRP3 (Figure 5-E) or of caspase- 1 (Figure 5-F) strongly abrogated IL-1β production by BMDMs infected with *M. tuberculosis* isolate 4I2. Inhibition of NLRP3 or caspase-1 during infection of macrophages with the severe-TB causing isolate (6C4) also decreased IL-1β secretion (Figure 5-G and -H). Therefore, the activation of NLRP3 is critical for the secretion of IL-1β by BMDMs infected with either *M. tuberculosis* isolate, but isolate 4I2 shows an enhanced ability to activate the inflammasome.

To unveil the bacterial determinants of differential inflammasome activation, we firstly addressed possible differences in the cell wall lipidic fraction. The whole-genome sequencing data indicated that strain 6C4 had SNPs in genes encoding two key *M. tuberculosis* lipids involved in virulence: Sulfolipid-I and Phthiocerol dimycocerosate, suggesting a potential change in the profiles of these lipids. Lipid extracts of either bacteria were prepared and analysed by TLC. With exception of free mycolic acids that seemed to be present in relatively higher amounts in *M. tuberculosis* isolate 4I2, no major differences were observed in the lipid profiles of the two strains (Supplemental Figure 5-D). To investigate if differences in the amount of cell-wall components might explain the differential IL-1β response by infected cells, we infected macrophages with increasing doses of *M. tuberculosis* isolate 6C4. Even when a MOI of 10 was used, the secretion of IL-1β was still low (Figure 5-I), whereas that of TNF increased steadily, showing that the macrophages were upregulating their response to infection with higher doses of *M. tuberculosis* 6C4 (Supplemental Figure 5-E).

To assess for a possible active inhibition of the inflammasome and IL-1β production in cells infected with *M. tuberculosis* isolate 6C4, we next performed mixed infections of BMDMs. In these infections, the total MOI was maintained at 2, but the amount of *M. tuberculosis* isolate 4I2 was progressively replaced by isolate 6C4. Independently of the amount of isolate 6C4 present in culture, the response of BMDMs to *M. tuberculosis* isolate 4I2 was not inhibited (Figure 5-J). Furthermore, no inhibition was seen in BMDMs infected with isolate 4I2 in the presence of supernatant from cells infected with isolate 6C4 (Supplemental Figure 5-F). Infection of BMDMs with *M. tuberculosis* isolate

6C4 did also not inhibit the transcription of key inflammasome components, as revealed through the RNA-Seq data (Supplemental Figure 5-G). Finally, if BMDMs infected with isolate 6C4 were provided with an inflammasome activator (as ATP), an upregulation of IL-1β was readily observed (Figure 5-K). Together, this set of experiments indicate that an intrinsic property of *M. tuberculosis* isolate 6C4 underlies the failure of this isolate to activate the inflammasome, which is not related to the amount of bacteria required nor to a direct inhibition of this pathway.

M. tuberculosis isolate 6C4 evades the macrophage cytosolic surveillance pathways

Previous reports showed that the activation of the inflammasome by *M. tuberculosis* requires a competent ESX system (Koo et al. 2008; Mishra et al. 2010; Orgeur and Brosch 2018). Through this system, *M. tuberculosis* DNA and RNA are exported to the cell cytosol, respectively activating cGAS (Wassermann et al. 2015; Collins et al. 2015; Watson et al. 2015) and RIG-I (Cheng and Schorey 2018). This, together with the fact that the low IL-1β-inducing *M. tuberculosis* isolates accumulated mutations in the ESX components or regulators (Figure 2-D), led us to interrogate the global transcriptomic data for differences in the activation of hallmark intracellular pathways in macrophages infected with either *M. tuberculosis* isolate. Heatmaps for cGAS (Figure 6-A), RIG-I (Supplemental Figure 6-A), TLR3 (Supplemental Figure 6-B) or TLR7/8/9 (Supplemental Figure 6-C) pathways showed a higher upregulation of the genes associated with these pathways in BMDMs infected with *M. tuberculosis* isolate 4I2. Furthermore, hierarchical clustering for cGAS and RIG-I pathways showed higher similarity between BMDMs infected with *M. tuberculosis* isolate 6C4 and non-infected cells, than between the two infections (Figure 6-A; Supplemental Figure 6-A). The enhanced transcription of the cGAS-associated genes *Ifnβ*, *Il10*, *Ccl2* and *Cxcl10* in BMDMs infected with *M. tuberculosis* isolate 4I2 was validated by real-time PCR (Figure 6-B) and found to depend on the activation of cGAS (Figure 6-C). Chemical inhibition of cGAS led to lesser alterations on *Tnf* transcription (Figure 6-C). Together, these data suggest a differential activation of cGAS in macrophages infected by *M. tuberculosis* isolate 4I2 versus isolate 6C4. The cGAS pathway is activated by bacterial DNA, which is also a ligand for AIM2, a member of the inflammasome family known to contribute to the secretion of IL-1β (Wassermann et al. 2015; Collins et al. 2015; Watson et al. 2015). Therefore, it is possible that the activation of AIM2 in BMDMs infected by *M. tuberculosis* isolate 4I2 is contributing to maximal IL-1β secretion. *Aim2*$^{-/-}$ BMDMs were thus generated

and infected with either isolate. The impact of AIM2 deficiency in the secretion of IL-1β by macrophages infected with isolate 4I2 was more pronounced than that seen in the case of infection with 6C4 (Figure 6-D). Phagosome rupture by mycobacteria triggers several other molecular signals perceived by the NLRP3 inflammasome, among which potassium efflux and cathepsin B release (Dorhoi et al. 2012; Amaral et al. 2018). In further support of a more active or efficient phagosome rupture induced by *M. tuberculosis* isolate 4I2, chemical inhibition of potassium channels or of cathepsin B decreased the secretion of IL-1β by 4I2-infected macrophages, whilst not affecting 6C4 infections (Figure 6-E and -F).

Therefore, our data fit the model whereby the activation of cytosolic pathways is preferentially occurring in the context of a mild TB-associated *M. tuberculosis* isolate. Because IFN-β has been shown to downregulate IL-1β (Moreira-Teixeira et al. 2018), we questioned if this cross-regulation was still in place in the case of *M. tuberculosis* isolate 4I2. To answer that question, we generated and infected *Ifnar*$^{-/-}$ BMDMs and measured their ability to secrete IL-1β as compared to WT cells. Absence of IFNAR further increased IL-1β secreted by BMDMs infected with isolate 4I2, but did not impact IL-1β secretion upon infection with 6C4 (Supplemental Figure 6-D). However, enhancing IFN-β production by activating BMDMs infected with *M. tuberculosis* isolate 6C4 with a RIG-I agonist decreased the secretion of IL-1β by the infected cells (Supplemental Figure 6-E). Therefore, independently of the activation of the host cytosolic surveillance pathways, the mechanisms regulating the secretion of IL-1β and IFN-β are still coupled.

Our genomic, transcriptional and functional analyses led to the hypothesis that the differential response of macrophages to infection with *M. tuberculosis* isolates 4I2 and 6C4 relies on differential activation of cytosolic surveillance pathways, possibly due to differences in the efficiencies of phagosome rupture and export. Blocking bacterial phagocytosis using cytochalasin D significantly compromised the secretion of IL-1β by BMDMs infected with either *M. tuberculosis* isolate (Figure 6-G), showing that intracellularly localized bacteria are required for IL-1β secretion. Of note, the phagocytosis of the two *M. tuberculosis* isolates by BMDMs occurred in a similar way (Supplemental Figure 6-F), demonstrating that differential IL-1β induction did not result from differences in bacterial internalization. Next, each isolate was exposed to a high dose of rifampicin, to block the transcriptional activity of the bacteria. Rifampicin treatment of *M. tuberculosis* isolate 4I2 decreased the secretion of IL-1β by BMDMs, but had no effect in the case of 6C4 isolate (Figure 6-H). In both cases, rifampicin treatment did not impact the transcription of *Il1b* by infected BMDMs (Figure 6-I), but decreased the transcription of the *Ifnb* gene induced by *M. tuberculosis* isolate 4I2 (Figure 6-J). In line with our hypothesis that by certain isolates of *M. tuberculosis* modulate their access to the macrophage cytosol, internalization of live,

transcriptionally competent bacteria is required to induce maximal production of IL-1β and IFN-β.

Discussion

M. tuberculosis, a leading pathogen of humans, has been traditionally considered a clone. This long-last prevailing dogma is no longer accepted, with several lines of evidence showing genetic diversity within TB-causing bacteria (Gagneux 2018). *M. tuberculosis* diversity is acknowledged to affect bacteria virulence, transmission potential, progression to disease and host-pathogen interactions (Coscolla and Gagneux 2014; Bastos et al. 2017). Here, we reveal important new insights into the functional relevance of *M. tuberculosis* intra-pathogen diversity, linking variations in the infecting bacteria to the modulation of host immune responses and clinical outcomes of TB.

Focusing on pulmonary TB patients without classical risk factors for TB, we report the existence of different severities of disease, in line with the recent concept that TB outcomes are heterogeneous and form a spectrum of disease (Lin and Flynn 2018; Cadena, Fortune, and Flynn 2017). Within the infecting bacteria, we found a high predominance of L4, particularly of the generalist sublineage L4.3/LAM, a genetic structure compatible with a high local adaptation between *M. tuberculosis* and the human population, paralleling studies in metropolitan cities (Hirsh et al. 2004; Baker et al. 2004; Reed et al. 2009). Remarkably, across a range of different host cells, *M. tuberculosis* isolates recovered from severe TB cases induced lower cytokine responses as compared to those recovered from mild TB patients. This finding raises the hypothesis that *M. tuberculosis* evolved to fine-tune the immune response, ultimately modulating the pathogenesis of TB. Therefore, the reason why some individuals develop severe TB, whilst others do not, may also be explained by the characteristics of the infecting bacteria. Consequently, understanding the underlying bacteria molecular bases will prove important from a clinical standpoint. So far, we did not identify a SNP or group of SNPs associated with the immunological phenotype or the TB severity at the genome-wide level, suggesting that the ability of closely related *M. tuberculosis* to modulate the host response results from diverse events. Reflecting that variety of pathways, we have identified signatures of positive selection when comparing to a large global strain database. We have also detected an accumulation of SNPs in genes encoding components or regulators of the ESX-1 secretion system in isolates associated with low IL-1β induction and high TB severity. This may hence be a hot-spot pathway explored by the bacteria to thwart full macrophage activation. Previous studies show that lack of the ESX-1 secretion system is characteristic of avirulent Mycobacteria and that

abrogation of this system renders *M. tuberculosis* avirulent (Lewis et al. 2003). We now disclose further layers of complexity to this aspect of *M. tuberculosis* biology, showing that variations in the efficiency within ESX-1 are likely to exist in highly related isolates, with profound implications for the pattern recognition pathways triggered in the infected macrophage and possibly for the outcome of disease. By restricting phagosome rupture and export of bacterial material to the cell cytosol, low cytokine inducing/severe TB *M. tuberculosis* isolates prevent the activation of the inflammasome, cGAS, RIG-I and other intracellular signaling cascades as revealed by our global transcriptomic analysis. This is in turn reflected in the lower induction of IL-1β and type I IFN.

Decreased IL-1β responses is compatible with more severe TB outcomes. In humans, low IL-1β production has been associated with increased susceptibility of TB (Carvalho et al. 2018; Waitt et al. 2015). Similarly, in experimental mouse models, genetic abrogation of IL-1β or IL-1R drives susceptibility to TB, specifically increased mortality, uncontrolled bacteria growth in the lung and enhanced tissue damage (Yamada et al. 2000; Juffermans et al. 2000). The protective role of IL-1 in TB is now known to extend beyond the control of intracellular bacterial replication via enhancing the macrophage antimicrobial mechanisms. Recent studies position IL-1β as one of the drivers of ILC3 responses required for early protection (Ardain et al. 2019). Furthermore, the relocalization of AMs from the alveolar space to lung interstitium required both IL-1R and bacterial ESX-1 (Cohen et al. 2018). Finally, a role for IL-1R signaling in limiting the proportion of infected cells and dissemination and thus affording protection has been recently revealed (Bohrer et al. 2018). It is therefore not surprising that a professional pathogen such as *M. tuberculosis* manipulates the IL-1 pathway to delay protective mechanisms, and possibly promote dissemination and/or more severe lung lesions, ultimately increasing virulence in the human host. Interestingly, the patient infected with the low IL-1β-inducing *M. tuberculosis* isolate 6C4 had extensive lung pathology, which included pleural involvement, thus suggesting initial dissemination events. Importantly, excessive IL-1β production may also be detrimental to the host and has been associated with more severe TB disease and increased lung damage (Mishra et al. 2017; Mishra et al. 2013; Zhang et al. 2014). From the pathogen's perspective, *M. tuberculosis* isolates that trigger high IL-1β responses would face the risk of being eliminated during early stages of infection, thus failing later transmission. It is thus tempting to speculate that the host-*M. tuberculosis* parallel evolution has perfected a mechanism that simultaneously controls IL-1β and its regulator IFN-β, which ultimately benefits both the host by keeping the infection and associated immune pathology at check, and the pathogen, by ensuring transmission. This mechanism relies on the activation of macrophage intracellular pathways leading to both IL-1β and IFN-β production.

Our findings have implications for the understanding of *M. tuberculosis* transmission. Efficiency of transmission within a host population is a reasonable measure of the pathogen success, as it ensures its persistence. In the case of *M. tuberculosis*, transmission is linked to lung damage, namely cavitation (Furin, Cox, and Pai 2019). Thus, isolates associated with milder lung pathology, such as the isolate 4I2, are likely to produce less cavitation and less exacerbated disease, but their infectiousness period maybe longer, which increases the chances to transmit to more hosts. On the other hand, *M. tuberculosis* isolates causing severe disease, as isolate 6C4, may induce the necessary pathology for transmission, but compromise the time of transmission by prematurely killing the host. Thus, it is tempting to propose that *M. tuberculosis* continuously adapts to new hosts, converging at the micro-evolutionary level to fine tune the immune response to a threshold that allows pulmonary pathology needed for transmission. In this sense, the more successful *M. tuberculosis* isolates may be those associated with moderate forms of TB, as supported by our data showing the highest percentage of moderate TB cases in the study population. A link between transmission potential and type of disease has been proposed recently, as *M. tuberculosis* isolates with differential transmission potential impacted the course of infection in C3H/FeJ mice, in terms of bacterial burden and lung pathology (Verma et al. 2019). Although still challenging, it will be interesting to generate experimental models based on the findings presented here and to contrast the hypothesis against population-level datasets in epidemiological settings.

Our study bears important consequences for future TB research. Host genetic or secondary immune suppression have been associated with higher TB susceptibility. We now show that in immune-competent hosts the initial immune response is also modulated by subtle differences in closely related *M. tuberculosis*. This finding calls for the inclusion of *M. tuberculosis* genetic diversity and continuous evolution as part of human genetic association studies, as well as of vaccine design and as an important component to consider in the development of host-directed therapies, such as those that interfere with the levels of IL-1β or other cytokines.

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Author contributions

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Supervision: SG, JTG, NSO, IC, MS
Visualization: JS, BC, CM, HNB, NSO, IC, MS
Writing- original draft: JS, SG, AGC, HNB, NSO, IC, MS
Writing- review and editing: all authors

Figures

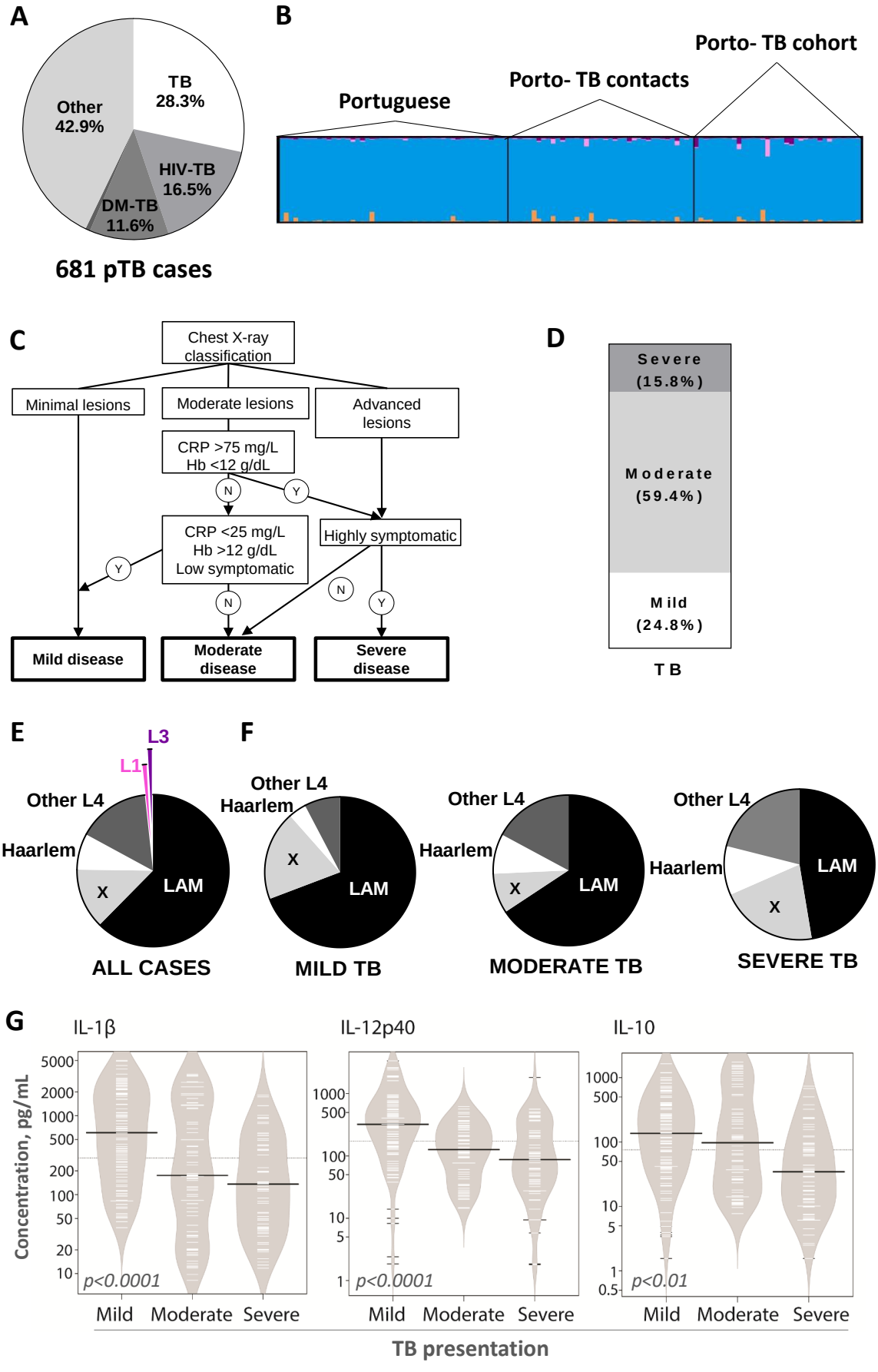

A
TB
28.3%
Other
42.9%
HIV-TB
16.5%
DM-TB
11.6%
681 pTB cases
B
Porto- TB cohort
Porto- TB contacts
Portuguese
C
Chest X-ray classification
Minimal lesions
Moderate lesions
Advanced lesions
CRP >75 mg/L
Hb <12 g/dL
N
Y
CRP <25 mg/L
Hb >12 g/dL
Low symptomatic
Highly symptomatic
Y
N
N
Y
Mild disease
Moderate disease
Severe disease
D
Severe (15.8%)
Moderate (59.4%)
Mild (24.8%)
TB
E
L3
L1
Other L4
Haarlem
X
LAM
ALL CASES
F
Other L4
Haarlem
X
LAM
MILD TB
Other L4
Haarlem
X
LAM
MODERATE TB
Other L4
Haarlem
LAM
X
SEVERE TB
G
IL-1β
IL-12p40
IL-10
Concentration, pg/mL
5000
2000
1000
500
200
100
50
20
10
p<0.0001
1000
500
100
50
10
5
1
p<0.0001
1000
500
100
50
10
5
1
0.5
p<0.01
Mild
Moderate
Severe
TB presentation

Figure 1 - *M. tuberculosis* clinical isolates from patients with severe forms of TB induce lower cytokine responses in infected human peripheral blood mononuclear cells.

(A) The clinical data for 681 adult pulmonary TB (pTB) cases selected from a cohort of 813 culture confirmed TB patients were reviewed and classified according to the absence (TB; 28.3%) or presence of comorbidities (HIV-TB, 16.5%; DM-TB, 11.6%; or Other, 42.9%). **(B)** The genetic ancestry for a group of TB patients was determined, together with TB contacts from the same area and a previously studied reference Portuguese population. Represented are European ancestry in blue, African in orange, East Asian in pink and Native American in purple. **(C)** A clinical decision system based on local and systemic parameters was developed to classify the severity of disease at presentation in mild, moderate or severe TB. **(D)** Patients who had no known comorbidities (n=133) were subjected to the clinical decision system and classified into mild, moderate or severe TB cases at presentation. **(E)** The infecting *M. tuberculosis* isolates were recovered for 117 of the 133 selected TB patients, their DNA isolated and used to determine the MTBC genetic population structure. *M. tuberculosis* isolates were classified into lineages and those belonging to lineage 4 (L4) were further classified into L4.3/LAM, L4.1.1/X, L4.1.2/Haarlem or other lineage 4 sublineages. **(F)** Mild, moderate and severe TB cases, as determined in **(D)**, were stratified according to the sublineage the infecting bacteria belonged to. Statistical analysis was performed with Fisher's exact test. **(G)** PBMC from 8-10 IGRA+ donors were infected with L4 *M. tuberculosis* isolated from mild (n=10), moderate (n=9), or severe (n=7) TB patients. An MOI of 1 was used and 24h post-infection the culture supernatants were harvested, filter-sterilized and the amounts of IL-1β, IL-12p40, IL-10 and IFN-β quantified by Multiplex assay. Represented are bean plots for each cytokine. Black lines show the medians; white lines represent individual data points; polygons represent the estimated density of the data. IFN-β was undetected for all conditions. Statistical analysis was performed with Pearson correlation; p values are indicated. CRP, C reactive protein; Hb, hemoglobin; N, no; Y, yes.

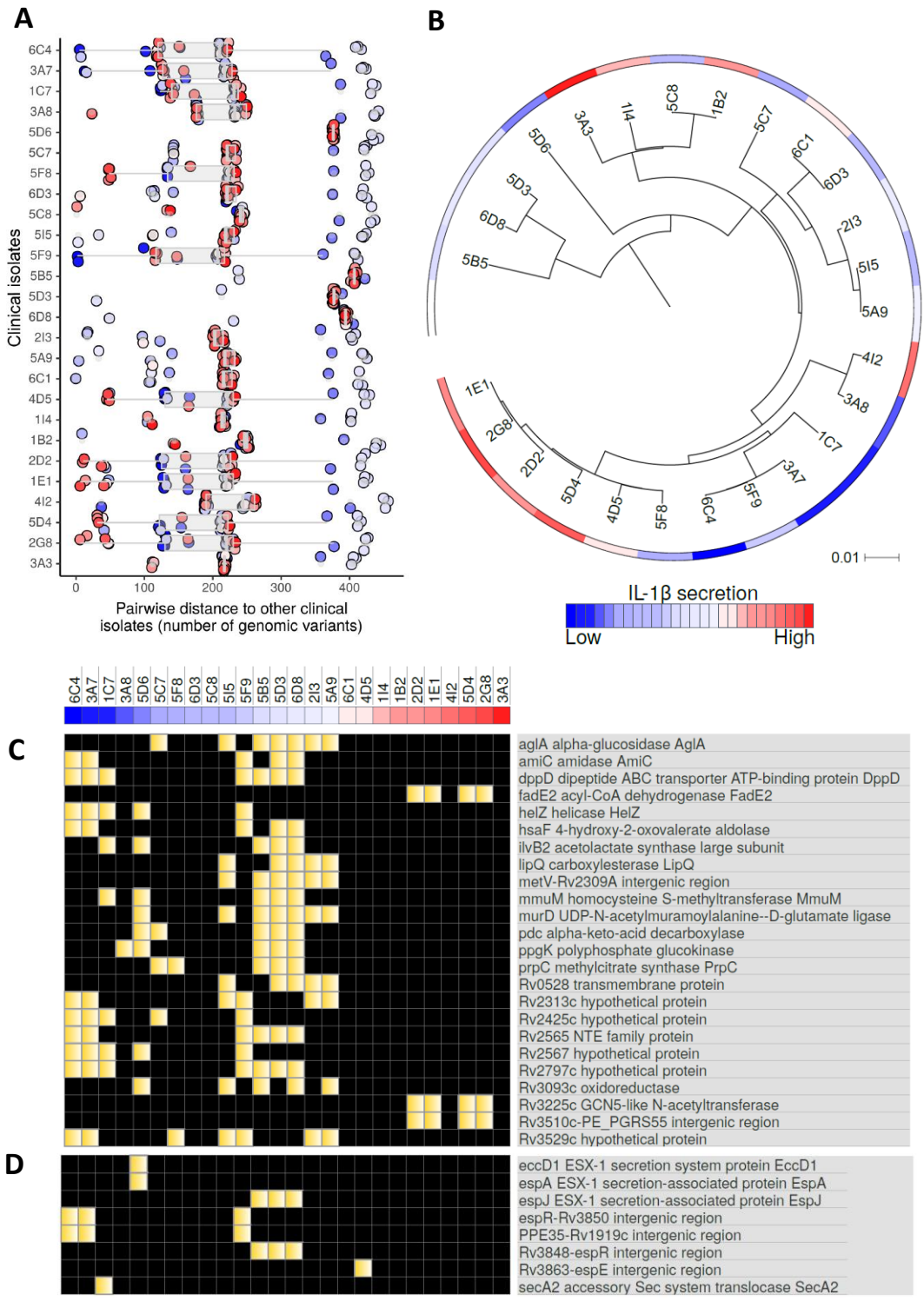


Figure 2 - Whole-genome analysis highlights common polymorphisms in groups of low cytokine-inducing *M. tuberculosis* clinical isolates.

Genome-wide analysis was performed for 26 *M. tuberculosis* clinical isolates with known IL-1β inducing profiles in human PBMCs. **(A)** Pairwise genomic distance (number of SNPs and InDels) between the different isolates. **(B)** Phylogenetic tree of the isolates constructed with genome-wide SNPs and using maximum likelihood. The colour gradient between dark blue (low) and dark red (high) indicates the level of IL-1β induced by each strain. **(C)** Genes with (yellow) or without (black) polymorphisms exclusively present in more than 30% of the *M. tuberculosis* isolates classified as low or high IL-1β inducers. **(D)** ESX-1 secretion system and associated regulators showing polymorphisms exclusively present in the low or high IL-1β inducing strains.

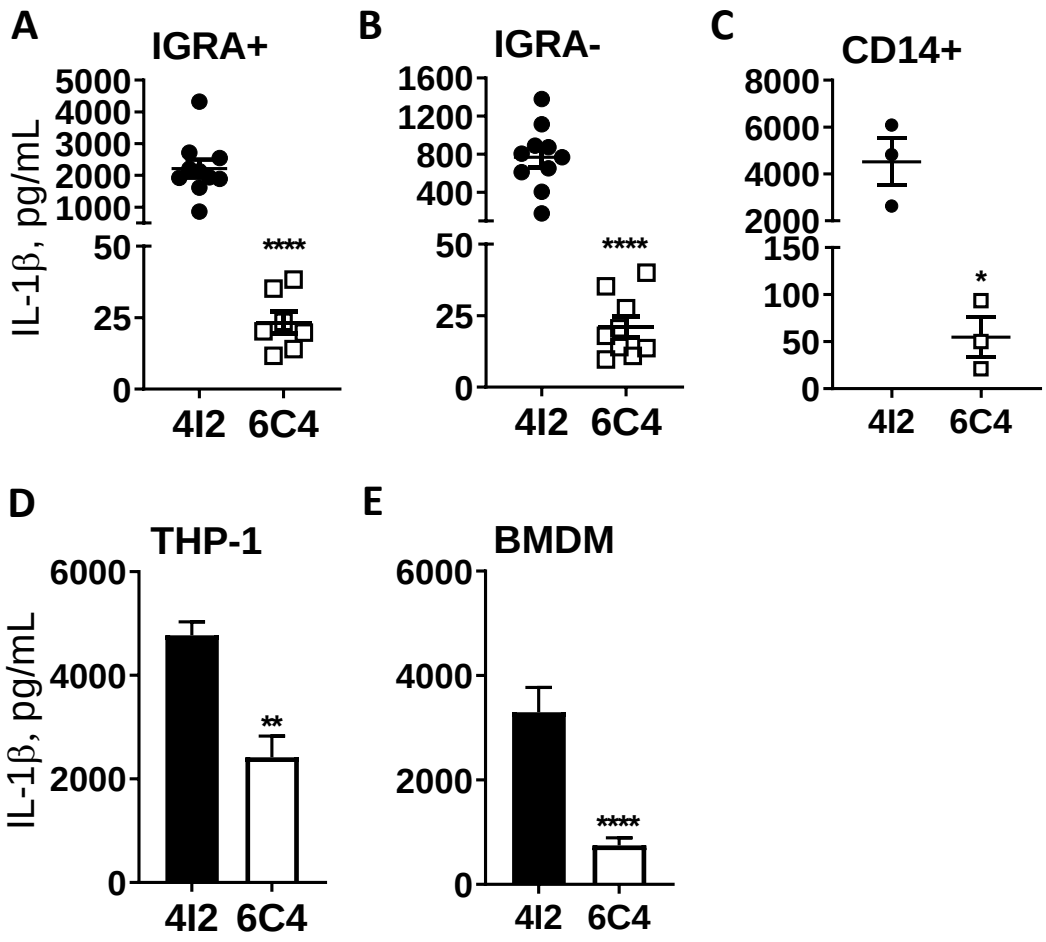
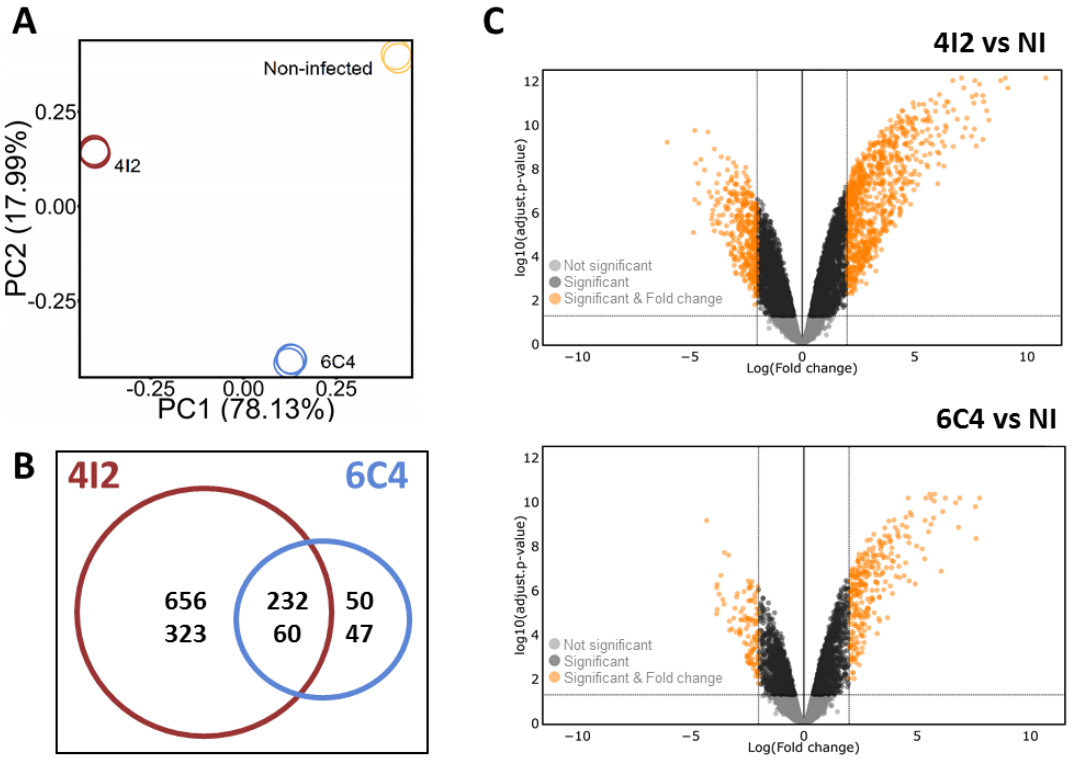
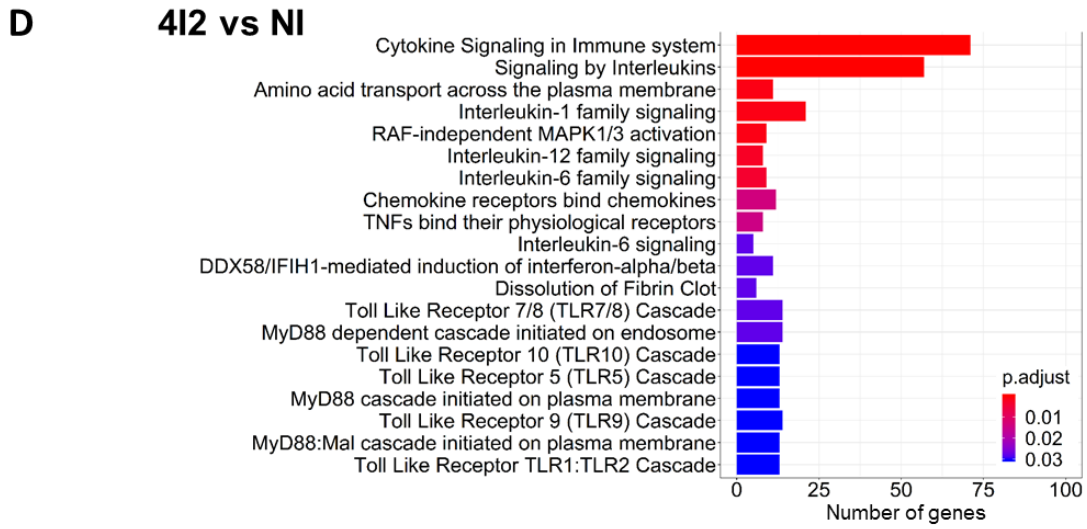


Figure 3 - *In clinico*-selected *M. tuberculosis* isolates associate with distinct IL-1β induction by monocytes or macrophages independently of the host TB status.

M. tuberculosis isolates 4I2 and 6C4 were selected based on the TB presentation, MTBC sublineage and intensity of cytokine induction in infected PBMCs. PBMCs from IGRA+ **(A)** or IGRA- **(B)** donors; CD14+ monocytes purified from IGRA- donors **(C)**; PMA-differentiated THP-1 cells **(D)** or C57BL/6 mouse BMDMs **(E)** were infected with *M. tuberculosis* isolate 4I2 (solid circles or bars) or 6C4 (open squares or bars) for 24h and the amount of secreted IL-1β quantified in the culture supernatants by immunoassay. MOIs of 1 **(A-D)** or 2 **(E)** were used for infection. Represented is the mean ± SEM of 8-10 **(A, B)** and 3 **(C)** donors or of triplicate wells from at least two **(D, E)** independent experiments. Statistical analysis was performed using Student's t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; and ****, $p<0.0001$).


A
Non-infected
4I2
6C4
PC2 (17.99%)
PC1 (78.13%)
0.25
0.00
-0.25
-0.25
0.00
0.25
B
4I2
6C4
656
323
232
60
50
47
C
4I2 vs NI
log10(adjust.p-value)
Log(Fold change)
Not significant
Significant
Significant & Fold change
6C4 vs NI
log10(adjust.p-value)
Log(Fold change)
Not significant
Significant
Significant & Fold change


D
4I2 vs NI
Cytokine Signaling in Immune system
Signaling by Interleukins
Amino acid transport across the plasma membrane
Interleukin-1 family signaling
RAF-independent MAPK1/3 activation
Interleukin-12 family signaling
Interleukin-6 family signaling
Chemokine receptors bind chemokines
TNFs bind their physiological receptors
Interleukin-6 signaling
DDX58/IFIH1-mediated induction of interferon-alpha/beta
Dissolution of Fibrin Clot
Toll Like Receptor 7/8 (TLR7/8) Cascade
MyD88 dependent cascade initiated on endosome
Toll Like Receptor 10 (TLR10) Cascade
Toll Like Receptor 5 (TLR5) Cascade
MyD88 cascade initiated on plasma membrane
Toll Like Receptor 9 (TLR9) Cascade
MyD88:Mal cascade initiated on plasma membrane
Toll Like Receptor TLR1:TLR2 Cascade
p.adjust
0.01
0.02
0.03
0
25
50
75
100
Number of genes

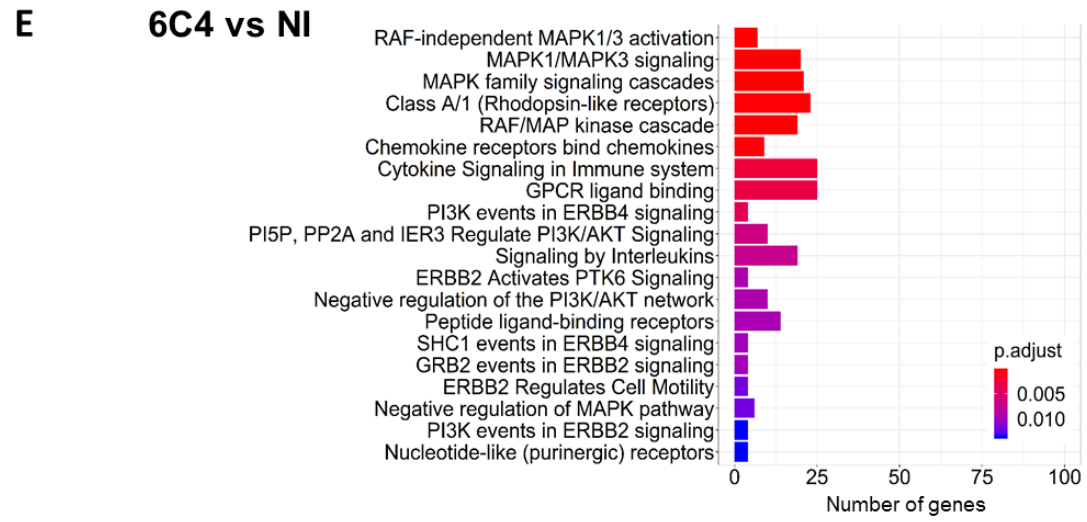

E
6C4 vs NI
RAF-independent MAPK1/3 activation
MAPK1/MAPK3 signaling
MAPK family signaling cascades
Class A/1 (Rhodopsin-like receptors)
RAF/MAP kinase cascade
Chemokine receptors bind chemokines
Cytokine Signaling in Immune system
GPCR ligand binding
PI3K events in ERBB4 signaling
PI5P, PP2A and IER3 Regulate PI3K/AKT Signaling
Signaling by Interleukins
ERBB2 Activates PTK6 Signaling
Negative regulation of the PI3K/AKT network
Peptide ligand-binding receptors
SHC1 events in ERBB4 signaling
GRB2 events in ERBB2 signaling
ERBB2 Regulates Cell Motility
Negative regulation of MAPK pathway
PI3K events in ERBB2 signaling
Nucleotide-like (purinergic) receptors
p.adjust
0.005
0.010
0
25
50
75
100
Number of genes

Figure 4 - Distinct global transcriptional changes are induced in macrophages by *M. tuberculosis* isolates associated with different TB severities.

BMDMs generated from C57BL/6 mice were infected with *M. tuberculosis* isolates 4I2 or 6C4 at a MOI of 2, or kept non-infected (NI). Six hours post-infection cell cultures were lysed, RNA extracted and subjected to targeted RNA-Seq. **(A)** Principal component (PC) analysis for data obtained from *M. tuberculosis* 4I2-infected (red), *M. tuberculosis* 6C4-infected (blue) and non-infected (NI, yellow) cells. Venn diagram **(B)** and volcano plots **(C)** representing the significantly differentially expressed genes (adjusted p-value ≤ 0.05 and log(fold change) ≥ 2 or ≤ -2) when comparing BMDMs infected with *M. tuberculosis* isolate 4I2 or 6C4 with NI cells. In **(C)** significantly differentially expressed genes (NI versus infected cells; adjusted p-value ≤ 0.05 and log(fold change) ≥ 2 or ≤ -2) are shown in orange. Significant genes (adjusted p-value ≤ 0.05 and log(fold change) between 2 and -2) are shown in black. Non-significant genes are displayed in light grey. Pathway analysis for significantly expressed genes detected in BMDMs infected with *M. tuberculosis* isolate 4I2 **(D)** or 6C4 **(E)** versus NI cells. Bar plots represent the number of genes present in each identified pathway and the colour codes reflect adjusted p-values. The 20 most affected pathways are represented for each condition.

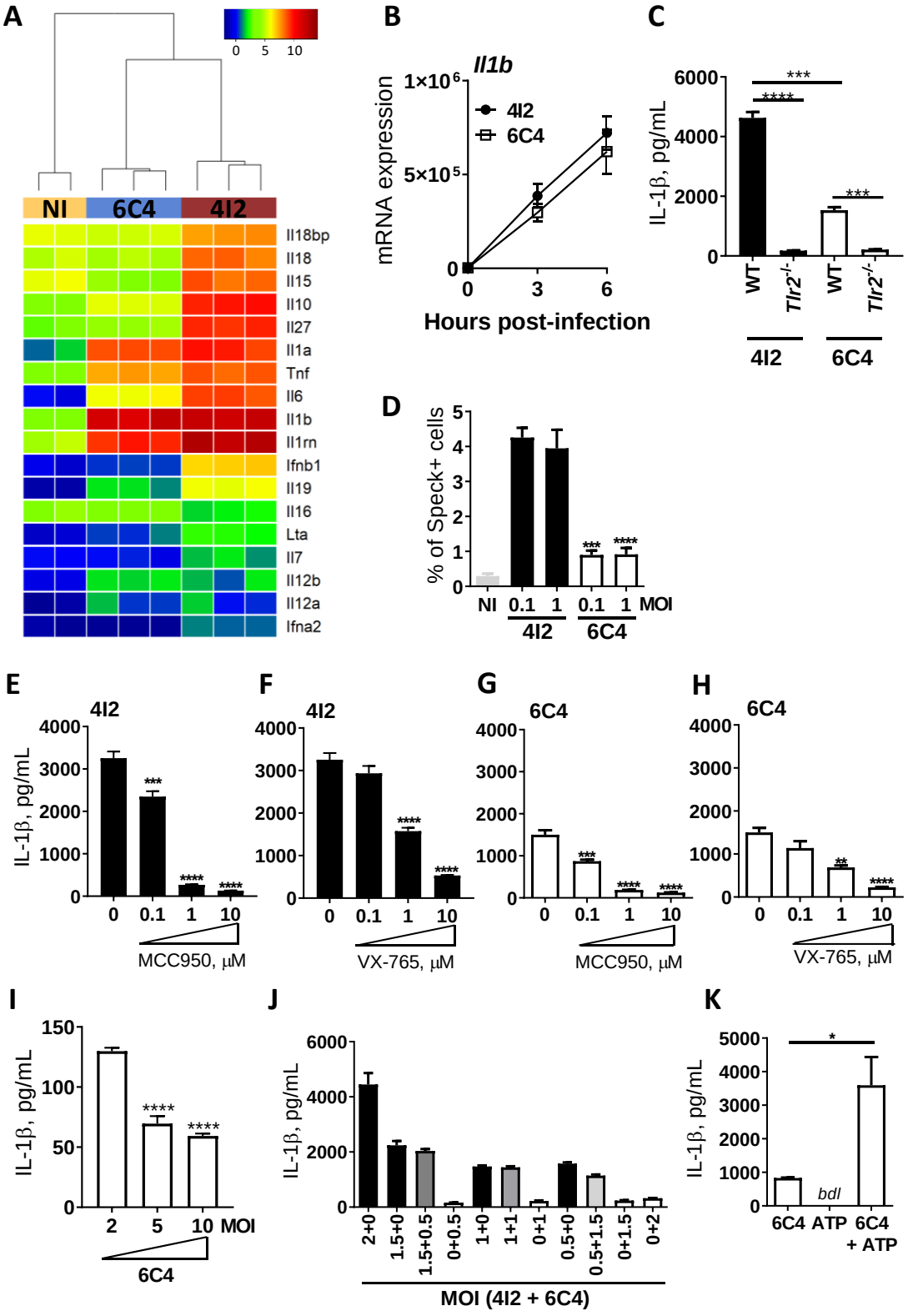

A
0 5 10
NI
6C4
4I2
Il18bp
Il18
Il15
Il10
Il27
Il1a
Tnf
Il6
Il1b
Il1rn
Ifnb1
Il19
Il16
Lta
Il7
Il12b
Il12a
Ifna2
B
Il1b
mRNA expression
1×10^6
5×10^5
0
4I2
6C4
0 3 6
Hours post-infection
C
IL-1β, pg/mL
6000
4000
2000
0
***
****
***
WT
Tlr2-/-
WT
Tlr2-/-
4I2
6C4
D
% of Speck+ cells
5 4 3 2 1 0
***
****
NI 0.1 1 0.1 1 MOI
4I2
6C4
E
4I2
IL-1β, pg/mL
4000 3000 2000 1000 0
***
****
****
0 0.1 1 10
MCC950, μM
F
4I2
4000 3000 2000 1000 0
****
****
0 0.1 1 10
VX-765, μM
G
6C4
4000 3000 2000 1000 0
***
****
****
0 0.1 1 10
MCC950, μM
H
6C4
4000 3000 2000 1000 0
**
****
0 0.1 1 10
VX-765, μM
I
IL-1β, pg/mL
150 100 50 0
****
****
2 5 10 MOI
6C4
J
IL-1β, pg/mL
6000 4000 2000 0
2+0
1.5+0
1.5+0.5
0+0.5
1+0
1+1
0+1
0.5+0
0.5+1.5
0+1.5
0+2
MOI (4I2 + 6C4)
K
IL-1β, pg/mL
5000 4000 3000 2000 1000 0
*
bdl
6C4
ATP
6C4 + ATP

Figure 5 - Enhanced activation of the inflammasome by macrophages infected with *M. tuberculosis* 4I2 isolate underlies increased IL-1β secretion.

(A) Gene expression heatmap representing cytokine expression for non-infected BMDMs (NI, yellow) and BMDMs infected with *M. tuberculosis* isolate 4I2 (dark red) or *M. tuberculosis* isolate 6C4 (blue). Gene expression levels are colour coded below the heatmap. **(B)** C57BL/6 WT BMDMs were generated and infected with *M. tuberculosis* isolates 4I2 (solid circles) or 6C4 (open squares), and at the indicated time points, the cell cultures were lysed, RNA extracted, converted to cDNA and subjected to real-time PCR. **(C)** C57BL/6 WT or *Tlr2*-deficient (-/-) BMDMs were generated and infected with *M. tuberculosis* isolates 4I2 (solid bars) or 6C4 (open bars). Twenty-four hours post-infection the culture supernatants were harvested and the amount of IL-1β quantified by ELISA. **(D)** THP-1-ASC-GFP monocytes were PMA-differentiated and left uninfected (NI) or infected with *M. tuberculosis* isolates 4I2 (solid bars) or 6C4 (open bars) at different MOI, as indicated. Six hours post-infection, cells were fixed and stained with DAPI prior to image acquisition. The percentage of Speck positive cells was determined for the different conditions. C57BL/6 WT BMDMs were generated and infected with *M. tuberculosis* isolates 4I2 **(E, F)** or 6C4 **(G, H)** in the absence (0) or presence of increasing doses of NLRP3 (MCC950; **E, G**) or Caspase 1 (VX-765; **F, H**) inhibitors. BMDMs were infected with increasing MOI of *M. tuberculosis* isolate 6C4 **(I),** with a combination of both isolates at different doses **(J)** or with *M. tuberculosis* isolate 6C4 in the absence (6C4) or presence (6C4+ATP) of 5mM of ATP **(K)**. **(E-K)** Twenty-four h post-infection the culture supernatants were harvested and the amount of IL-1β quantified by ELISA. Unless indicated, an MOI of 2 was used for infections. Represented is the mean ± SEM of triplicate wells of at least two independent experiments. Statistical analysis was performed using Student's t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; and ****, $p<0.0001$).

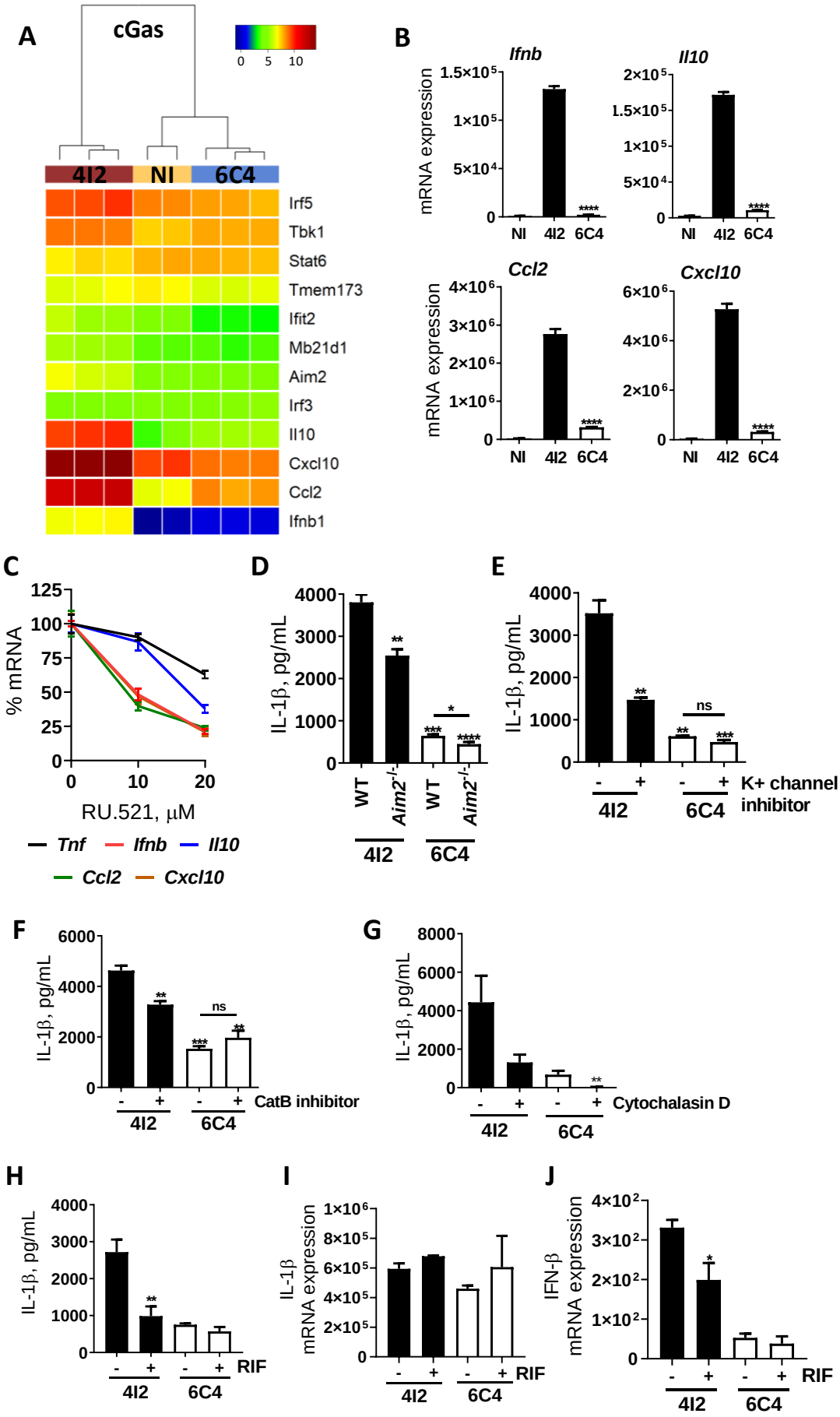
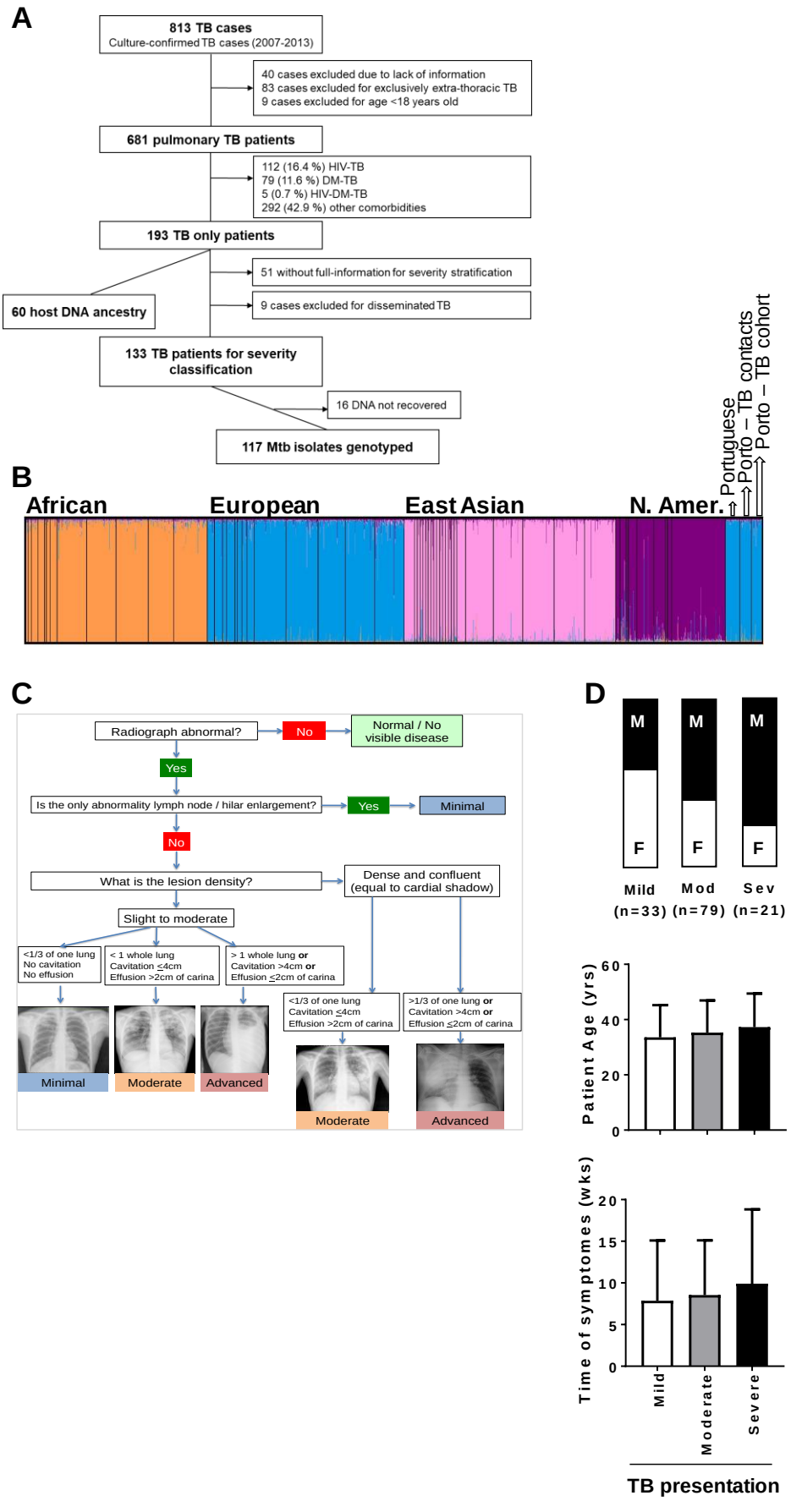

A
cGas
0 5 10
4I2
NI
6C4
Irf5
Tbk1
Stat6
Tmem173
Ifit2
Mb21d1
Aim2
Irf3
Il10
Cxcl10
Ccl2
Ifnb1
B
Ifnb
Il10
Ccl2
Cxcl10
mRNA expression
NI 4I2 6C4
C
% mRNA
RU.521, µM
Tnf
Ifnb
Il10
Ccl2
Cxcl10
D
IL-1β, pg/mL
WT
Aim2-/-
4I2
6C4
E
IL-1β, pg/mL
ns
K+ channel inhibitor
F
IL-1β, pg/mL
CatB inhibitor
G
IL-1β, pg/mL
Cytochalasin D
H
IL-1β, pg/mL
RIF
I
IL-1β mRNA expression
RIF
J
IFN-β mRNA expression
RIF

Figure 6 - *M. tuberculosis* isolate 6C4 evades the macrophage cytosolic surveillance pathways.

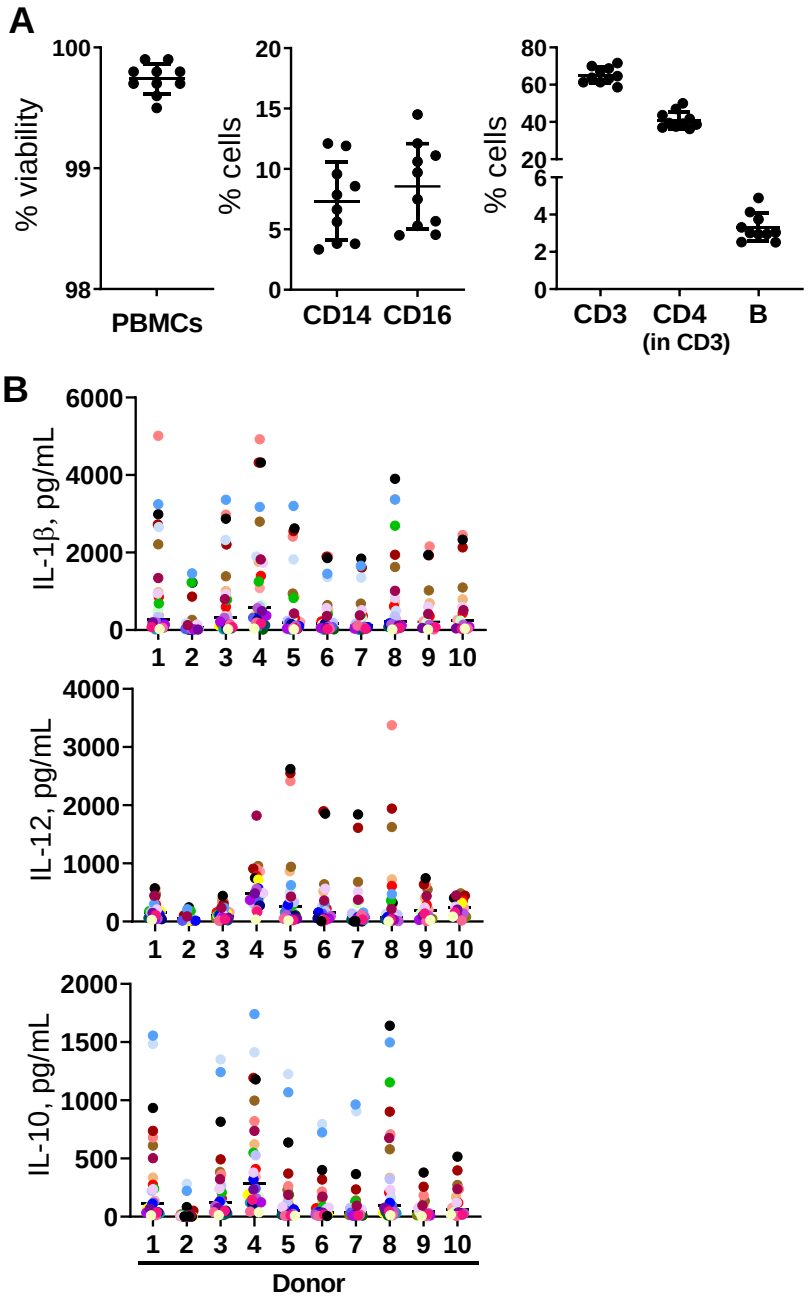
(A) Heatmap representing the expression of genes associated with the cGAS pathway in non-infected BMDMs (NI; yellow) and BMDMs infected with *M. tuberculosis* isolate 4I2 (dark red) or M. *tuberculosis* isolate 6C4 (blue). Gene expression levels are colour coded below the heatmap. C57BL/6 WT BMDMs were generated and infected with **(B)** *M. tuberculosis* isolates 4I2 (solid bars) or 6C4 (open bars) or **(C)** with *M. tuberculosis* isolate 4I2 in the absence (0) or presence of different doses of the cGAS inhibitor RU521. Six hours post-infection, the cell cultures were lysed, RNA extracted, converted to cDNA and subjected to real-time PCR to quantify the expression of the indicated genes. **(D)** C57BL/6 WT or *Aim2*-deficient (-/-) BMDMs were generated and infected with *M. tuberculosis* isolates 4I2 (solid bars) or 6C4 (open bars). Infection of C57BL/6 WT BMDMs in the absence (-) or presence (+) of **(E)** a K^+ (potassium) channel inhibitor (glybenclamide; 25µg/mL), **(F)** a cathepsin B (CatB) inhibitor (ZRLR; 10µM) or **(G)** a phagocytosis inhibitor (cytochalasin D; 5µM). (H-J) C57BL/6 WT BMDMs were generated and infected with live or rifampicin (RIF)-treated *M. tuberculosis* isolates 4I2 (solid bars) or 6C4 (open bars). **(D-H)** Twenty-four h post-infection the culture supernatants were harvested and the amount of IL-1β quantified by ELISA. **(I, J)** Six hours post-infection the cell cultures were lysed, RNA extracted, converted to cDNA and subjected to real-time PCR to quantify the expression of the indicated genes. Represented is the mean ± SEM of triplicate wells of at least two independent experiments **(B, D-J)**. **(C)** Represented is the % of inhibition for the expression of each gene in BMDMs infected with *M. tuberculosis* isolate 4I2 in the presence of RU.521 relatively to those infected in the absence of the cGAS inhibitor. An MOI of 2 was used for all infections. Statistical analysis was performed using Student's t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; and ****, $p<0.0001$).

Supplemental information



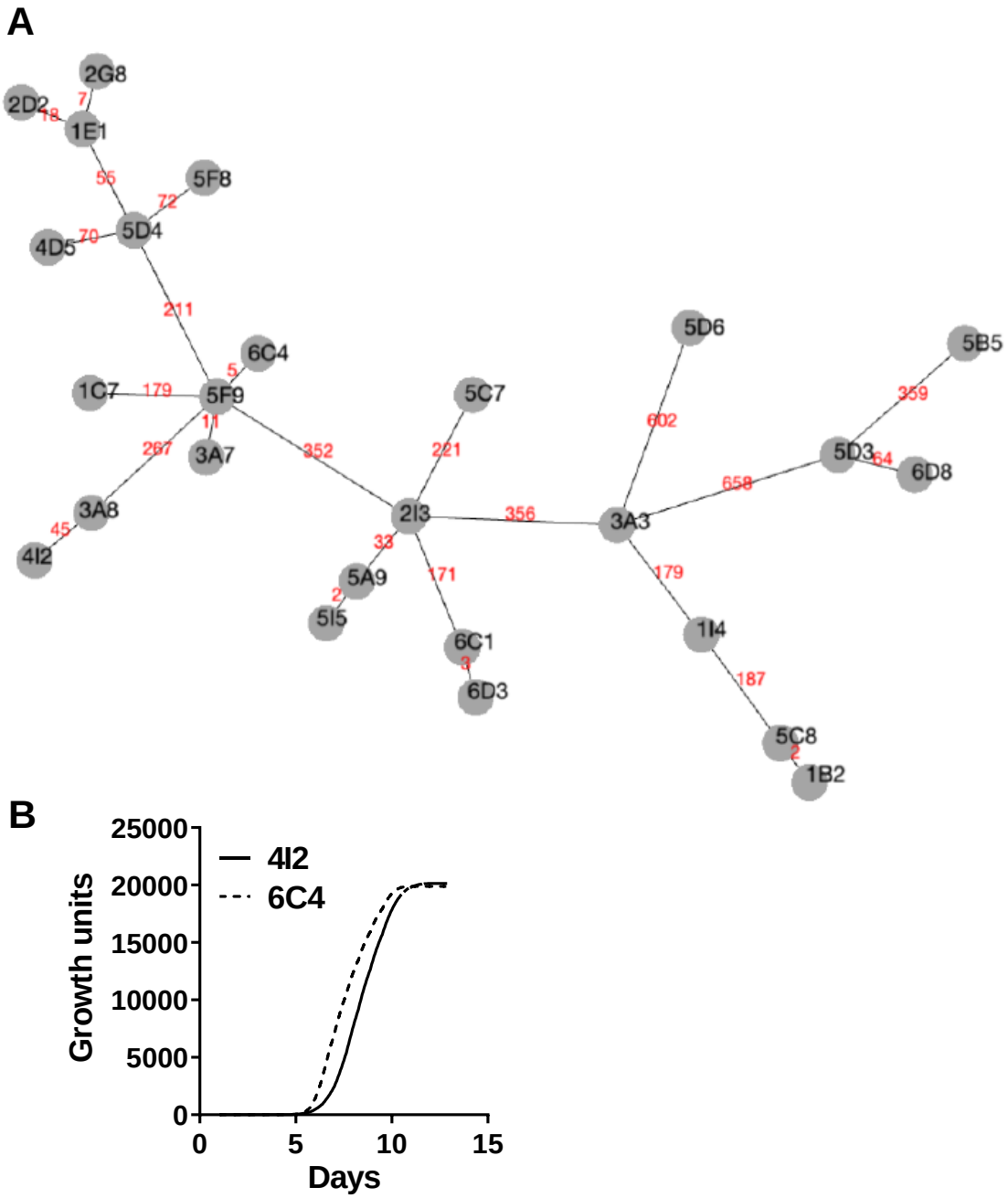
Supplemental Figure 1 (related to Figure 1) - Overview of the study population and characteristics of the selected TB patients.

(A) Overview of the study population included in the different phases of the work. **(B)** Genetic makeup through a validated panel of autosomal ancestry-informative markers (AIMs) of reference populations of African, European, East Asian and Native American biogeographical origin. Populations, from left to right: Africans (Bantu South Africa, Bantu Kenya, Mandenka, Yoruba, San, Mbuti Pygmy, Biaka Pygmy, LWK, ESN, YRI, MSL and GWD); Europeans (Basque, French, Italian, Sardinian, Tuscan, Orcadian, Adygei, Russian, TSI, IBS, GBR, CEU and FIN); East Asians (Han, Han-NChina, Dai, Daur, Hezhen, Lahu, Miao, Oroqen, She, Tujia, Tu, Xibo, Yi, Mongola, Naxi, Cambodian, Japanese, CDX, KHV, CHS, CHB and JPT); Native Americans (Pima, Maya, Colombian, Guainía, Motilón-Bari, Emberá-Chamí, Surui, Karitiana, Asháninka; Portuguese, Portuguese controls and TB patients (this study). **(C)** Schematic representation of the chest X-ray classification method based on lymph node/ hilar abnormality and lesion density. **(D)** Distribution of gender (male, M; female, F), age and time of symptoms in mild, moderate and severe TB groups.



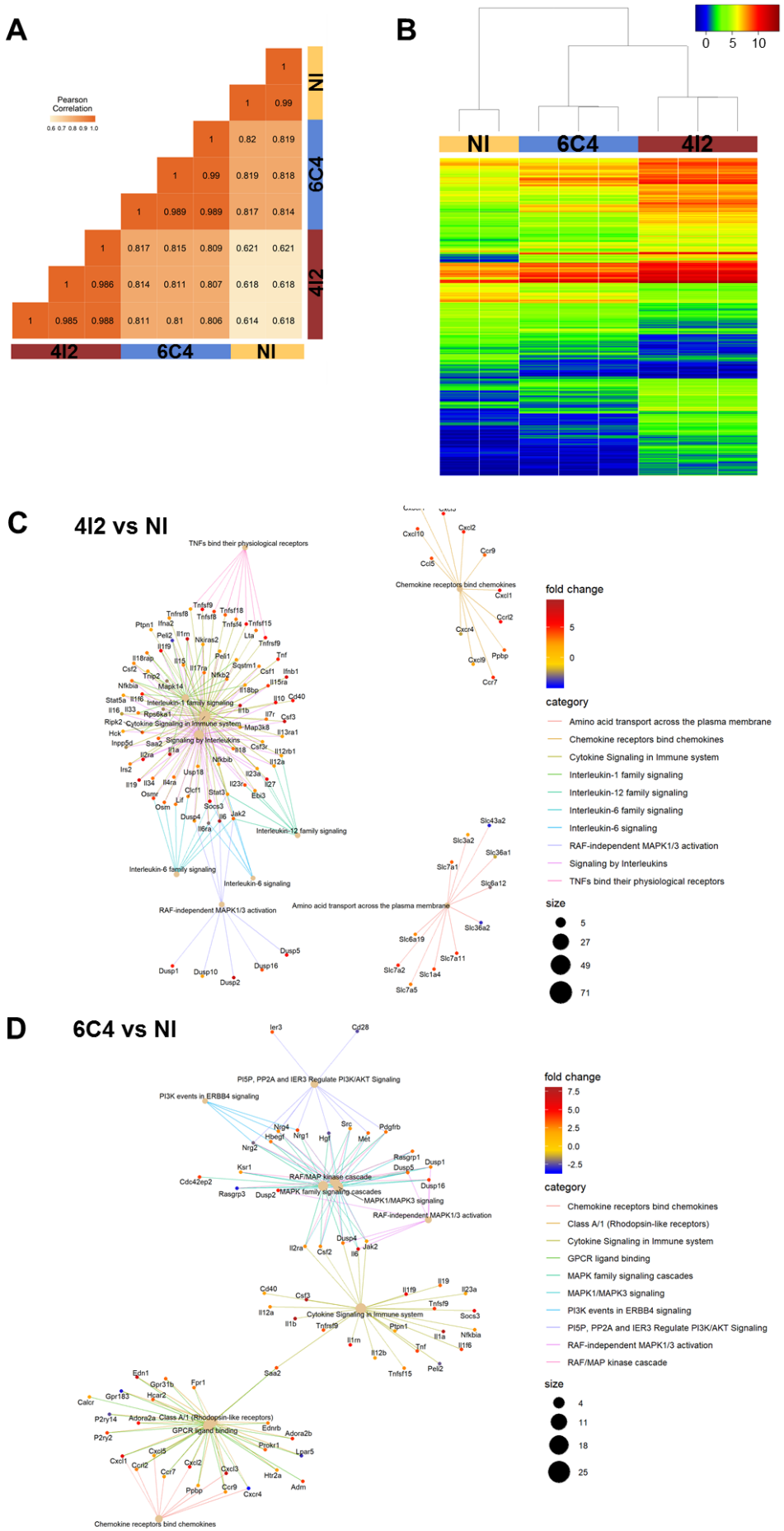
Supplemental Figure 2 (related to Figure 1) - Highly related *M. tuberculosis* isolates modulate cytokine production by infected PBMCs.

(A) Viability and cellular composition of the PBMCs isolated from IGRA+ donors and used for the initial infections with 26 clinical *M. tuberculosis* isolates associated with different TB severities. Represented are the mean ± SEM for 10 donors; each dot represents one donor. **(B)** Cytokine production by PBMCs infected for 24h with 26 clinical *M. tuberculosis* isolates associated with different TB severities. Each number represents one donor and each dot one isolate. The same isolate is represented with the same colour across the different donors. An MOI of 1 was used for infection.



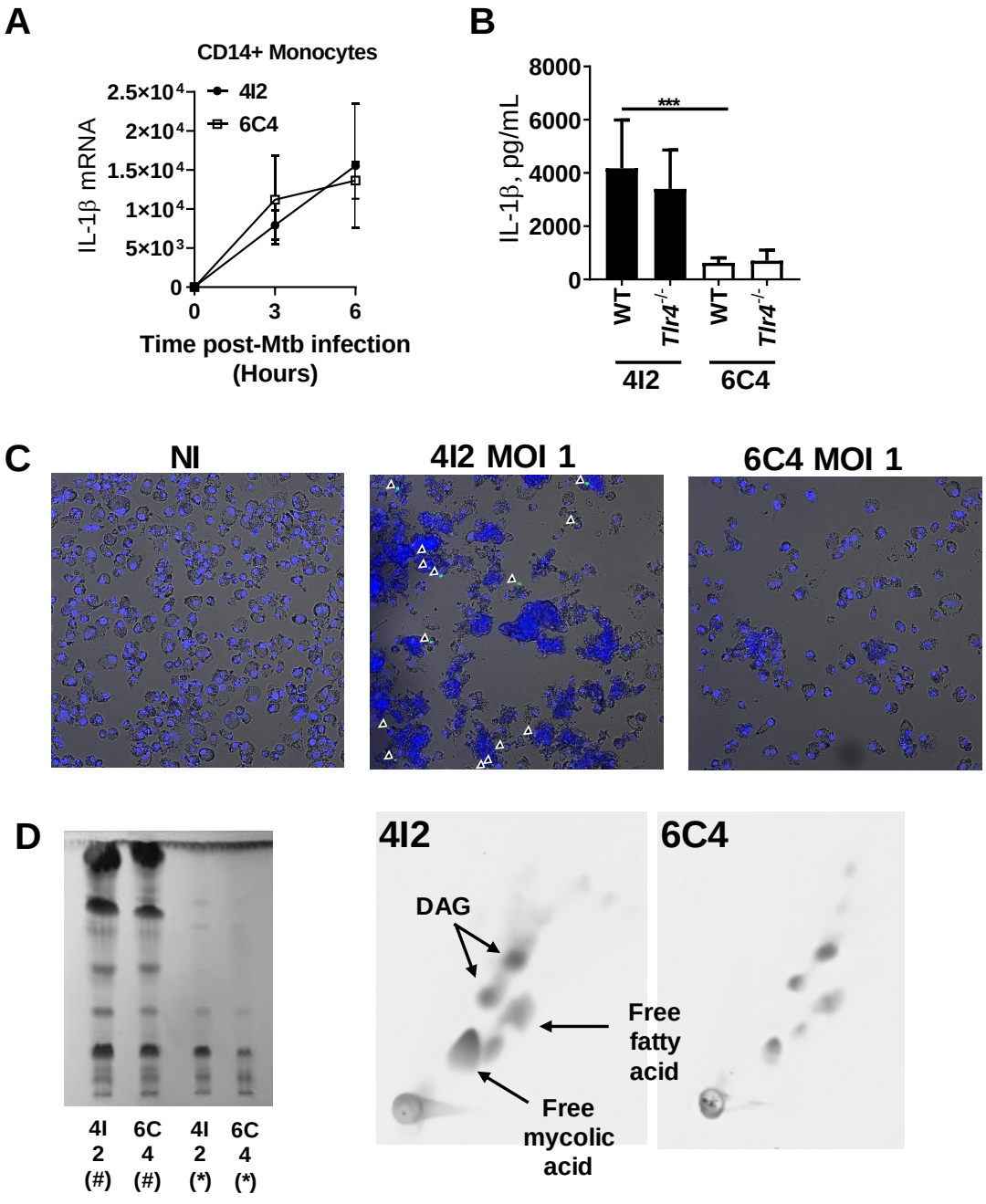
Supplemental Figure 3 (related to Figure 2) - Five possible transmission clusters identified among the selected isolates. Growth profiles for the selected *M. tuberculosis* isolates 4I2 and 6C4.

(A) Minimum Spanning Tree used for visualizing the possible evolutionary relationships and number of pairwise SNPs (in red) between the studied clinical isolates. Clinical isolates separated by less than 12 SNPs were considered part of transmission clusters or pairs. **(B)** *M. tuberculosis* isolates 4I2 (solid line) and 6C4 (dashed line) were grown from standardized inocula in a Bactec MGIT 960 (Gehre et al. 2013). The growth units are plotted versus time (days). Time to positivity was determined as the time taken to detect 75 growth units and the doubling time calculated during the exponential phase of growth.


A
Pearson Correlation
0.6 0.7 0.8 0.9 1.0
NI
6C4
4I2
B
0 5 10
NI
6C4
4I2
C
4I2 vs NI
TNFs bind their physiological receptors
Chemokine receptors bind chemokines
Cytokine Signaling in Immune system
Signaling by Interleukins
Interleukin-1 family signaling
Interleukin-12 family signaling
Interleukin-6 family signaling
Interleukin-6 signaling
RAF-independent MAPK1/3 activation
Amino acid transport across the plasma membrane
fold change
5
0
category
Amino acid transport across the plasma membrane
Chemokine receptors bind chemokines
Cytokine Signaling in Immune system
Interleukin-1 family signaling
Interleukin-12 family signaling
Interleukin-6 family signaling
Interleukin-6 signaling
RAF-independent MAPK1/3 activation
Signaling by Interleukins
TNFs bind their physiological receptors
size
5
27
49
71
D
6C4 vs NI
PI5P, PP2A and IER3 Regulate PI3K/AKT Signaling
PI3K events in ERBB4 signaling
RAF/MAP kinase cascade
MAPK family signaling cascades
MAPK1/MAPK3 signaling
RAF-independent MAPK1/3 activation
Cytokine Signaling in Immune system
GPCR ligand binding
Class A/1 (Rhodopsin-like receptors)
Chemokine receptors bind chemokines
fold change
7.5
5.0
2.5
0.0
-2.5
category
Chemokine receptors bind chemokines
Class A/1 (Rhodopsin-like receptors)
Cytokine Signaling in Immune system
GPCR ligand binding
MAPK family signaling cascades
MAPK1/MAPK3 signaling
PI3K events in ERBB4 signaling
PI5P, PP2A and IER3 Regulate PI3K/AKT Signaling
RAF-independent MAPK1/3 activation
RAF/MAP kinase cascade
size
4
11
18
25

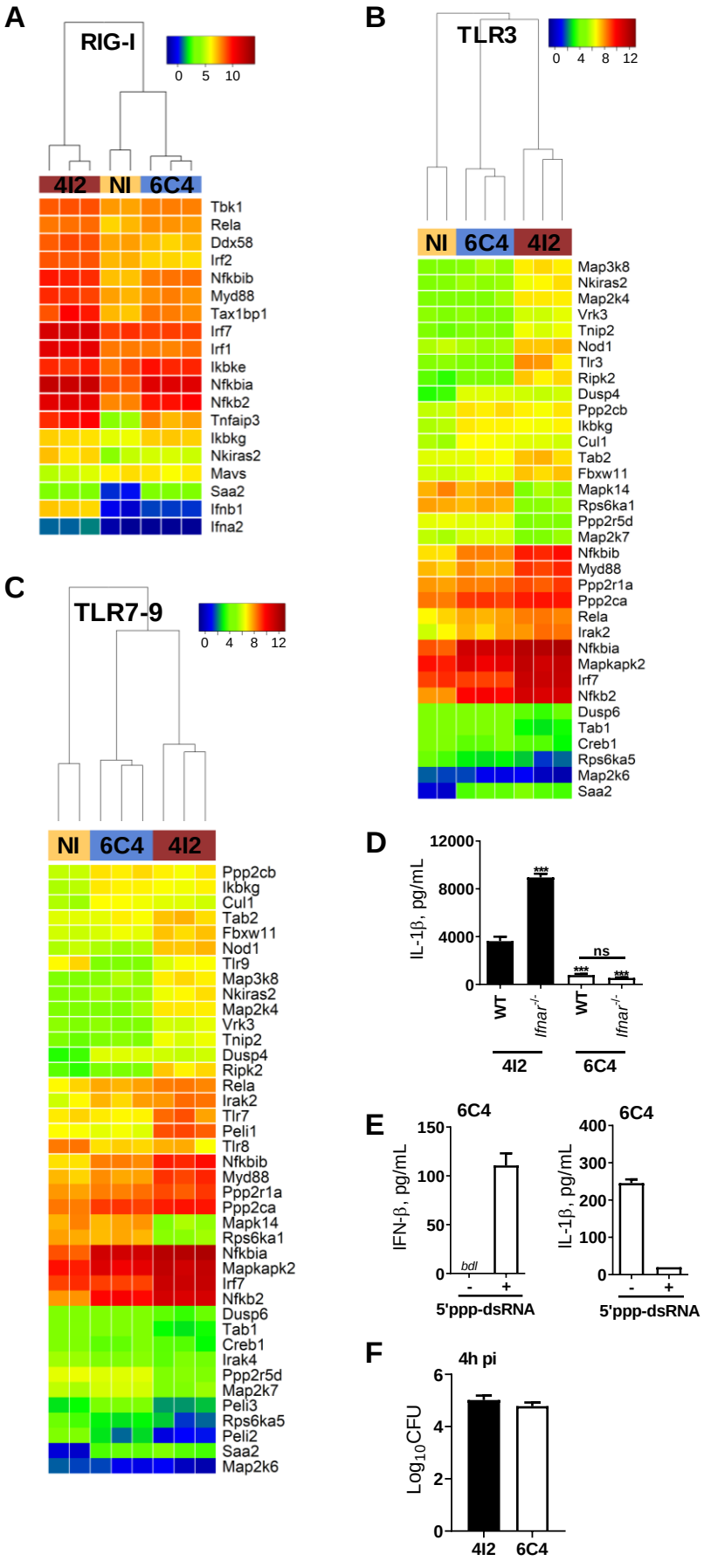
Supplemental Figure 4 (related to Figure 4) - Global transcription analysis of BMDMs infected with *M. tuberculosis* isolates 4I2 or 6C4.

(A) Correlation heatmap generated from gene expression data for each of the non-infected BMDMs, BMDMs infected with *M. tuberculosis* isolate 4I2, and BMDMs infected with *M. tuberculosis* isolate 6C4 replicates. Pearson correlation coefficient is colour coded and indicated on each cell. **(B)** Heatmap representing unsupervised hierarchical clustering of sample distribution and expression levels of differentially expressed genes (adjusted p-value ≤0.05, up- (≥2) or down-regulated (≤-2) log fold). In total, 1366 genes were found to be significantly differentially regulated in one or both conditions: BMDMs infected with *M. tuberculosis* isolate 4I2 (dark red) or *M. tuberculosis* isolate 6C4 (blue) versus non-infected BMDMs (NI, yellow). **(C, D)** Plots representing the ten pathways most affected in BMDMs infected with *M. tuberculosis* isolates 4I2 **(C)** or 6C4 **(D)**. Each pathway is represented by a beige node, connected to each gene associated with it by coloured lines. Node size is associated with number of genes significantly expressed in the pathway. The legend indicates the colour codes for each pathway/gene set. Fold change is used as a measure for significance in the altered genes, and it is colour coded in each gene node. An MOI of 2 was used for infection.

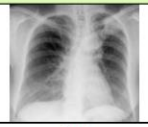
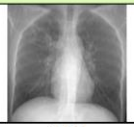
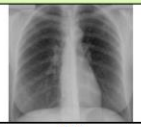
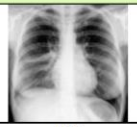
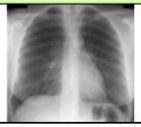
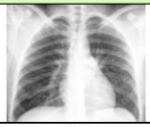
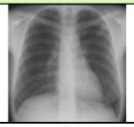
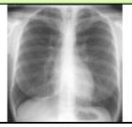
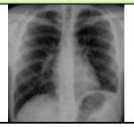
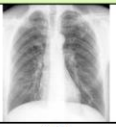
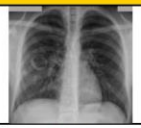
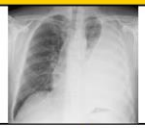
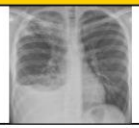
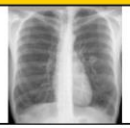
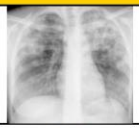
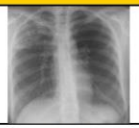
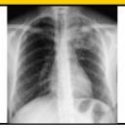
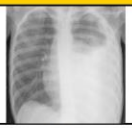
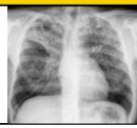
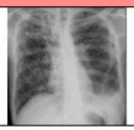
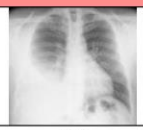
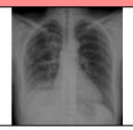
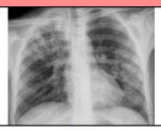
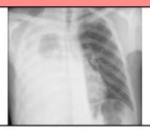
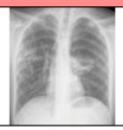

A
CD14+ Monocytes
IL-1β mRNA
2.5×10⁴
2×10⁴
1.5×10⁴
1×10⁴
5×10³
0
4I2
6C4
0
3
6
Time post-Mtb infection
(Hours)
B
IL-1β, pg/mL
8000
6000
4000
2000
0
***
WT
Tlr4-/-
WT
Tlr4-/-
4I2
6C4
C
NI
4I2 MOI 1
6C4 MOI 1
D
4I 2 (#)
6C 4 (#)
4I 2 (*)
6C 4 (*)
4I2
6C4
DAG
Free fatty acid
Free mycolic acid
E
TNF, pg/mL
1000
800
600
400
200
0
***
***
2
5
10
MOI
6C4
F
IL-1β, pg/mL
3000
2000
1000
0
****
****
4I2
6C4
4I2
NI
+ SN 6C4
G
Inflammasome
2
6
10
NI
6C4
4I2
Pycard
Aim2
Casp1
Nlrc4
Nlrp1a
Nlrp1b
Nlrp3

Supplemental Figure 5 (related to Figure 5)

(A) CD14+ monocytes enriched from PBMCs of 5 donors using MACS beads, were infected with *M. tuberculosis* isolates 4I2 (solid circles) or 6C4 (opened squares) at a MOI of 1. At the indicated time points, the cell cultures were lysed, RNA extracted, converted to cDNA and subjected to real-time PCR. C57BL/6 WT **(B, E, F, G)** and *Tlr4*-deficient (-/-) **(B)** BMDMs were generated and infected with *M. tuberculosis* isolates 4I2 or 6C4 as indicated. In **(E)** different MOI of bacteria were used, and in **(F)** the infection with *M. tuberculosis* isolate 4I2 was combined with conditioned supernatant from BMDM cultures infected with *M. tuberculosis* isolate 6C4 for 24h. **(B, E, F)** Twenty-four h post-infection the culture supernatants were harvested and the indicated cytokines measured by ELISA. **(C)** THP-1-ASC-GFP cells were PMA-differentiated and infected with either *M. tuberculosis* isolate as indicated. Speck positive cells (white arrows) were identified and the percentage of Speck positive cells determined for the different conditions. **(D)** TLC of apolar (#) or polar (*) cell wall lipidic extracts obtained from *M. tuberculosis* isolates 4I2 and 6C4 were run in a system consisting of chloroform/methanol/water (60:16:2) (Left). Free and apolar cell wall lipidic extracts were run in a 2D system B (direction 1: Petroleum ether 60-80/ acetone (92:8) – 3 runs; direction 2: Toluene/acetone (95:5) – 1 run) (Right). **(G)** Heatmap representing the expression of genes associated with the inflammasome pathway in non-infected BMDMs (NI; yellow) and BMDMs infected with *M. tuberculosis* isolate 4I2 (dark red) or *M. tuberculosis* isolate 6C4 (blue) for 6h. Gene expression levels are color coded below the heatmap. A MOI of 2 **(A, B, F)** or of 1 **(C)** was used for infection. **(A, B, E, F)** Represented are the mean ± SEM for different donors **(A)** or for triplicate wells from at least 2 independent experiments **(B, E, F)**. Statistical analysis was performed using Student's t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; and ****, $p<0.0001$).


A
RIG-I
0 5 10
4I2 NI 6C4
Tbk1
Rela
Ddx58
Irf2
Nfkbib
Myd88
Tax1bp1
Irf7
Irf1
Ikbke
Nfkbia
Nfkb2
Tnfaip3
Ikbkg
Nkiras2
Mavs
Saa2
Ifnb1
Ifna2
B
TLR3
0 4 8 12
NI 6C4 4I2
Map3k8
Nkiras2
Map2k4
Vrk3
Tnip2
Nod1
Tlr3
Ripk2
Dusp4
Ppp2cb
Ikbkg
Cul1
Tab2
Fbxw11
Mapk14
Rps6ka1
Ppp2r5d
Map2k7
Nfkbib
Myd88
Ppp2r1a
Ppp2ca
Rela
Irak2
Nfkbia
Mapkapk2
Irf7
Nfkb2
Dusp6
Tab1
Creb1
Rps6ka5
Map2k6
Saa2
C
TLR7-9
0 4 8 12
NI 6C4 4I2
Ppp2cb
Ikbkg
Cul1
Tab2
Fbxw11
Nod1
Tlr9
Map3k8
Nkiras2
Map2k4
Vrk3
Tnip2
Dusp4
Ripk2
Rela
Irak2
Tlr7
Peli1
Tlr8
Nfkbib
Myd88
Ppp2r1a
Ppp2ca
Mapk14
Rps6ka1
Nfkbia
Mapkapk2
Irf7
Nfkb2
Dusp6
Tab1
Creb1
Irak4
Ppp2r5d
Map2k7
Peli3
Rps6ka5
Peli2
Saa2
Map2k6
D
IL-1β, pg/mL
12000
8000
4000
0
***
***
***
ns
WT
Ifnar-/-
WT
Ifnar-/-
4I2
6C4
E
6C4
IFN-β, pg/mL
150
100
50
0
bdl
- +
5'ppp-dsRNA
6C4
IL-1β, pg/mL
400
300
200
100
0
- +
5'ppp-dsRNA
F
4h pi
Log10CFU
6
4
2
0
4I2
6C4

Supplemental Figure 6 (related to Figure 6) - *M. tuberculosis* isolate 4I2 leads to an overall higher expression of genes in the RIG-I and TLR3/7/8/9. IL-1β secretion by BMDM infected with *M. tuberculosis* isolate 4I2 is regulated by IFN-β.
IL-1β secretion by BMDMs infected with *M. tuberculosis* isolate 4I2 is regulated by IFN-β. Heatmaps representing the expression of genes associated with the RIG-1 **(A),** TLR3 (B), TLR7, TLR8 and TLR9 **(C)** pathway in non-infected BMDMs (NI; yellow) and BMDMs infected with *M. tuberculosis* isolate 4I2 (dark red) or *M. tuberculosis* isolate 6C4 (blue) for 6h. Gene expression levels are colour coded below the heatmap. C57BL/6 WT **(D, E, F)** and *Ifnar* deficient (-/-) **(D)** BMDMs were generated and infected with *M. tuberculosis* isolates 4I2 or 6C4 as indicated. In **(E)** the infection with *M. tuberculosis* isolate 6C4 was combined with 1µg/mL of the RIG-I agonist 5'ppp-dsRNA mixed with a transfection agent. **(D, E)** Twenty-four h post-infection the culture supernatants were harvested and the indicated cytokines measured by ELISA. **(F)** Four h post-infection, the cell cultures were extensively washed with PBS, the cells lysed and the intracellular bacteria quantified by CFU enumeration. A MOI of 2 was used for infection. Represented are the mean ± SEM for triplicate wells from at least 2 independent experiments. Statistical analysis was performed using Student's t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; and ****, $p<0.0001$).

MILD										
Chest X-ray										
Code	**6D8**	**3A3**	**1I4**	**6D3**	**2I3**	**5A9**	**4I2**	**5F8**	**5D4**	**2D2**
Sublineage	L4/X	L4/LAM	L4/LAM	L4/LAM	L4/LAM	L4/LAM	L4/LAM	L4/LAM	L4/LAM	L4/LAM
Gender	Female	Female	Female	Female	Female	Male	Female	Female	Male	Male
Age (years)	44	35	39	30	39	25	34	26	28	45
Time sympt. (w)	10	2	10	24	12	5	1	8	26	13
Clinical features	Moderate lesions, low sympt., no anemia, low CRP	Minimal lesion, low sympt., no anemia, medium CRP	Minimal lesions, low sympt., no anemia, low CRP	Moderate lesions, low sympt., no anemia, low CRP	Minimal lesions, low sympt., no anemia, low CRP	Minimal lesions, low sympt., no anemia, low CRP	Moderate lesions, low sympt., no anemia, low CRP	Minimal lesions, low sympt., no anemia, low CRP	Minimal lesion, low sympt., no anemia, medium CRP	Minimal lesions, low sympt., no anemia, low CRP
MODERATE										
Chest X-ray										
Code	**5D3**	**1B2**	**5I5**	**3A8**	**1C7**	**3A7**	**5F9**	**1E1**	**2G8**	
Sublineage	L4/X	L4/LAM	L4/LAM	L4/LAM	L4/LAM	L4/LAM	L4/LAM	L4/LAM	L4/LAM	
Gender	Male	Male	Male	Female	Female	Female	Female	Female	Male	
Age (years)	22	35	22	31	20	28	50	27	25	
Time sympt. (w)	15	3	13	9	17	6	11	2	4	
Clinical features	Mod. lesions, low sympt., no anemia, medium CRP	Advanced lesions, no respir. failure, low sympt.	Moderate lesions, low sympt., no anemia, high CRP	Moderate lesions, low sympt., anemic, low CRP	Moderate lesions, low sympt., anemic, high CRP	Moderate lesions, low sympt., anemic, low CRP	Advanced lesions, no respir. failure, low sympt.	Advanced lesions, no respir. failure, low sympt.	Advanced lesions, no respir. failure, low sympt.	
SEVERE										
Chest X-ray										
Code	**5B5**	**5D6**	**5C8**	**5C7**	**6C1**	**6C4**	**4D5**			
Sublineage	L4/X	L4/other L4	L4/LAM	L4/LAM	L4/LAM	L4/LAM	L4/other L4			
Gender	Male	Male	Male	Female	Male	Male	Male			
Age (years)	54	43	42	49	19	20	19			
Time sympt. (w)	3	10	1	10	7	3	9			
Clinical features	Advanced lesions, no respir. failure, high sympt. / CRP	Advanced lesions, no respir. failure, high sympt. / CRP, anemic	Advanced lesions, no respir. failure, high sympt. / CRP	Advanced lesions, no respir. failure, high sympt. / CRP, anemic	Advanced lesions, no respir. failure, high sympt. / CRP, anemic	Advanced lesions, no respir. failure, high sympt. / CRP	Advanced lesions, no respir. failure, high sympt. / CRP			

Supplemental Table 1 - General characteristics and clinical features associated with selected *M. tuberculosis* isolates.

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Chapter IV – Final Discussion

The immune response to *M. tuberculosis* infection is a highly regulated process. The capacity of the immune cells to control and eliminate the pathogen relies on an equilibrium between pro- and anti-inflammatory responses dictated by cell interactions and molecular signaling pathways. The data presented here highlight the fact that *M. tuberculosis* strain diversity plays a key role in regulating the immune response, by differently activating and modulating the host cell response to infection. The individual characteristics of different *M. tuberculosis* strains opens new and exciting questions on the main players in TB immunity, namely the bacterial influence on the host and immune response.

Using a clinical isolate of *M. tuberculosis* that induces high production of IFN-β in macrophages, we demonstrate in Chapter III, section I, that the production of type I IFN, at specific time and context, can be protective in TB. This adds to the complex biology of type I IFN in TB, showing that a dual role for this cytokine is in place. Understanding the protective role for type I IFN in TB may have translational implications, for example in the context of defective or decreased IFN-γ responses. This is the case of individuals presenting mutations for the IFN-γR, or of HIV-positive patients who present a decrease of IFN-γ-producing $CD4^+$ T cells. Thus, the new data herein disclosed contribute to a better understanding of the role of type I IFN in TB, as well as to the future development of host-directed therapies in TB based on administration of recombinant cytokine to the specific risk groups above mentioned.

During many years, the role of *M. tuberculosis* diversity in regulating the host immune response has been underestimated. Now, our results presented in Chapter III, section II, show how different strains of *M. tuberculosis* modulate the immune response in favour of a specific outcome of disease. Our findings demonstrate that *M. tuberculosis* isolated from patients with mild disease are strong inducers of the immune response, through high production of cytokines and activation of cytosolic surveillance systems in macrophages. Meanwhile, *M. tuberculosis* isolated from patients with severe disease poorly activated the immune response. We thus propose that individual characteristics of the pathogen, probably resulting from recent adaptation to its host, may influence a specific outcome of disease.

Complementary research will need to be performed to fully understand the impact of *M. tuberculosis* diversity on the immune response. Thus, we propose to in the future test whether other immune cell populations will respond differently to the selected *M. tuberculosis* isolates, and to determine the *M. tuberculosis* transcriptome during infection to unveil new mechanisms of virulence. Additionally, it will be interesting to test whether other severe *M. tuberculosis* isolates present the same evasion mechanism described for *M. tuberculosis* isolate 6C4. Furthermore, the impact of our findings in TB pathogenesis also warrants further research. A first step towards that will be the study of the immune response

to different *M. tuberculosis* isolates in the context of an *in vivo* model. Then, the lung bacterial burdens and pathology, as well as the dissemination of the bacteria to other organs will be addressed. For that, we propose to use in the future C57BL/6 and C3H/FeJ mice (which are described to present lung pathology more similar to the human case), to study the course of disease, pathology, and susceptibilities to specific *M. tuberculosis* isolates. Finally, we would like to investigate how monocytes or macrophages from individuals with comorbidities such as HIV, diabetes and pharmacologic immunosuppression will respond to infection to different *M. tuberculosis* isolates.

Our findings may be relevant for the development of novel diagnosis methods based on specific bacterial gene markers to identify patients at increased risk of developing severe TB disease. This remains a poorly explored area, as most efforts are devoted to reveal factors that influence the progression from latent to active forms of disease. Additionally, host-direct therapies based on the controlled stimulation of the immune response to counteract the immune evasion strategies of certain *M. tuberculosis* isolates may be a possibility to improve the condition of patients with severe TB, by restoring a protective environment. To continue this line of research, and validate the development of new bacteria-based methods for detecting risk factors of TB severity, we will need to increase our cohort and *M. tuberculosis* collection to validate in a larger scale our results.

To conclude, my thesis bears key contributions for TB. From a fundamental point of view, we highlight the fact that some molecules, such as type I IFN, may have a protective role during infection. We also show that different *M. tuberculosis* isolates regulate the immune response contributing to specific TB severities, and offer the modulation of macrophage cytosolic surveillance systems as an underlying mechanism. From a clinical standpoint, we open new avenues for the development of TB prognostic tools. Most importantly, we call the attention for the need to incorporate the diversity of *M. tuberculosis* in all aspects of TB research.