Functional impact of differential IL-1 signaling in tuberculosis

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Todas as correções determinadas
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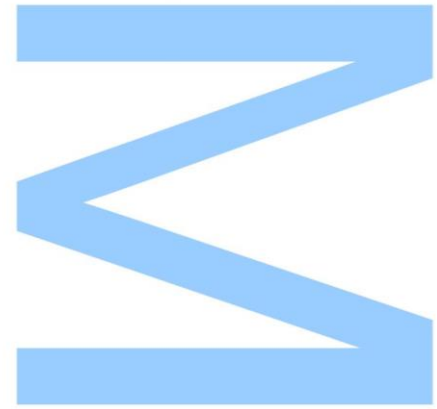

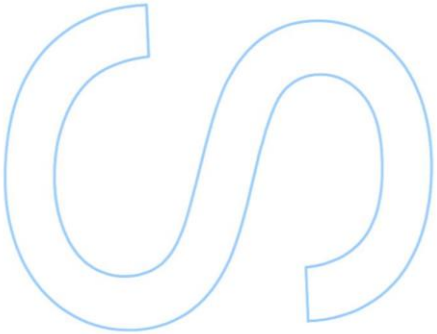

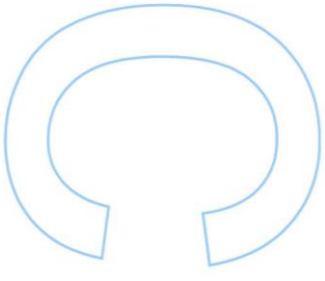

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Resumo

A tuberculose continua a ser uma grande problemática de saúde a nível mundial, sendo responsável por mais de 1 milhão de mortes por ano. Além disso, estima-se que o agente infecioso responsável pela tuberculose, *Mycobacterium tuberculosis*, infete de forma latente cerca de um quarto da população mundial. A variabilidade de manifestações da doença é uma das mais importantes características da tuberculose, e esta variabilidade pode-se relacionar tanto com fatores do hospedeiro, incluindo a sua variabilidade genética, estilo de vida e estado imunitário, como com a variabilidade genética entre as estirpes bacterianas.

Num estudo a decorrer no nosso laboratório a caracterização da estrutura filogeográfica dos isolados clínicos de *M. tuberculosis* responsáveis por casos de tuberculose na área geográfica do Porto está a ser investigada. A interação destes isolados com células mononucleares do sangue periférico está também a ser estudada. Através deste trabalho, ficou claro que alguns isolados de *M. tuberculosis* são fortes indutores de produção de citocinas por células infetadas, enquanto que outros apenas induziam uma produção modesta, por vezes no limite de deteção, das mesmas citocinas. O facto de estas observações serem independentes do hospedeiro, sugere fortemente que fatores da bactéria influenciam a intensidade de resposta à infeção por parte das células infetadas. Este projeto centrou-se em investigar em mais detalhe esta hipótese. Para isso, e com o objetivo de eliminar a variabilidade do hospedeiro, recorremos a um modelo murino de infeção, tanto *in vitro* como *in vivo*, e a dois isolados clínicos de *M. tuberculosis* diferentes, um forte indutor e um fraco indutor de produção de citocinas em células infetadas.

Começámos por infetar macrófagos derivados da medula óssea de ratinhos C57BL/6 com os dois isolados clínicos de *M. tuberculosis* selecionados. Semelhante ao observado na infeção de células humanas, a infeção de macrófagos de ratinho com cada um dos isolados levou a enormes diferenças na produção de IL-1β e IL-1α. De forma a procurar uma explicação mecanística para estas diferenças começámos por analisar a transcrição de ambas as citocinas por macrófagos infetados com cada um dos isolados. Surpreendentemente, apesar de haver diferenças na quantidade de transcritos de mRNA, estas diferenças não eram suficientemente notórias ao ponto de explicar as proeminentes diferenças na produção de IL-1β e IL-1α. A IL-1β precisa de ser processada antes de ser secretada na sua forma ativa, processamento que resulta

da ativação do inflamassoma. Desta forma, decidimos avaliar a contribuição do inflamassoma para a produção diferencial de IL-1β induzida pelos diferentes isolados em macrófagos infetados. De facto, observámos uma redução significativa na produção de IL-1β por macrófagos infetados com o isolado altamente indutor de produção de citocinas, e, para além disso, conseguimos aumentar a produção de IL-1β após infeção pelo isolado menos inflamatório, através da ativação do inflamassoma. Estes resultados indicam que uma ativação diferencial do inflamassoma pela bactéria deverá ter um papel fundamental no balanço da resposta imune contra *M. tuberculosis*.

Investigámos também diferenças entre os isolados relativamente ao crescimento intracelular em macrófagos. O crescimento intracelular foi medido 3 dias após a infeção. Observou-se um crescimento diferente entre os dois isolados, sugerindo a existência de fatores de virulência diretamente relacionados com o crescimento bacteriano. Adicionalmente, observámos que diferentes mecanismos microbicidas ganham preponderância consoante o isolado que é utilizado na infeção.

Realizámos ainda uma experiência *in vivo* com um período de 90 dias, para obter dados relativos a carga bacteriana, histologia, transcrição e recrutamento de células no contexto complexo de infeção num organismo completo. Os resultados obtidos no dia 30 após infeção demonstraram a existência de uma maior carga bacteriana nos pulmões de ratinhos infetados com o isolado mais inflamatório, no entanto, verificou-se uma maior disseminação pelo organismo do isolado menos inflamatório. Além disso, verificou-se um maior recrutamento de linfócitos B para os pulmões de animais infetados com o isolado mais inflamatório. Observámos também que ambos os isolados provocaram dano tecidular em extensão semelhante, e que os animais não apresentavam diferentes sinais de doença após infeção por cada um dos isolados.

Finalmente, comparámos o genoma dos dois isolados para desvendar potenciais candidatos responsáveis pelas diferenças fenotípicas em termos de interação hospedeiro-patogénio, e descobrimos que a maioria dos polimorfismos que diferem os dois isolados estão relacionados com atividade catabólica e metabolismo do ATP, bem como com a síntese da parede bacteriana. Além disso, verificámos que inativando os isolados através da temperatura abolimos as diferenças na produção de IL-1β, sugerindo que as diferenças fenotípicas são mais prováveis de advir de um impacto funcional de diferenças genotípicas entre os isolados, do que de um impacto estrutural.

Este trabalho demonstrou que mesmo pequenas variações genotípicas entre isolados de *M. tuberculosis* podem levar a drásticas diferenças na resposta imune. Desta forma, estudar o genoma da bactéria e relacioná-lo com o impacto funcional ao nível da resposta imune e da manifestação clínica da tuberculose poderá apontar novos alvos nos quais podemos intervir para criar novas terapias para prevenir e/ou combater a tuberculose.

Abstract

Tuberculosis remains a major health concern worldwide, causing over 1 million deaths per year. Moreover, it is estimated that about one-fourth of the world population is latently infected with *Mycobacterium tuberculosis*. The variability of the disease outcomes is one of the most striking features of tuberculosis, and it can be related both to host factors, including genetic variability, lifestyle and balanced/unbalanced immune state, and to the genetic variability within the bacteria strains.

In an on-going project in our lab, clinical isolates of *M. tuberculosis* causing tuberculosis in the Porto geographic area are being studied at the genomic level. Their interactions with human peripheral blood mononuclear cells are also being investigated. From this work, it became clear that some *M. tuberculosis* isolates were strong inducers of cytokine responses by infected cells, whereas others induced only a modest, sometimes barely detectable production of the same cytokines. As these observations were independent of the host, they strongly suggest that bacterial factors influence the intensity of the cell response to infection. This project was centred in further investigating this hypothesis. For this, and with the aim of eliminating host variability, we resorted to the mouse model of infection, both *in vivo* and *in vitro*, and to two distinct isolates, a high and a low cytokine inducer in infected cells.

We started by infecting bone-marrow derived macrophages obtained from C57BL/6 mice with the two selected clinical isolates of *M.tuberculosis*. As observed upon infection of human mononuclear cells, infection of mouse macrophages with either isolate led to striking differences in the production of IL-1β and IL-1α. To search for a mechanistic explanation for this, we started by analysing the transcription of both cytokines by macrophages infected with either isolate. Surprisingly, although there were mRNA transcriptional differences, they did not appear profound enough to explain the outstanding differences in cytokine production. IL-1β needs to be processed before being secreted in its active form, which results from the activation of the inflammasome. Thus, we next evaluated the contribution of the inflammasome for the differential production of IL-1β induced by either isolate in infected macrophages. Indeed, we observed that there was a significant reduction on the production of IL-1β by BMDMs upon infection with the strong cytokine inducer isolate, and by activating the inflammasome we could lead to a higher production of this cytokine upon infection with the less inflammatory isolate, indicating that the modulation of the inflammasome activation by the bacteria might have a key role on the immune response balance against *M. tuberculosis*.

We also investigated whether the growth of either *M. tuberculosis* isolate in infected macrophages was different. The intramacrophage bacterial growth was measured 3 days post-infection. Different bacterial growth was observed between the two isolates, suggesting the existence of virulence factors directly related with bacterial growth. We could also observe triggering of different microbicidal mechanisms upon infection with either isolate.

Additionally, we performed a 90-day *in vivo* experiment, to obtain bacterial burden, histological, transcriptomic and cell recruitment data of infection in the complex context of a whole organism. At day 30 post-infection, we observed that lungs of animals infected with the higher inflammatory isolate had a higher bacterial burden, but the less inflammatory isolate presented a higher dissemination throughout the organism. Furthermore, we could observe a higher recruitment of B-cells to the lungs of animals infected with the strong cytokine inducer isolate. We also observed a similar extent of lung damage caused by both isolates, and that the animals showed no differential signs of disease upon infection with either isolate.

Finally, we compared the whole genome of the two isolates to uncover potential candidates underlying the phenotypic differences in terms of host-pathogen interactions, and we found that most of the single nucleotide polymorphisms that differed between the two isolates are related with catabolic activity and ATP metabolism, and with the cell wall synthesis. Furthermore, we found that heat-killing the isolates abolished the differences in IL-1β production, which suggests that the observed phenotypic differences might come from a functional impact of genotypic differences between isolates, rather than a structural one.

This work has shown that even small genotypic differences between *M. tuberculosis* isolates may lead to drastic differences in the immune response. Therefore, studying the bacterial genome and relating it to a functional impact in the host, in terms of immune response and tuberculosis manifestation, might unveil new targets in which we can intervene to create novel approaches and therapies to fight this devastating disease.

Keywords

Tuberculosis, *Mycobacterium tuberculosis*, *Mycobacterium tuberculosis* complex, Interleukin-1, Inflammasome, NLRP3, Host-Pathogen Interactions, Experimental infection

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List of Abbreviations

Ab	Antibody
AIDS	Acquired Immunodeficiency Syndrome
AIM2	Absent in Melanoma 2
ALR	AIM-2-like Receptor
BCG	Bacillus Calmette-Guérin
BMDM	Bone Marrow-Derived Macrophage
BSL3	Biosafety Level 3
cDMEM	Complete Dulbecco's Modified Eagle Medium
CFU	Colony Forming Unit
cGAS	Cyclic GMP-AMP Synthase
CRP	C-Reactive Protein
DAMP	Danger Associated Molecular Pattern
DC	Dendritic Cell
DHE	Dihydroethidium
DHR	Dihydrorhodamine 123
dsDNA	Double-strand DNA
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FSC	Forward-scattered
HIV	Human Immunodeficiency Virus
HSJ	Hospital de S. João
IFNAR	Interferon alpha/beta Receptor
IL	Interleukin
IL-1R	Interleukin-1 Receptor
IL-1Ra	Interleukin-1 Receptor Antagonist
iNOS	Inducible Nitic Oxide Synthase
LAM	Latin America/Mediterranean
LCCM	L-929-cell Conditioned Media
LRR	Leucine-rich Repeat
LSP	Large Sequence Polymorphism
MAF	*Mycobacterium africanum*
MDM	Monocyte-derived Macrophage
MDR	Multidrug Resistant
miRNA	Micro-RNA

MOI	Multiplicity of Infection
Mtb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
NLR	NOD-like Receptor
NLRP3	NACHT, LRR and PYD domains Containing Protein 3
NO	Nitric Oxide
NOD	Nucleotide-binding Oligomerization Domain
OADC	Oleic Albumin Dextrose Catalase
P2X7	P2X Purinoceptor
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PFA	Phosphate-buffered Formalin
PRR	Pattern Recognition Receptor
RD	Region of genomic Difference
ROS	Reactive Oxygen Species
siRNA	Small Interfering RNA
SNP	Single Nucleotide Polymorphism
SSC	Side-scattered
STING	Stimulator of Interferon Genes
TB	Tuberculosis
Th	T-helper
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
WGS	Whole Genome Sequencing
XDR	Extensively Drug Resistant

Chapter I: Introduction

Introduction

1.1. Tuberculosis, a historical and still a current global health concern

Tuberculosis (TB) is a lung disease that has afflicted Humankind for a long time and is still a devastating condition. TB-like symptoms and disease have been reported for centuries. Indeed, individuals with bone TB over a 4000 year-period have been identified, which allowed to infer that TB was a very common disease in ancient Egypt[1]. Beyond the physical evidences for the disease, there are also reported cases of patients coughing blood as far as the seventh century B.C.[2] Apart from these ancient reports of TB-like symptoms, several TB epidemics in Europe in the 16th and 17th centuries, and a peak of the disease in the first half of the 19th century, when an estimated one-quarter of the Europeans died of TB, have been described[3]. A period of decrease in mortality due to TB was observed in the second half of the 19th century, due to the improvement of the sanitary and housing conditions. This pattern was maintained during the 20th century in developed countries, due to general better health practices, but also to the introduction and the implemented use of the Bacillus Calmette-Guérin (BCG) vaccine. The development of efficient anti-TB drugs on the beginning of the 1950s also led to an improvement on the mortality rates due to the disease[4]. The picture was again torn apart in the 1980s, when a new increase on TB rates was observed, due to a higher rate of homelessness and poverty, and especially, due to the emergence of the human immunodeficiency virus (HIV) and the resulting acquired immunodeficiency syndrome (AIDS). This led to a huge expense of funds to reverse this increase in TB cases in developed countries, but still TB remains a striking health problem[5]

TB has recently surpassed HIV as the most killing disease caused by a single biological agent[6]. TB causes around 1,5 million deaths per year, and an estimate of 10 million new cases are registered each year, especially on the southern part of the African continent and southeast Asia (Figure 1)[7, 8]. In countries like South Africa and Swaziland, for example, it is estimated that at least 1 in every 100 people develops TB each year[9].

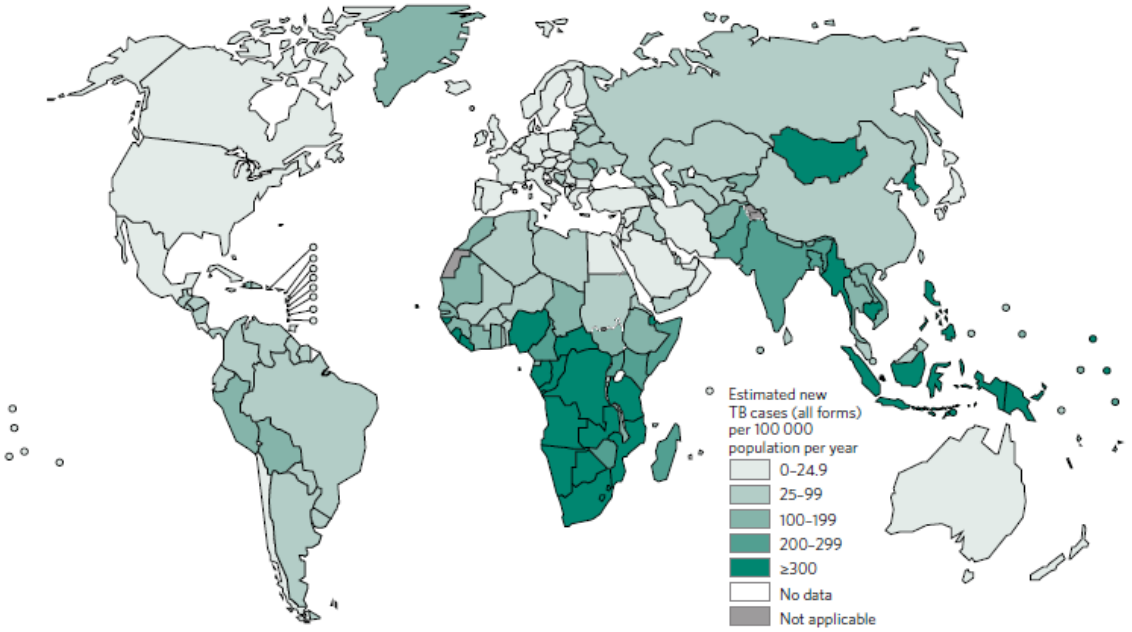

Figure 1. Global distribution of TB cases, according to World Health Organization 2016 Global TB Report[6].

Furthermore, *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, is thought to latently infect 2 billion people worldwide, and, most importantly, it is predicted that 5 to 10% of these individuals will eventually develop active disease in any point of their life, making the latent individuals a very concerning reservoir of TB[10].

Further aggravating the TB scenario is the emergence of strains resistant to the first-line antibiotics, isoniazid and rifampicin (Multidrug Resistant strains – MDR)[11, 12]. Even more concerning is the development of Extensively Drug Resistant (XDR) strains, which are resistant to fluoroquinolones and second-line injectable drugs like kanamycin or capreomycin, or even to the two newest anti-TB drugs, delamanid and bedaquiline[13, 14]. Altogether, this makes TB a very delicate subject and a great matter of concern for the health community, not only because of the high costs that the disease represents, but also because of the growing number of cases for which there is no efficient treatment available[8, 13, 14].

1.2. Mtb and the *Mycobacterium tuberculosis* complex

Mtb (Figure 2), the pathogen responsible for TB, is a member of the *Mycobacterium* genus, that comprises acid-fast bacteria with a thick and specific cell wall containing mycolic acids[15, 16].

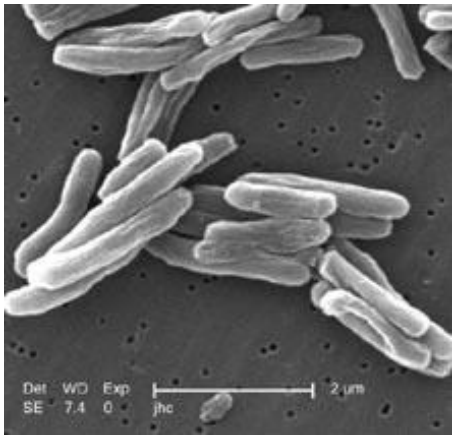

Figure 2. Scanning electron micrograph picture of Mtb under a 15549x amplification. By *J. Carr* from Centers for Disease Control and Prevention Public Health Image Library (PHIL) by Janice Carr.

Mtb belongs to the *Mycobacterium tuberculosis* complex (MTBC) along with Mycobacteria infecting other mammalian hosts, that include, for instance, *Mycobacterium bovis*, *Mycobacterium caprae*, *Mycobacterium pinnipedii* and *Mycobacterium microti*[17].

A whole variety of molecular and bioinformatic techniques have been used in the past years to study the MTBC and identify both the origins and the genomic differences within the complex[18]. The first insights on this field came from studies exploring how different types of highly polymorphic and repetitive DNA elements could be used as genetic markers for differentiating strains, and these techniques include spolygotyping or IS6110 fingerprinting[19, 20]. With advances on molecular and bioinformatic technologies, techniques like analysis of regions of genomic differences (RDs)/large sequence polymorphisms (LSPs)[21], multilocus sequence typing[22], single nucleotide polymorphisms (SNP) typing[23] and, most recently, whole-genome sequencing (WGS)[24], provided a clear picture of the origin and differences within the MTBC. The current idea is that Mtb has most likely been infecting humans for at least 70 000 years, during which time it has been adapting constantly to the differences within the host[24]. This adaptation resulted in the arise of seven different MTBC lineages[24]. These lineages have a very clear and distinct geographical distribution, with the most ancestral lineages 1,5,6 and 7 mostly found in Africa, and more recent lineages 3, centred in Asia, and 2 and 4 with a higher intercontinental distribution. The last branch of the MTBC includes species adapted to other mammalian hosts (Figure 3)[18].

Strong phylogeographical relationships are also seen at the sublineage level. This is the case for lineages 2 and 4, with for instance, Beijing sublineage of lineage 2 being mainly prevalent in patients from Eastern Asia[25].Similarly, some sublineages of

lineage 4 are connected to certain regions in Europe (sublineage Haarlem), western Africa (sublineages Ghana and Cameroon), and Latin America/Mediterranean region (LAM sublineage) [26-29].

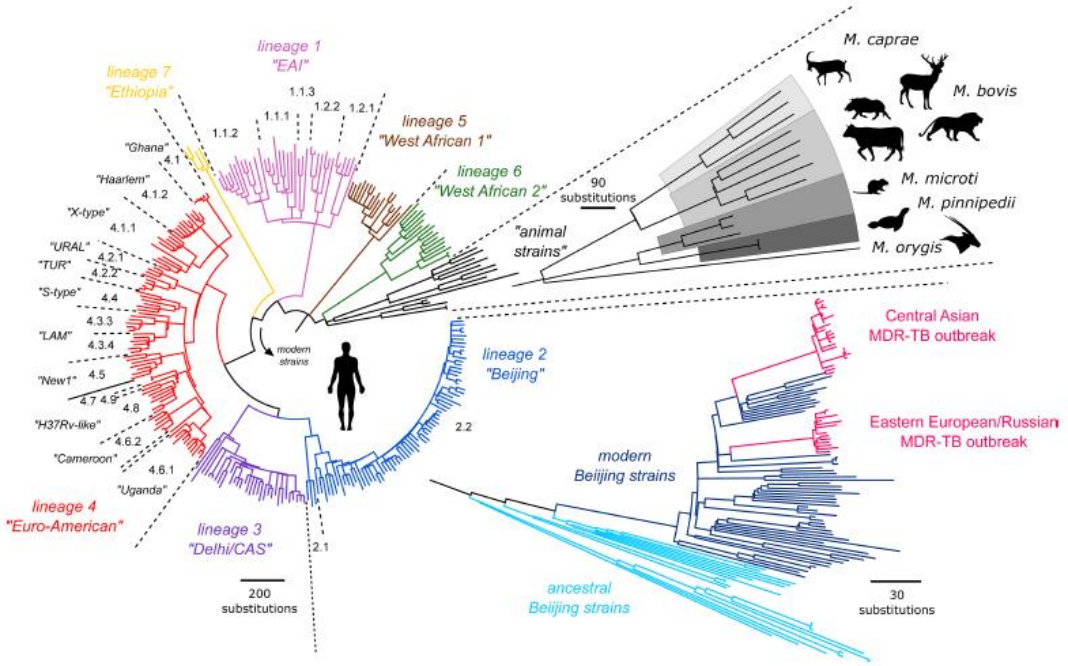

Figure 3. Global stratification and phylogenetic relationships of the MTBC complex. Image obtained from Niemann et al, 2016[18].

Of all the MTBC lineages, clearly lineage 4 exists at high frequencies in all continents, and it is taken as the most widespread lineage causing human TB[30, 31]. A recent study by Stucki *et al* focused on the global success of this lineage compared to the other MTBC lineages that are clearly more restricted[30]. This study showed that this success came from the different distribution and specialization of the sublineages of lineage 4. It was demonstrated that within lineage 4 there are sublineages that can be classified either as ecologically generalists (a wider geographical range), including Haarlem, LAM and PGG3 sublineages, or ecologically specialists (geographically restricted), including Ghana, Uganda and Cameroon sublineages. These sublineages displayed a different evolutionary strategy which is thought to be the reason of the global success of lineage 4. Very importantly, the origin of sublineage LAM, the most widespread, was traced as European, being disseminated along with the Portuguese and Spanish explorations and conquests in the different continents, mainly America[32].

The evident phylogenetic and phylogeographic relationships and restrictions between MTBC members and their hosts are a likely sign of the host-pathogen coevolution[33]. Also, there is compelling evidence of parallels between evolutionary

history and the human population demographic changes, with deriving lineages and sublineages appearing along with great migration events out of the common and most believed original continent of Mtb, Africa[24]. Furthermore, in cosmopolitan urban centres, it is observed that sympatric strain-host transmission occurs more frequently than allopatric strain-host combinations[34, 35]. More importantly, this sympatric association is not so strict for immunocompromised individuals, which suggests that an impairment in the host immunity can lead to an increased vulnerability for infections with allopatric bacteria[35, 36]. This pinpoint the stratification of the Mtb lineages and the respective populations they infect, thus further supporting coevolution events.

1.3. MTBC diversity and clinical outcomes

One of the most striking features about TB is its notorious heterogeneity, with some patients only presenting small clinical manifestations, and others severe symptoms, such as hypoxemic respiratory failure or involvement of both lungs[37]. For many years it was thought that only the host contributed to the different outcomes of disease. This was due to observations that socio-epidemiological factors including poverty and malnutrition, had a negative impact and contributed to higher rates of disease and most aggravating outcomes[38]. Also, the emergence of HIV and its role on the magnification of the TB burden, especially in sub-Saharan Africa, highlighted the key role of the host immunity[39]. Furthermore, this host-centred concept for the outcome of disease was somehow reinforced by the first molecular exploration studies that showed a very low rate of polymorphisms within structural genes among the MTBC[40], thus minimizing a potential role for the bacteria diversity.

However, the studies stated in the previous section that contributed to stratify and divide the components of the MTBC in different lineages, also contributed to the recognition that even slight differences in the bacteria genome can contribute to phenotypically different bacteria and thus to a different severity of disease. Several functional studies started to unveil that different genetic backgrounds or lineages may contribute to a significantly different outcome of disease. For instance, Mtb from the Beijing lineage 2 was shown to cause very aggravating disease symptoms and outcomes[41]. On the other hand, *Mycobacterium africanum* (MAF) from lineages 5 and 6 only causes a mild TB[42]. In fact, there seems to be a difference in virulence between the ancient lineages and the modern ones, with the modern lineages generally inducing a lower cytokine response and causing more aggravating symptoms of disease[43]. A study by Portevin *et al* showed that human monocyte-derived macrophages (MDMs)

infected with 28 different MTBC strains produced a significantly differential level of cytokines, with the modern strains inducing a lower amount of proinflammatory cytokines compared to ancient strains[44]. An older study also showed a higher virulence of MTBC strains from the UK when compared to strains from South India, which today we know that belong respectively to lineage 4 (modern) and lineage 1 (ancient)[45]. Furthermore, a study relying on TB patients from the Gambia, that followed their household contacts for 2 years showed that the contacts exposed to modern Mtb strains were more likely to develop active disease than the ones exposed to ancient strains[42].

1.4. The immune response to Mtb: an overview

Mtb is naturally spread by aerosol particles, which are formed within an infected individual with active TB and released while these individuals cough (one of the hallmark symptoms of TB)[46]. Once these aerosol particles are inhaled by another individual, resident cells of the lung, usually macrophages, phagocyte the bacteria inside a phagosome that later fuses with a lysosome, forming the phagolysosome, a structure typically involved in acidic killing of intracellular pathogens[47, 48]. However, in the context of Mtb infection, often the bacterium can block the endosome maturation[49, 50]. An initial innate response is still mounted in these conditions, and eventually the infected macrophages produce cytokines and chemokines responsible for triggering the adaptive immune response, attracting monocytes, lymphocytes and neutrophils to the site of infection[51].

The action of effector cells is, yet, not very efficient, and in most of the cases these cells are not capable of fully eliminate the pathogen[52]. Instead, a typical TB structure is formed, the granuloma, a lesion composed by a caseous centre with infected macrophages killed upon cellular immune response, surrounded by fibroblasts, lymphocytes and blood-derived monocytes[53]. Mtb bacilli are thought to be capable of surviving in a dormant state inside granulomas for decades[5]. Whether the bacteria pass this stage and lead to active disease or not is determined by the strength of the host adaptive immune response. In immunocompetent people, the infection may be permanently detained at this point, with the granuloma eventually healing, only remaining small fibrous and calcified lesions[54]. However, if at any point of their lives the infected people become immunocompromised, the granuloma centre can become liquefied, leaving the bacteria with a rich medium to uncontrollably replicate and escape the granuloma, spreading within the lungs and possibly to other organs, leading the host to contract active disease and be infectious[54]. T cells, in particular T helper (Th) 1 cells,

are critical for the maintenance of the granuloma[55]. This is demonstrated by the essential role of the interleukin (IL)-12/interferon (IFN)-γ axis in conferring resistance to TB[56]. Clinical observations have shown a high susceptibility of children with genetic deficiencies in the IL-12/IFN-γ pathway to TB, and also studies in murine models show an essential role for these cytokines in the context of Mtb infection[56, 57].

As mentioned before, the triggering of the immune response that culminates in granuloma formation relies on the activation of innate immune cells. Events as early as recognition of bacterial products by pattern recognition receptors (PRRs), like toll-like receptors (TLRs) or nucleotide-binding oligomerization domain (NOD) - like receptors (NLRs) are often decisive in the outcome of disease[58]. The intricate relationship between PRRs and Mtb is especially noticeable in the case of TLRs. To date, TLR2, TLR4 and TLR9 have been shown to recognize Mtb constituents or products[58]. TLR2 not only triggers proinflammatory cytokine production and induces apoptosis, but also activates autophagy, a protective host mechanism against Mtb infection[59, 60]. However, Mtb can change the balance between pro and anti-inflammatory cytokine production, by producing the protein hsp60, that binds TLR2 but leads to production of IL-10, thus exploiting TLR2 signalling in its favour[61]. TLR4 can trigger two different signalling pathways upon recognition, one dependent on MyD88, and another one that leads to production of IFN-β[62]. Mtb can exploit this duality in TLR4 signalling, and lead to a higher IFN-β production that can contribute to pathogenesis[63].

However, a lot of controversy still exists in the literature on how fundamental the presence of TLRs is for the course of infection *in vivo*. For instance, while one study showed that TLR2 has a critical role in Mtb infection[64], another showed that TLR2 knock-out (KO) mice depicted no survival differences when compared to wildtype mice[65]. Nevertheless, in a paradoxical way, mice lacking MyD88, the usual adaptor for TLRs and IL-1 receptor (IL-1R), rapidly succumb to aerosol infection with Mtb[66-68].

Other innate immune mediators like tumor necrosis factor (TNF)–α play an essential role on protection against Mtb infection. Studies have shown that TNF-α KO mice have a higher susceptibility to Mtb infection[69]. Also, patients with Chron's Disease and rheumatoid arthritis treated with anti-TNF-α antibodies (Abs) display a higher reactivation of TB[70]. In this work, we will focus on another prominent player in the defence against Mtb infection: the IL-1 family.

1.5. IL-1 and the Inflammasome complex

1.5.1 The dual role of IL-1 in TB

The IL-1 cytokine family has been gaining a lot of focus in the context of TB in the past years. A growing body of evidences has pointed to this IL-1 preponderance. Studies on IL-1α and IL-1β double KO mice, and IL-1R type-1-deficient mice showed a higher susceptibility of these mice to infection by Mtb [71, 72]. Furthermore, mice deficient in IL-1β alone have been shown to be highly susceptible to infection by Mtb independently of TLR signalling[73]. These data clarified the apparent paradox between the lack of susceptibility to infection seen in TLR deficient mice and the high susceptibility observed in MyD88 deficient mice, as MyD88 is a common adaptor for TLR and IL-1R signalling.

Several mechanistic studies explain the protective effects of IL-1β in the context of Mtb infection. In the early stages of infection, IL-1β can initiate and propagate an inflammatory reaction characterized by intravascular coagulation, tissue edema and neutrophil infiltration[74]. These roles of IL-1β come from its ability to regulate the adhesion of leukocytes to the endothelium, and induce the production of chemokines that can guide the chemotaxis of neutrophils to the zone of tissue damage[74].

At later stages of infection, an uncontrolled IL-1β production is dangerous for the host, as the continuous influx of neutrophils to the site of infection not only promotes tissue damage, but also provides a niche of cells for Mtb to continually grow and spread[75, 76]. In support of the detrimental role of IL-1β in TB, several polymorphisms associated with overproduction of IL-1β have been associated with susceptibility for TB. A rs16944 SNP in the IL1B locus has been reported to be associated with susceptibility to TB in a Gambian population[77]. Other 3 SNPs on this locus, rs1143629, rs1143643 and rs3917368, have been reported to cause susceptibility to TB in a Japanese population[78]. A recent study has also associated a genetic polymorphism rs1143627 on the promoter region of the IL1B gene that increases the cytokine expression to susceptibility to TB and poor clinical outcome[79]. Together, these observations pinpoint the IL-1 dual role in TB context.

1.5.2 The regulation of IL-1 production

Given the aforementioned dual role of IL-1β in TB, it is not surprising that the production of this cytokine needs to be tightly regulated. IL-1β is regulated both at the level of transcription and post-transcriptionally.

The transcription of IL-1β is stimulated by the transcription factor NF-κB. NF-κB is triggered upon PRR activation, leading to transcription of pro-IL-1β mRNA. NF-kB is downstream of TLR and IL1-R signalling pathway, thus, transcription of IL-1 can be further activated by IL-1 itself [80]. Regulation of IL-1 transcription is performed by different mechanisms. The most common inhibitor of the IL-1 pathway is the IL-1R antagonist (IL-1Ra). Thus, regulation of IL-1β transcription is usually achieved by binding of the IL-1Ra to the IL-1R, shutting down the cascade of events that initiates IL-1 transcription[81]. In the context of TB, IL-1β regulation is also achieved by type-I IFN and IL-10 production[68, 82]. Among its other anti-inflammatory functions, IL-10 inhibits the IL-1 signalling pathway that culminates in IL-1β transcription[83].

The key post-transcriptional step regulating IL-1 production results from the fact that IL-1β is translated as a pro-protein, thus requiring a proteolytic cleavage to become active. This cleavage constitutes the second level of regulation of the IL-1β levels[84, 85]. The maturation and release of IL-1β is performed by the inflammasome. The inflammasome is a high-molecular-weight proteic complex residing on the cytosol of stimulated immune cells, whose main function is the activation of proinflammatory caspases[86]. Many types of inflammasomes have been described to date, and they are classified according to the receptor protein that assembles the inflammasome, and these include NODs, leucine-rich repeat (LRR)-containing protein, proteins absent in melanoma 2 (AIM2) and pyrin[87, 88]. Inflammasomes have been linked as being important upon infection with Mtb, with several studies demonstrating that in their absence decreased levels of both IL-1β and IL-18 are present, and, in this way, increased susceptibility to infection is observed[89-91].

1.5.3 The importance of the inflammasome in TB

In the context of Mtb infection, at least two types of inflammasomes are activated: the NACHT, LRR and PYD domains-containing protein 3 (NLRP3)[92], and AIM2 inflammasomes[91] (Figure 4).

NLRP3 is by far the most described inflammasome and the one with a wider range of responses to stimuli[93]. The activation of this complex consists in a two-step process. The first, and one quite unique, is the activation the NF-κB signalling pathway by TLR ligands or IL-1 itself, that leads to an upregulation of the transcription of NLRP3 and pro-IL-1β[94, 95]. Once this prime signal is present, the activation and assembly of the NLRP3 inflammasome is achieved by a bacterial[96-98], fungal[99] or viral[100] signal, as well as danger associated molecular patterns (DAMPs) of various natures[96], environmental crystals[101] and ionophores, like nigericin[102]. The inflammasome can directly sense the cytosolic presence of these diverse agonists or, and most probably, taking into account the immense diversity of its agonists, respond to stress signals induced by the infectious agents[103]. For instance, cardiolipin, which is released upon mitochondrial damage induced by infectious agents, is able to activate NLRP3 inflammasome assembly[104, 105]. NLRP3 is also regarded as dependent on the efflux of K^+, since many studies show that administrating K^+ to cell culture media and, therefore, abolishing the release of potassium by the cells, prevents the activation of caspase-1 by the NLRP3 inflammasome[102, 106-108] The importance of NLRP3 inflammasome activation in the context of TB is well reported in the literature. Studies have shown that macrophages and dendritic cells (DCs) lacking NLRP3 do not secrete IL-1β and IL-18 upon Mtb infection[89, 90]. Furthermore, a study showed that human macrophages with a gain of function mutation in NLRP3 have a better capacity to control Mtb growth[109].

AIM2 inflammasome is a member of the HIN200 family/AIM-2-like receptor (ALR) family, which are characterized by a N-terminal PYD and the presence of one or two DNA-binding Hin200 domains[110]. The assembly of the AIM2 inflammasome is triggered by the binding of double-strand DNA (dsDNA) from bacterial or viral origin to the Hin200 domain[111-113], endowing myeloid cells with capacity to produce mature IL-1β and IL-18, and induce pyroptosis when viral or bacterial nucleic acids are detected in the cytosol of infected macrophages[114, 115]. The importance of the AIM2 inflammasome on Mtb infection is highlighted by a study from Saiga *et al* of 2012 that shows that upon infection with Mtb, AIM2 KO mice presented decreased levels of IL-1β and IL-18 in the lungs, accompanied by higher bacterial burdens, atypical granuloma formation and lower survivability[91].

1.5.4. The regulation of the inflammasome in TB.

As aforementioned, IL-1β has a major importance upon infection by Mtb. However, an overproduction of IL-1β is highly detrimental for the infection by heightening

tissue damage and pathology[75]. Therefore, the inflammasome activation and consequent mature IL-1β production must be tightly regulated.

In the context of TB, one of the most important inflammasome regulatory pathways is held by type-I IFNs. Evidence for this comes from the observation that type-I IFNs have a detrimental role in the context of TB[116] and that during infection with Mtb, IL-1 production is negatively regulated by type-I IFNs in human and murine DCs and macrophages[68, 117, 118]. Interestingly, it was also observed that DCs from IFN alpha/beta receptor 1(IFNAR1)-deficient animals have increased expression of IL-1β *in vivo*[68], and a mechanism by which this downregulation is achieved is by a modulation of the transcription of the IL-1β mRNA by type-I IFNs[118]. Furthermore, it is also established that type-I IFNs negatively regulate the inflammasome-mediated IL-1β maturation[83]. Interestingly, it was reported that Mtb itself is capable of triggering the production of type-I IFNs. This is achieved by binding of Mtb dsDNA or cyclic dinucleotides to the stimulator of IFN genes (STING). STING, on its turn, activates the cyclic GMP-AMP synthase (cGAS) that leads to stimulation of the type-I IFN pathway[119-121]. Different TLR signalling also plays a role on this regulation. Carmona *et al* showed in 2013 that different strains within the Beijing sublineage elicited different degrees of cytokine response that could be more, or less protective for the host, and these differences were due to differential activation of TLRs. Importantly, this study showed that TLR4 recognition of Mtb 02-171, a clinical isolate more virulent than the reference Mtb strain H37Rv, is associated with a higher production of type-I IFN[122], which is in line with some reports that show that the virulence of some Mtb strains are intimately related with their capability to induce type-I IFNs *in vivo*[123, 124]. This further highlights the intricate host-pathogen interaction, and further show how Mtb can shape the regulation of host-cell innate immune response.

IFN-γ has also been reported to supress inflammasome activation during infection in a murine model, by activating the inducible nitric oxide synthase (iNOS), and thereby leading to the production of nitric oxide (NO). NO on its turn performs a S nitrosylation of NLRP3, making it unable to assembly the inflammasome complex and, therefore, unable to activate the caspase-1 for pro-IL-1β cleavage[75]. This is extremely important in the context of Mtb infection, since it prevents an exacerbated neutrophil recruitment, that could provide a nutritive milieu for Mtb to grow, and thus leading to higher bacterial burdens. Thus, NO exerts its protective role by inhibiting the formation a granulocytic inflammation environment that would otherwise enable a higher growth of Mtb[76].

Finally, during TB, modulation of NLRP3 transcription through micro-RNAs (miRNA) or regulation of the inflammasome complexes by autophagy pathways can also influence the activation of the inflammasomes[125, 126].

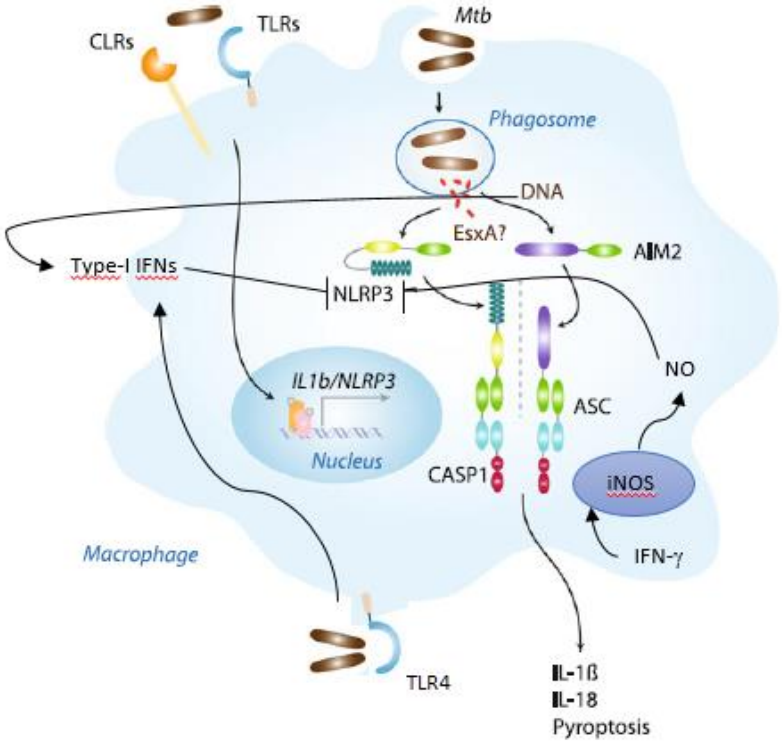

Figure 4. Inflammasomes NLRP3 and AIM2 pathway activation pathway, and respective inhibitory and control mechanisms. Image adapted from Ablasser and Dorhoi, 2016[127].

1.6. A Phylogeny-Based Approach to understand the Dichotomy Host-Pathogen in TB

To study the host-pathogen dichotomy in TB, taking into consideration the host and bacteria diversity and mutual adaptation described above, our group has taken a phylogeny-based approach. For this, a cohort of TB patients from Hospital de S. João (HSJ) was studied and the clinical isolates of TB were collected from these patients. The clinical history and the progress and features of the disease were reviewed for each patient and the TB manifestation classified as mild, moderate, severe or extremely severe (Figure 5). In this way, each Mtb isolate is mapped to a specific patient and a specific TB manifestation.

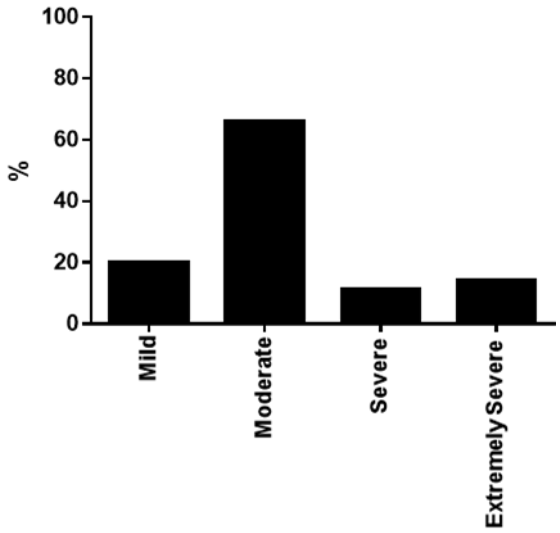

Figure 5. Distribution of TB manifestations. Figure kindly provided by Hélder Bastos, from our group.

The Mtb isolates were genotyped to assess the lineages and sublineages to which they belonged. We observed that most of the isolates were from lineage 4, in line with previous data showing that this is the most prominent lineage of TB in Western Europe[32]. Furthermore, we also observed that the most represented sublineage was LAM, in line with this being the most representative sublineage in Portugal, as in Europe[32] (Figure 6).

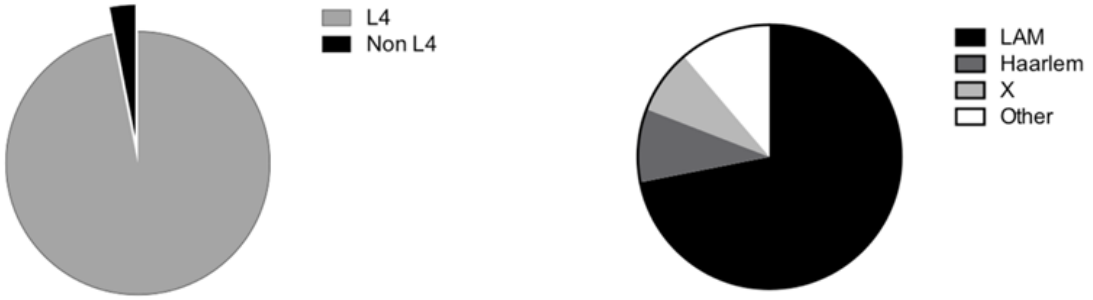

Figure 6. Overall distribution of the lineages of the isolates of the cohort (on the left) and sublineages within lineage 4 (on the right). The data was kindly provided by Baltazar Cá, from our group.

In all, these studies offered a global picture of the TB presentation in our cohort and the phylogenetic distribution of the infecting isolates. Next, we selected 16 Mtb isolates, 8 associated with mild TB presentation and 8 with severe and infected peripheral blood mononuclear cells (PBMCs) from healthy donors of the same geographic area. In this way, we can study host-pathogen relationships respecting the mutual adaptation likely to have occurred over the years. The cytokine production profile of the infected cells was assessed 24 hours after infection (Figure 7). It was clear from

this set of experiments that there was a very heterogenous distribution of the Mtb isolates regarding their capacity to induce cytokine production by infected PBMCs, with some isolates leading to a very strong cytokine response, and others only to a barely detectable one (Figure 7). Interestingly a certain degree of association between mild TB and higher cytokine response, and conversely severe TB and lower cytokine response, was in place.

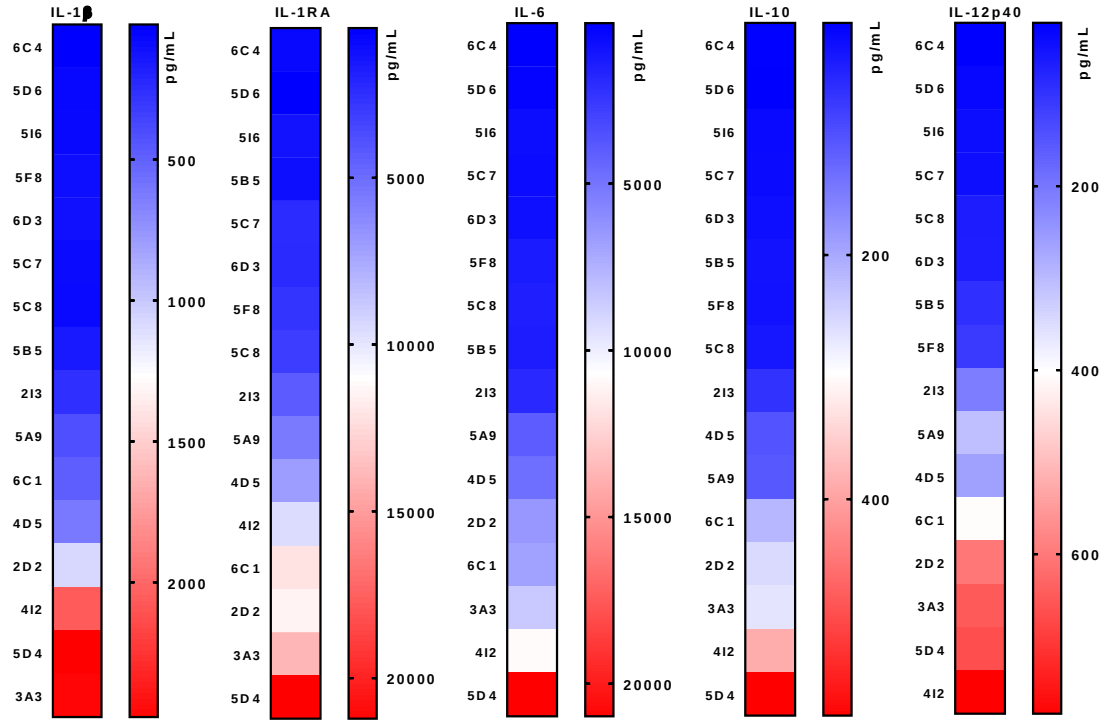

Figure 7. Cytokine production levels in human PBMCs from healthy donors upon infection with different clinical isolates of Mtb. The data was kindly provided by Jeremy de Sousa, from our group.

This pattern was observed independently of the donor from whom PBMCs were collected, which highlights the role of the bacterial diversity in shaping the host cells immune response, and ultimately on the outcome of infection and disease. This calls for a deeper understanding of the bacterial factors that modulate the host immune response and may impact the outcome of disease. Addressing these issues were the focus of my thesis.

Chapter II:
Aims

Aims

This study aimed to investigate the molecular mechanisms explored by certain Mtb clinical isolates to modulate the cytokine production by host cells. For this, our strategy was to select two Mtb clinical isolates that differ in their cytokine induction by infected cells: 4I2 (potent cytokine inducer) and 6C4 (weak cytokine inducer). The specific goals for the thesis were to:

1. Investigate the cytokine production profile of bone marrow-derived macrophages (BMDMs) upon infection with either Mtb clinical isolate.
2. Find a mechanistic explanation for differences in cytokine production.
3. Relate differences in immune response against each clinical isolate with intracellular growth in BMDMs.
4. Study the differences in outcome of disease caused by either clinical isolate in a whole organism, resorting to a mouse model of infection.
5. Analyse differences in the genome of the two clinical isolates in order to find possible targets related to phenotypic differences observed in infection by either isolate.

Overall, we expect to, in the long run, elucidate how Mtb shapes the host immune system, and how does that contribute to the outcome of disease. This knowledge will allow for the definition of better correlates of protection, and for the development of novel strategies to prevent or treat TB.

Chapter III:
Material and Methods

Material and Methods

3.1. Ethics Statement

The study protocol was approved by the Health Ethics Committees of the HSJ (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.

3.2. Preparation of the Clinical Isolates

Bacterial samples of the selected 384 subjects were recovered from stored primary cultures of Mtb clinical isolates at HSJ. Two hundred µL of inoculum were plated and smeared uniformly on solid Mycobacteria 7H11 agar supplemented with Oleic Albumin Dextrose Catalase Growth Supplement (OADC) and Panta antibiotic mixture (BD Biosciences). 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) complemented with OADC, 2% glycerol and 0.5% Tween® 80 (Sigma-Aldrich). Alternatively, the stored primary cultures were re-grown in MGIT tubes using a Bactec apparatus and once a positive signal was obtained, transferred to 20 mL of Middlebrook 7H9 liquid medium, as stated before. All cultures were incubated at 37°C with constant 120 rpm shaking for an additional 7–10 days, to increase the bacterial biomass.

3.3. BMDM Culture

Wildtype C57BL/6 mice were humanely euthanized and the femur and tibias were collected and cleaned. Bone marrow was aseptically collected from these bones to complete Dulbecco's Modified Eagle Medium (cDMEM), which was prepared by complementing DMEM with 10% fetal bovine serum (FBS), 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 1% Sodium Pyruvate and 1% L-glutamine (all from GIBCO). BMDMs were generated by plating the cells from the bone marrow in cDMEM supplemented with 20% of L-929-cell conditioned media (LCCM). On the day of collection (day 0), $4x10^6$ cells in 8 mL were plated in each Sterilin Petri dish and incubated at 37º C and 5% CO_2. On day 4, 10 mL of fresh cDMEM supplemented

with 20% LCCM were added to each plate. BMDMs were obtained on the seventh day of culture, counted, and plated at the desired concentration for each experiment.

3.4. *In vitro* infection of BMDMs

BMDMs were plated in 24-well plates at a concentration of $5x10^5$ cells/well and incubated for at least one hour at 37º C and 5% CO_2 atmosphere. Cells were infected with clinical isolates of Mtb at a multiplicity of infection (MOI) of 2 bacteria:1 cell in cDMEM. Infections for cytokine analysis were performed in a total volume of 500 μL. Infected cells were collected in TRizol for RNA analysis at different time-points post infection. The supernatants of the infected cultures were collected at 24h post-infection, filter-sterilized and stored at -80C.

3.5. Determination of Intracellular Mtb in BMDMs

For colony forming unit (CFU) determination, the infection of BMDM was performed in 200 μL. The concentration of intracellular Mtb was determined 4h, and 3 or 4 days post-infection by CFU enumeration. After a 4-hour incubation, the infected cells were washed 4 times with apyrogenic phosphate-buffered saline (PBS) and further incubated in cDMEM. At the intended timepoint, each well was incubated for 10 minutes with saponin (0,1% in cDMEM or PBS) for macrophage lysis. The resulting suspension was serially diluted and plated onto Middlebrook 7H11 agar (BD Biosciences) plates containing OADC, and colonies were counted after 3 or 4 weeks of incubation at 37º C.

3.6. Enzyme-Linked Immunosorbent Assay (ELISA)

IL-1β, IL-1α, TNF-α, IL-10 and IL-12p40 concentrations on supernatants collected from infected BMDM were analyzed by ELISA using eBioscience Ready Set-Go® kits, according to the manufacturer's instructions.

3.7. Measure of NO Production

Cell culture supernatants were used to measure the production of NO by the Griess reaction. Absorbance was measured at 550 nm using a Bio Tek MQX200 Spectometer, and NO concentration was assessed using a sodium nitrite standard curve (0–100 μM analyzed in duplicate).

3.8. Reactive Oxygen Species (ROS) Measurement

Superoxide and hydrogen peroxide were measured by intracellular staining of cells with dihydroethidium (DHE) and dihydrorhodamine 123 (DHR) probes (Thermo Fisher Scientific), respectively. Cells were incubated with 10 μM of DHE for 10 minutes, or 30 minutes with 10 μM of DHR, at 37ºC. Cells were then fixed with 4% phosphate-buffered formalin (PFA) for 20 minutes, and the probes signal and respective mean fluorescence intensity (MFI) was measured by flow cytometry in a FACS Canto II (BD Immunocytometry Systems) cytometer.

3.9. Quantitative Real-Time Polymerase Chain Reaction (real-time PCR) Analysis

RNA from infected BMDMs was extracted using 250 μL of Triple Xtractor reagent (Grisp). The RNA was converted to cDNA using GRS cDNA Synthesis Kit (Grisp) according to the manufacturer's instructions. Target genes mRNA expression was assessed by real-time PCR and normalized to Ubiquitin mRNA levels. mRNA expression was quantified using SYBR Green (Thermo Scientific) and specific oligonucleotides (Invitrogen):

Gene	Sequence	
IL-1β	Forward	5' - GTG CTG TCG GAC CCA TAT GAG - 3'
	Reverse	5' - CAG GAA GAC AGG CTT GTG CTC - 3'
IL-1α	Forward	5' – GGG AAG ATT CTG AAG AAG AG – 3'
	Reverse	5' – GAG TAA CAG GAT ATT TAG AGT CG – 3'
TNF-α	Forward	5′ - TGG CTA TTA ATT ATT CGG TCT GCAT-3′
	Reverse	5′ - TGA GGG TCT GGG CCA TAG AAC-3′
Ubiquitin	Forward	5 ′- TGG CTA TTA ATT ATT CGG TCT GCAT - 3′
	Reverse	5′ - GCA AGT GGC TAG AGT GCA GAG TAA - 3′

Table 1: List of real-time PCR SybrGreen Primers and the respective sequences.

The real-time PCR was performed under the following conditions: 15 min at 95 °C, followed by 40 cycles (95 °C denaturing for 15 s; 58 °C annealing for 20 s; 72 °C extension for 15 s), melting at 65 °C until 95 °C for 5 minutes, and finally cooling. The specificity of the SYBR green assays was confirmed by melting point analysis. Data were

normalized to ubiquitin mRNA levels, using the following equation: $2^{(\text{ct reference gene} - \text{ct target gene})} \times 100\,000$.

3.10. Mice

Infections were performed at the biosafety level 3 (BSL3) laboratory. Male C57BL/6 mice were transferred from the breeding facility to the BSL3 laboratory seven days before infection to allow the adaptation to the new environment. Mice were 7-8 weeks of age at the beginning of experiments.

At the BSL3 facility, mice were housed in environmentally controlled Techniplast ventilated polycarbonate cages under negative pressure with 40 air changes per hour. The temperature was maintained at 21–23˚C and the relative humidity at 50% ± 20% with a 12-hours light/dark cycle. The cages contained hard-wood bedding and provided with Mucedola Diet and fresh tap water, *ad libitum*, throughout the study.

3.11. *In vivo* infection

Wildtype C57BL/6 mice, at least 8 weeks old, were submitted to infection by 4I2 and 6C4 via the aerosol route using an inhalation exposure system (GlasCol). The inocula were diluted to a concentration of $2x10^6$ CFU/mL, to deliver 100-200 CFU to the lungs. The infection dose was confirmed by performing the CFUs of the lungs of 3 mice 3 days after infection.

3.12. Organ Harvesting and Cell Suspension Preparation

To collect the organs to perform the desired assays, mice were euthanized by CO_2 inhalation. After the euthanasia, the whole blood was collected by cardiac puncture. A small part of the blood was inserted in a hematocrit capillary and centrifuged for 6 minutes to determine the percentage of hematocrit. The rest of the blood was used to collect serum, by performing a first centrifugation at 5000 rpm for 2 minutes, and a second centrifugation of the supernatant at 10000 rpm for 10 minutes.

A fraction of the lungs of both infected and non-infected mice was collected to perform histological staining. Before fixing the organ, right after the euthanasia, each lung was washed with sterile PBS, and inflated with 4% PFA. After this, the fraction of the lung destined to histology was collected to a Falcon tube with 5 mL 4% PFA. The remaining fraction of each lung was incubated for 30 minutes at 37º C with 150 μg/mL of collagenase (Roche). After that, 10 mL of cDMEM were added to each tube, to inactivate the collagenase and cells were centrifuged for 6 minutes at 1200 rpm at 4ºC. The cells

were then resuspended in red blood cell lysis buffer with 0,87% amonium chloride. 2 minutes after, 10 mL of cDMEM were added to each tube and a centrifugation at 1200 rpm was made for 6 minutes at 4ºC. Finally, the cells were resuspended in 2 mL of cDMEM. From this suspension, 500 μL of cells were centrifuged at 1200 rpm for 6 minutes at 4º C, and then resuspended in 250 μL of TRizol to collect RNA. 1 mL of cell suspension was counted and $1x10^7$ cells/mL were plated on a 96 well to posterior staining for flow cytometry.

3.13. Lung, Liver, and Spleen CFU Enumeration

The remaining 500 μL of lung cell suspension were used to assess the bacterial load. For that, after a 10-minute incubation with 10% saponin, serial dilutions were plated on Middlebrook 7H11 agar (BD Biosciences) supplemented with OADC and Panta (BD Biosciences). The plates were then incubated at 37º C for 3-4 weeks.

Bacterial load was also assessed in the liver and spleen of infected mice. For that, the organs were aseptically collected to a Falcon tube with 5 mL cDMEM, and then homogenized. After a 10-minute incubation with 10% saponin, serial dilutions were plated on Middlebrook 7H11 agar (BD Biosciences) supplemented with OADC and Panta (BD Biosciences). The plates were then incubated at 37º C for 3-4 weeks.

3.14. Flow Cytometry

Myeloid and lymphoid cell populations from infected lungs were characterized by flow cytometry. The Abs used for cell surface staining are listed in the table below. After a 30-minute staining at 4ºC, cells were fixed with 2% PFA. Samples were acquired on a FACS Canto II flow cytometer (BD Immunocytometry Systems) with FACSDiva software (BD Bioscience). Data were analysed using FlowJo software (TreeStar).

4I2 Infection Experiment				
Antigen	**Color**	**Brand**	**Clone**	**Cat. Number**
CD8	FITC	Biolegend	5H10-1	100804
Ly6C	PE	Biolegend	HK1.4	128008
Ly6G	PerCpCy5.5	Biolegend	1A8	127616
CD11c	PE Cy7	Biolegend	N418	117318
7CD19	APC	Biolegend	6D5	115512
CD4	APC Cy7	Biolegend	GK1.5	100414
CD3	Pacific Blue	BD	500A2	558214

CD11b	V500	BD	M1/70	562128
6C4 Infection Experiment				
Antigen	**Color**	**Brand**	**Clone**	**Cat. Number**
CD8	FITC	Biolegend	5H10-1	100804
CD19	PE	Biolegend	6D5	115508
CD3	PerCpCy5.5	Biolegend	145-2C11	100328
CD11c	PE Cy7	Biolegend	N418	117318
CD4	APC	Biolegend	GK1.5	100412
Ly6G	APC Cy7	Biolegend	1A8	127624
Ly6C	Pacific Blue	Biolegend	HK1.4	128014
CD11b	V500	BD	M1/70	562128

Table 2: List of Abs used on the flow cytometry experiments, with the respective color, brand, clone and catalogue number.

3.15. Histology

Before collection, lungs were perfused in situ with PBS. For histology, the right upper lobe of infected and non-infected lungs was excised and fixed with 4% PFA for 1 week. The tissue was then embedded in paraffin and cut into 3-mm-thick sections. The samples were stained with H&E and subjected to microscopic morphological analysis.

3.16. Statistical analysis

Data are shown as Mean ± SD. Statistical tests used to compare experimental groups are described in the figure legends, and include Student's t-test, One-way ANOVA, or Two-way ANOVA. A p value < 0.05 was considered statistically significant. The graphs were prepared and analyzed using GraphPad Prism 5 (GraphPad).

Chapter IV:

Results

Part of this chapter was presented as a poster at the XLIII SPI Annual Meeting, June 2017, Porto

"Functional Impact of differential IL-1 signalling in tuberculosis"

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Results

4.1. Differential induction of cytokines by Mtb clinical isolates is reproduced in mouse cells

According to the data obtained from human PBMCs (Figure 7), the Mtb clinical isolate 4I2 was a much stronger inducer of cytokine production than 6C4. We were interested to understand the bacterial molecules or molecular mechanisms that contributed to such a difference. To that end, the mouse model of infection would be clearly advantageous over human cells, as the host-associated variability would be eliminated. Thus, we started by infecting mouse BMDMs with either 4I2 or 6C4 Mtb isolate and measured the cytokines on the supernatants of the infected cell cultures 24 hours post-infection, by immunoassay. As observed in human PBMCs, the Mtb isolate 4I2 induced a much stronger cytokine response by infected BMDM than that of 6C4, with significantly higher levels of IL-1β (Figure 8A), IL-1α (Figure 8B), TNF-α (Figure 8C), IL-10 (Figure 8D), and IL-12p40 (Figure 8E). This difference was especially evident in the cases of IL-1β and IL-1α, with 4I2 infection resulting in approximately 10 times higher cytokine induction than that observed upon 6C4 infection. Our findings show that the differences observed previously in the cytokine response of human PBMCs to Mtb isolates 4I2 and 6C4 are reproduced in mouse cells. Therefore, for the rest of the study, we used mouse macrophages as a platform to understand the bacterial molecular bases underlying the observed differences.

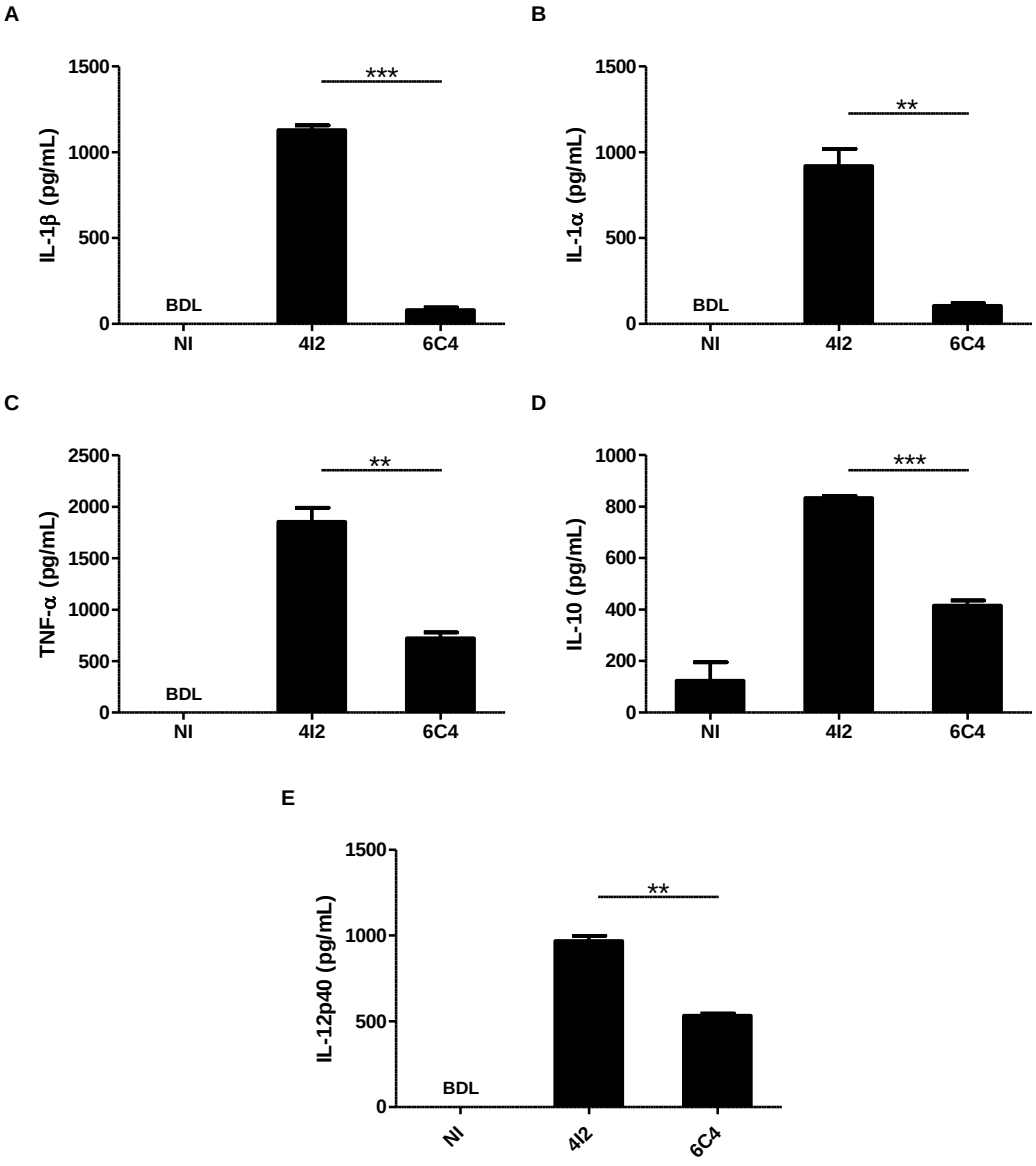


Figure 8. Different Mtb isolates induce a differential cytokine production by infected BMDM. WT mouse BMDMs were infected with either clinical isolate 4I2 or 6C4 at a moi of 2. Twenty-four hours post-infection supernatants were harvested and the production of IL-1β (A), TNF-α (B), IL-1α (C), IL-10 (D) and IL-12p40 (E) was assessed by ELISA. Each bar represents Mean ± SD of triplicate wells. BDL, below detection level. The statistical analysis, determined by Student's t-test reflects the differences between clinical isolates 4I2 and 6C4. ** $p < 0,01$; *** $p < 0,001$.

4.2. Differential cytokine transcription is induced by the two Mtb clinical isolates in infected mouse macrophages

To investigate the molecular mechanisms underlying the differences in cytokine production at 24h post-infection with Mtb isolates 4I2 versus 6C4, we first questioned if these differences were due to a differential induction of cytokine gene transcription. Thus, we quantified, by real-time PCR, the levels of mRNA of IL-1β, IL-1α and TNF-α at 1, 3

and 6h after infection of BMDMs with either isolate. We chose these cytokines as both IL-1β and IL-1α belong to the same transcriptional locus, whereas TNF-α is independent of the IL-1 genetic locus, thus being a control cytokine. We observed that infection with Mtb isolate 4I2 induced a significantly higher transcription of IL-1β (Figure 9A) and IL-1α (Figure 9B) than did infection with isolate 6C4, specifically at 1 and 6h post-infection. For TNF-α (Figure 9C) the same trend was observed, but the differences did not reach statistical significance. In all, the results indicated that infection with clinical isolate 4I2 triggers a higher transcription of IL-1β and IL-1α than that observed upon infection with isolate 6C4. However, these differences alone might not be sufficient to explain the differences observed for the levels of these secreted cytokines upon infection with either clinical isolate.

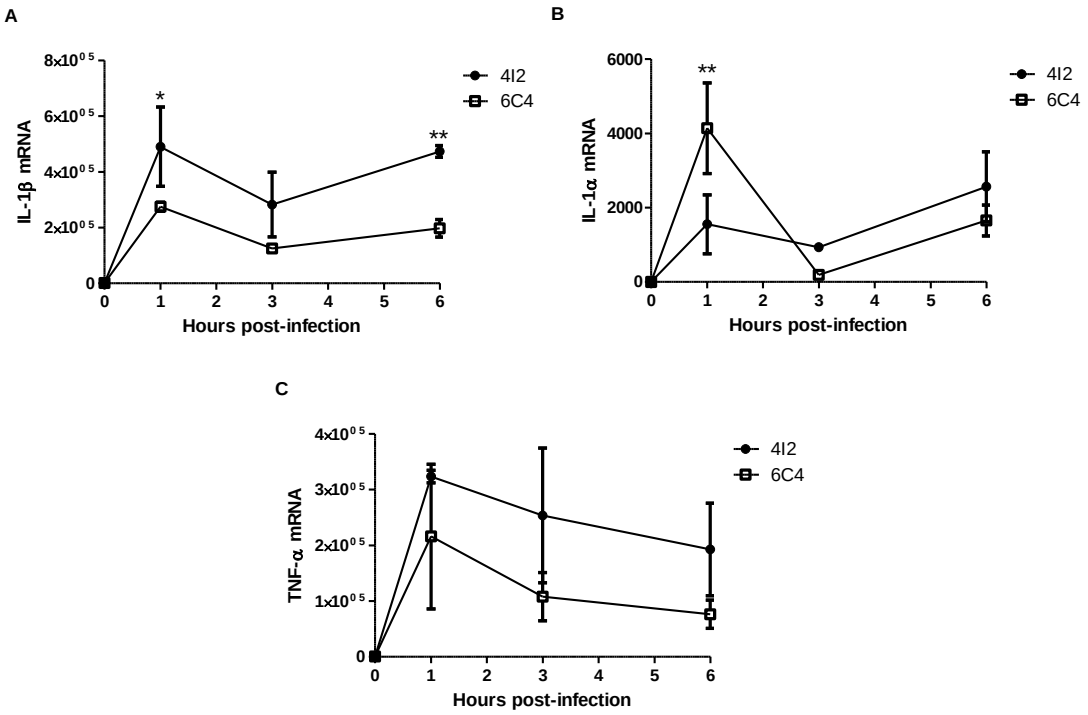


Figure 9. Distinct levels of transcription are induced by the two Mtb clinical isolates in infected BMDM. WT mouse BMDMs were infected with either clinical isolate 4I2 or 6C4 at a moi of 2, and 24 hours after infection 250 μL TRizol reagent was added to the wells to extract the mRNA from infected cells. IL-1β (A), IL-1α (B), and TNF-α (C) mRNA transcripts were quantified by real-time PCR relatively to ubiquitin. Each timepoint represents Mean ± SD of triplicate wells. The statistical difference between the infection with either clinical isolate was analysed by two-way ANOVA. * $p < 0,05$; ** $p < 0,01$.

4.3. Clinical isolates 4I2 and 6C4 differentially activate the inflammasome, resulting in distinct IL-1β production

The findings presented in the two previous sections suggest that although Mtb clinical isolate 4I2 is a more potent inducer of IL-1 transcription, the amount of protein detected 24h post-infection is much higher than the differences seen at the mRNA level. As mentioned in the introduction, the secretion of IL-1β by macrophages requires a series of steps, including its post-transcriptional cleavage by caspase-1[86]. This maturation process and caspase-1 activation result from the activation of the inflammasome complex[86]. Therefore, we assessed if the two clinical isolates could be inducing differential IL-1β secretion by infected macrophages, due to a differential activation of the inflammasome. To do so, we infected mouse macrophages with either isolate as before and in parallel chemically inhibited the NLRP3 inflammasome with isoliquiritigenin. Twenty-four hours after infection the supernatants of the cell cultures were collected and the production of IL-1β assessed by immunoassay. Interestingly, NLRP3 inhibition led to a marked decrease in the production of IL-1β upon infection by 4I2 and to an abolishment of its production upon 6C4 infection (Figure 10A). These findings suggest that both isolates trigger the inflammasome during infection of BMDM, but that the isolate 4I2 is a more potent activator of this complex. Furthermore, when the inflammasome is blocked, the isolate 4I2 is still a more powerful inducer of IL-1β, which is in line with the transcriptional differences found in Figure 9A. As controls, we also assessed the levels of IL-1α and TNF-α secreted by 4I2- or 6C4-infected macrophages upon treatment with the inflammasome inhibitor. The inhibition of the NLRP3 inflammasome, not only abolished IL-1β production by infected cells, but also led to a marked decrease in the levels of IL-1α (Figure 10C) and TNF-α (Figure 10D).

To further confirm the role of the inflammasome in the differential IL-1β production by macrophages infected with either 4I2 or 6C4 isolates, we next infected cells with Mtb isolate 6C4 in the presence or absence of nigericin, a known NLRP3 activator[102]. As shown in Figure 10B, the presence of the inflammasome activator rescued IL-1β production by macrophages infected with Mtb isolate 6C4. Together, these results strongly suggest a differential activation of the inflammasome during macrophage infection with Mtb isolate 4I2 or 6C4. They also suggest that a possible feedback loop maintained by high IL-1β secretion may be operating, which links high inflammasome activation to high IL-1β and to high secretion of other cytokines. These results lead us to

two main questions: what is the effect of these differences in the outcome of infection, and what are the bacterial determinants underlying these differences.

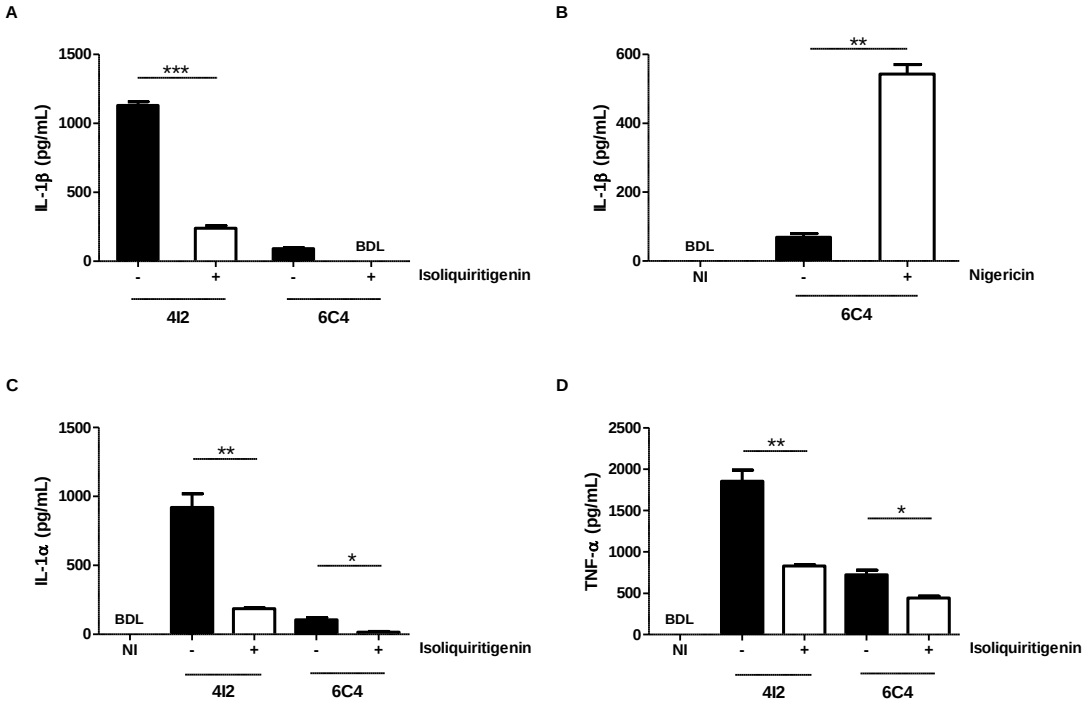


Figure 10. Differential inflammasome activation by either clinical isolate leads to different IL-1β production, and to a similar effect on other proinflammatory cytokines. WT mouse BMDMs were infected with either Mtb clinical isolate 4I2 or 6C4, and simultaneously treated or not with 10 μg/mL of isoliquiritigenin. Twenty-four hours after infection the supernatants were collected and the production of IL-1β (A), IL-1α (C) and TNF-α (D) was measured by ELISA. (B) WT mouse BMDMs were infected with Mtb clinical isolate 6C4, and simultaneously treated or not with 5 μg/mL of nigericin. Twenty-four hours after infection the supernatants were collected and the production of IL-1β was assessed by immunoassay. Each bar represents Mean ± SD of triplicate wells. Treated groups were compared to the respective untreated group of infection by Student's t-test. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$.

4.4. BMDMs are more efficient in controlling the intracellular growth of Mtb isolate 6C4 than that of 4I2

IL-1 signaling has been thoroughly described as a protective mechanism in TB, as it triggers several microbicidal mechanisms in the infected macrophage[127]. Thus, we sought to investigate if macrophages differ in their ability to control infection by either 4I2 or 6C4 clinical isolates. To investigate this issue, we infected macrophages with either clinical isolate and assessed the intracellular bacterial growth 3 days after infection, by CFU enumeration. Surprisingly, infected macrophages displayed a better control of Mtb isolate 6C4 as compared to 4I2 (Figure 11). Of note, in both cases, decrease of CFUs

between day 0 and day 3 was observed, suggesting activation of microbicidal mechanisms upon infection with Mtb isolates 6C4 and 4I2.

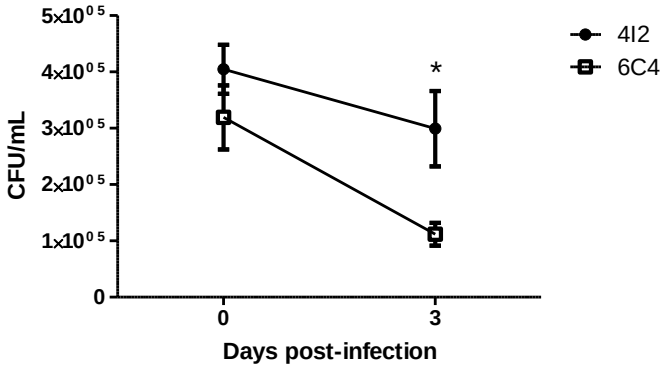


Figure 11. BMDMs are more efficient in controlling the intracellular growth of Mtb isolate 6C4 than that of 4I2. WT mouse BMDMs were infected with either Mtb isolate 4I2 or 6C4 at moi of 2. At 4 hours (day 0) and 3 days after infection, the intramacrophage CFUs were determined. Each timepoint represents Mean ± SD of 8 wells out of 2 experiments. The statistical analysis, determined by two-way ANOVA, reflects the difference between the two isolates. * $p < 0,05$.

4.5. Activation of different microbicidal mechanisms is triggered in BMDMs upon infection with the different clinical isolates.

To investigate the mechanisms underlying the better control of Mtb isolate 6C4 by infected macrophages, we started by quantifying a series of microbicidal mediators upon infection with either isolate, including NO, superoxide and hydrogen peroxide. Experimentally, we infected BMDMs with either clinical isolate 4I2 or 6C4 and measured the NO concentration 24 hours after infection by the Greiss method. Surprisingly, the production of NO was significantly higher upon infection with clinical isolate 4I2 as compared to isolate 6C4 (Figure 12A). However, and in line with a better control of infection, a higher quantity of superoxide was produced upon infection by isolate 6C4 as compared to 4I2, as observed in Figure 12B by a higher MFI of the DHR probe, which measures the production of this microbicidal anion. We also aimed to measure the hydrogen peroxide production by the DHE probe, but, due to technical issues, we were not able to evaluate this parameter. Together, these results suggest that different microbicidal mechanisms are triggered by infection with either clinical isolate, and that in some way the production of superoxide may help to control the infection by the 6C4

clinical isolate better than the production of NO. Further experiments are required to clarify this question.

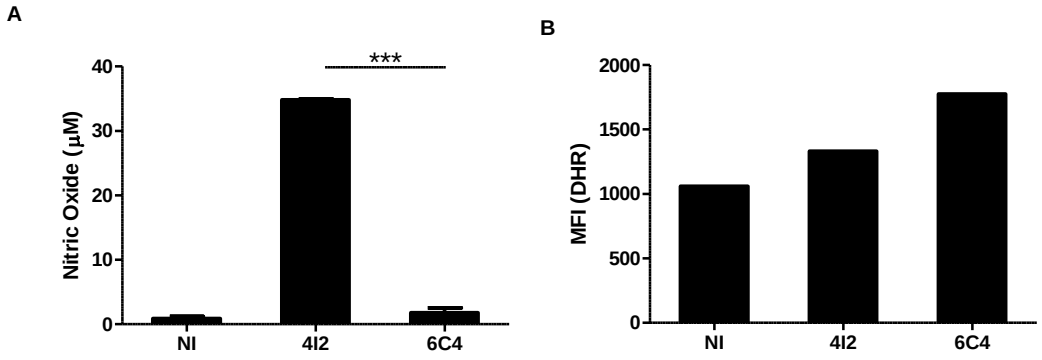


Figure 12. Differential activation of microbicidal mechanisms by 4I2 and 6C4 clinical isolates. (A) WT mouse BMDMs were infected with either clinical isolate 4I2 or 6C4. Twenty-four hours after infection supernatants were collected and the production of NO was measured by the Greiss method. Each bar represents the Mean ± SD of triplicate wells. The differences between the infected groups were assessed by Student's t-test. *** $p < 0,001$. (B) WT mouse BMDMs were infected with either clinical isolate 4I2 or 6C4. Twenty-four hours after infection cells from 2 wells were detached and pooled together, and incubated with 10 μM of DHR for 30 minutes at 37º C. After fixation with 4% PFA, the MFI of the DHR probe was assessed by flow cytometry, to evaluate superoxide production.

4.6. Differences in clinical isolates 4I2 and 6C4 impact the course of infection in an *in vivo* mouse model of experimental TB

Given the differences found for the macrophage response to infection with Mtb isolates 4I2 and 6C4, and given that the TB patients from where these two bacteria samples were isolated presented different clinical manifestations of disease, we next sought to compare the progress of infection associated with each Mtb isolate in a whole organism. For this, we resorted to the mouse model of experimental aerosol infection, previously used by our team[128]. So, we infected C57BL/6 mice with a low dose of either isolate by the aerosol route and started following them for a total period of 90 days after infection. First, we confirmed that mice were infected in an equal manner by each isolate, by assessing the CFUs at day 3 post-infection. Indeed, there was no difference in the bacterial burden on the lungs of mice infected by 4I2 or 6C4 at day 3 post infection (Figure 13). On days 30 and 90 post-infection, a group of animals were sacrificed and a series of infection read-outs were analysed. So far, only day 30 was performed, being the corresponding results described below.

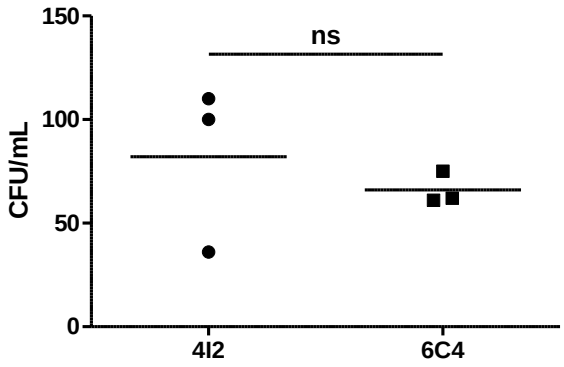


Figure 13. Aerosol infection of wild-type C57BL/6 mice showed similar initial doses for Mtb isolates 4I2 and 6C4. C57BL/6 mice were infected by the aerosol route with a low dose of either Mtb isolate 4I2 or 6C4. Three days after infection the lungs of 3 infected animals with either isolate were collected and bacterial burden was assessed by CFU enumeration. The different points in the graph represent each of the lungs infected with either isolate, and the horizontal lines represent the Mean. A Student's t-test was performed to evaluate differences in bacterial burden between the two groups and no statistical differences were observed between the two groups. ns, non-significant.

4.6.1. Infection with clinical Isolate 4I2 resulted in a higher burden in lungs, but that with isolate 6C4 showed a higher dissemination

On day 30 post-infection a group of infected mice, and a group of non-infected mice were euthanized. We assessed the bacterial burden in the lungs of the infected mice, the site of Mtb infection, and also in the liver and spleen to evaluate the dissemination of the bacteria. 4I2-infected mice had a significantly higher bacterial burden in the lungs when compared to 6C4-infected mice (Figure 14A). However, and very interestingly, the bacterial burden in the liver (Figure 14B) and spleen (Figure 14C) of 6C4-infected mice was higher than that of 4I2-infected animals. These results suggest that Mtb isolate 6C4 is better controlled than isolate 4I2 by immune mechanisms in the lung, but somehow evades these mechanisms and disseminates more efficiently to other organs.

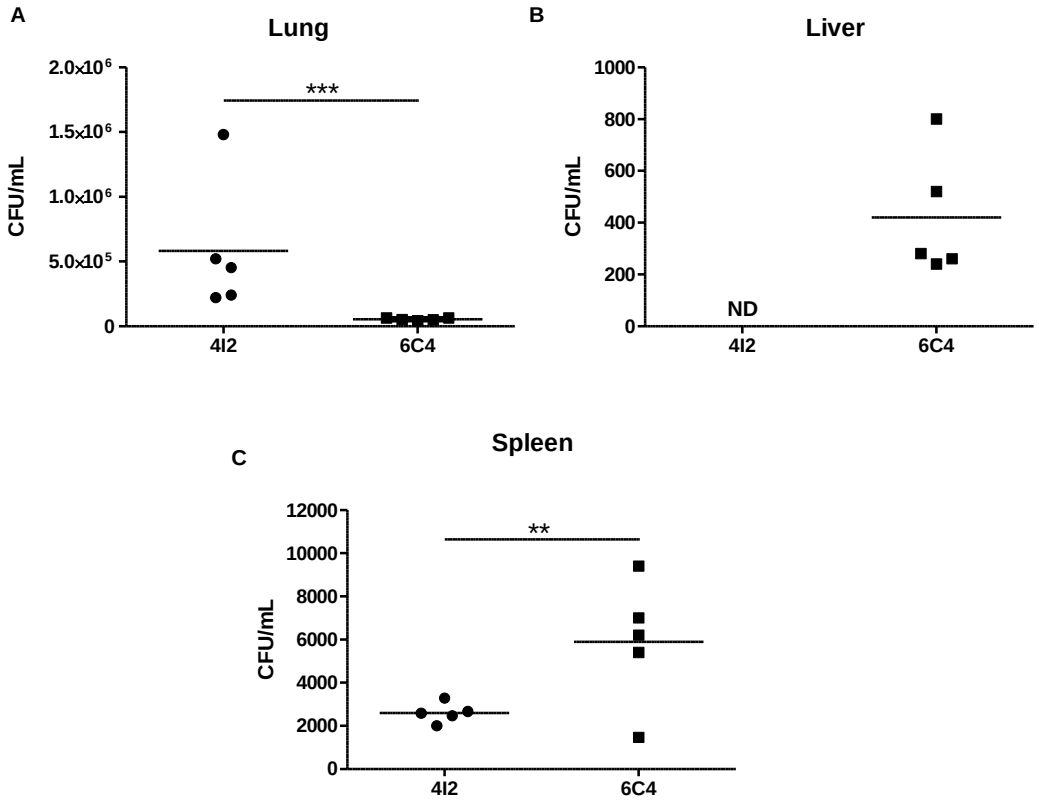


Figure 14. Infection with Mtb isolate 4I2 leads to a higher bacterial burden in the lungs, whereas that with isolate 6C4 results in a higher dissemination. C57BL/6 mice were infected by the aerosol route with a low dose of either Mtb isolate 4I2 or 6C4. Thirty days after infection the bacterial burden was determined for 5 infected animals in the lungs (A), liver (B) and spleen (C). The different points in the graph represent each of the organs infected with either isolate, and the horizontal lines represent the Mean of 5 animals of each group of infection. A Student's t-test was performed to evaluate differences in bacterial burden between the two groups of infection. ** $p < 0,01$; *** $p < 0,001$.

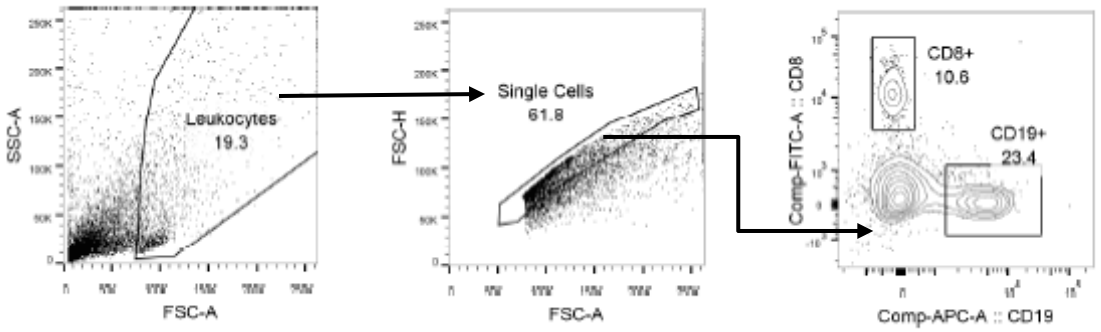
4.6.2. An adaptive immune response is well established at day 30 post-infection, with clinical isolate 4I2 leading to a higher B-cell recruitment

In addition to bacterial burden, we also analysed the dynamics of cell recruitment to the lungs of infected animals by flow cytometry. We used several surface markers specific for myeloid or lymphoid cells, including, respectively, CD11b, CD11c, Ly6G, Ly6C, and CD3, CD4, CD8 and CD19. On the flow cytometry analysis we started to gate the leukocyte population by forward-scattered (FSC) light and side-scattered (SSC) light, and within this population we excluded cell duplets an others, selecting only the single cell population (Figures 15A and B). We then gated the population of myeloid and lymphoid cells within the single cell population. Unfortunately, due to technical issues, we could not get a proper analysis for the myeloid cells. We could get a good analysis for the CD4 population, but only for the experiment with the isolate 6C4, therefore we

could not compare the populations of these cells between the infection with the two isolates. Therefore, we could only get a complete parallel analysis for both clinical isolates in what respects CD8 and CD19 cells.

Interestingly, 4I2-infected animals presented a significantly higher recruitment of $CD19^{+}$ B cells than 6C4-infected animals (Figures 15 A, B and C). On the other hand, during both infections, the recruitment of CD8+ T cells to the lungs was well visible (Figures 15 A, B and D). Altogether, there is an indication for an establishment of an adaptive immune response against the clinical isolates.

A



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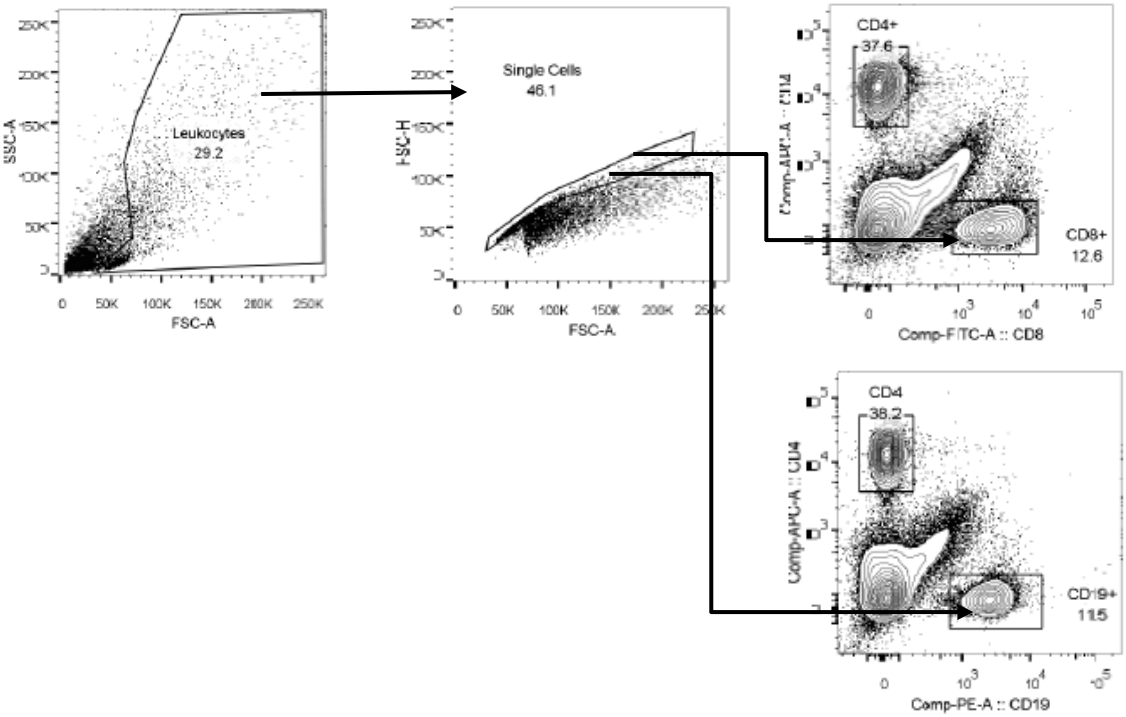

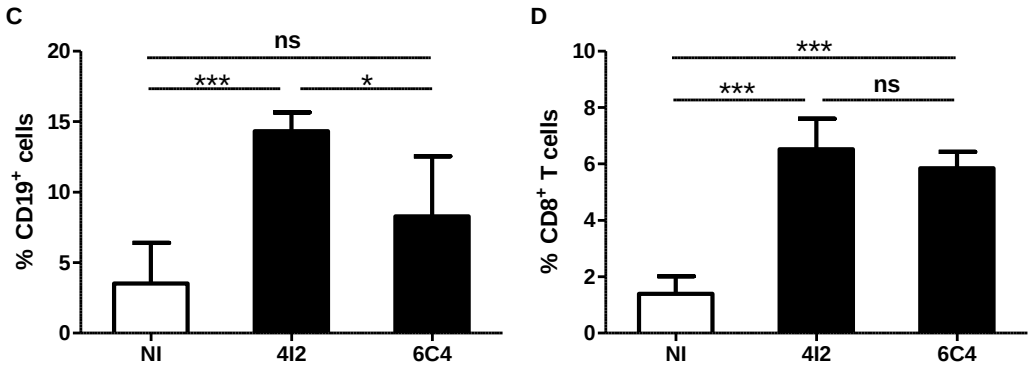

Figure 15. Establishment of adaptive immune response against Mtb isolates 4I2 and 6C4. Thirty days after low dose aerosol infection with either clinical isolate 4I2 or 6C4, lungs of 5 animals infected with either clinical isolates and 6 non-infected animals were collected. Cells of both infected and non-infected animals were stained with mixture of several myeloid and lymphoid markers for 30 minutes at 4ºC. After staining cells were fixed with 2% PFA for 20 minutes and then resuspended PBS with 2% FBS for analysis by flow cytometry. (A) (B) Representative image of the gating strategy for analysis of cell populations by flow cytometry in the lung of one animal infected by, respectively, clinical isolate 4I2 or 6C4. Images obtained by analysis on FlowJo v10.0.7r2 software. (C) (D) Percentage of $CD19^+$ B-cells and $CD8^+$ T-cells single cells, respectively, within the leukocyte population in the lungs of non-infected and 4I2 or 6C4-infected animals. Each bar represents the Mean ± SD of 5 infected animals in each group and 6 non-infected animals. The statistical analysis, performed by one-way ANOVA, refers to differences between all the experimental groups. ns, non-significant. * $p < 0,05$; *** $p < 0,001$

4.6.3. Both infected groups display inflammatory lesions in the lungs

Finally, we also evaluated the histological features of the lungs of infected mice and compared them with those of non-infected animals. There were clear inflammatory cell infiltrates on the lungs of mice infected by both 4I2 or 6C4 (Figures 16 A and B, respectively), whereas non-infected mice did not depict any signs of inflammatory cell infiltrates (Figure 16C).

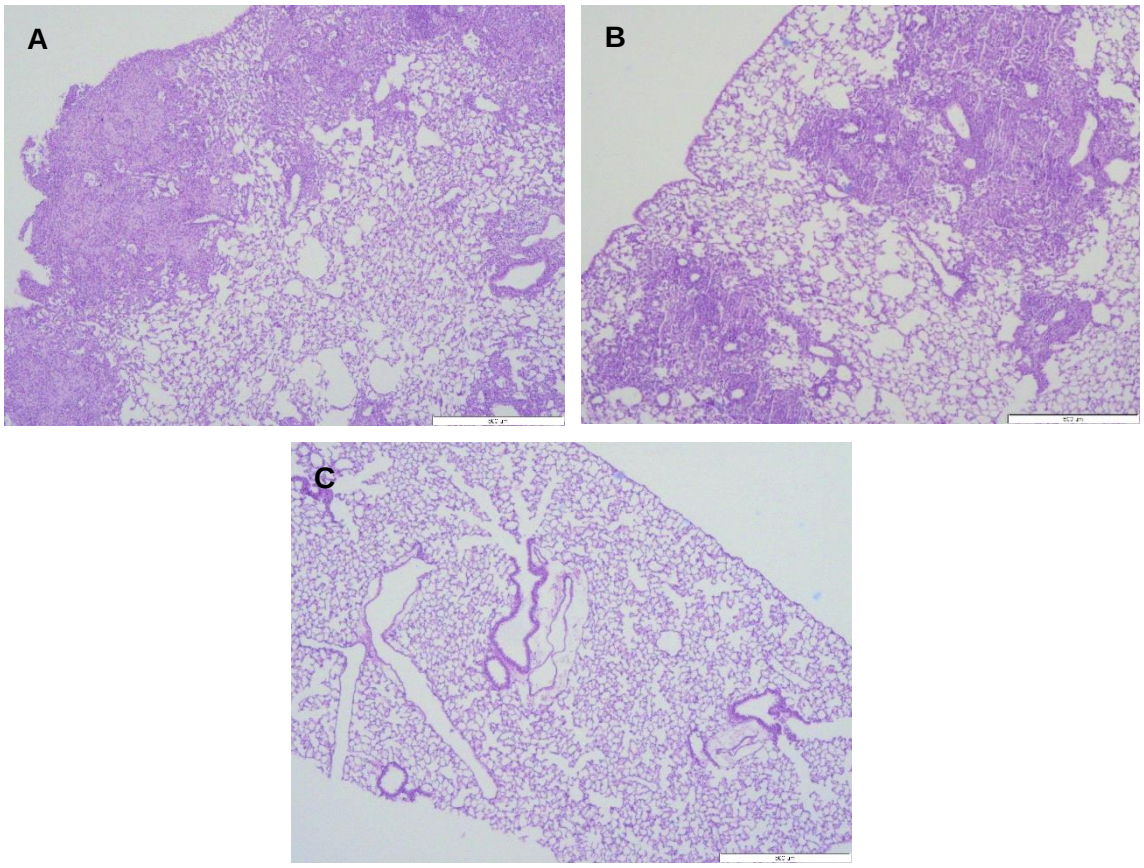

Figure 16. Mtb clinical isolates cause inflammatory lesions on lungs of infected mice. Thirty days after low dose aerosol infection with either clinical isolate 4I2 or 6C4, part of the lungs of animals from both infected groups and non-infected animals were collected and fixed for one week with 4% PFA. Afterwards, the tissue was embedded in paraffin and sections of the organ were obtained to perform an H&E staining. Images represent the microscopic observation of the lung of one animal infected with clinical isolate 4I2 (A) or 6C4 (B), and one non-infected animal (C), obtained at a 4x magnification.

4.6.4. Infected animals did not display differential signs of disease

To complete the analysis of the in vivo model of infection, we followed the weight of the animals over time and measure their hematocit on day 30 post-infection as disease metrics. So far, we observed a general gain of weight during the course of infection (Figure 17A), suggesting that the animals are coping well with the infection. In what respects any signs of anaemia, on day 30 post-infection, we observed no differences between infected and non-infected animals, nor between animals infected with the different isolates (Figure 17B). Together, this shows that, although the animals were sustained the infection and supporting bacterial growth at the organ level, they were still able to control the infection showing to general signs of disease progression.

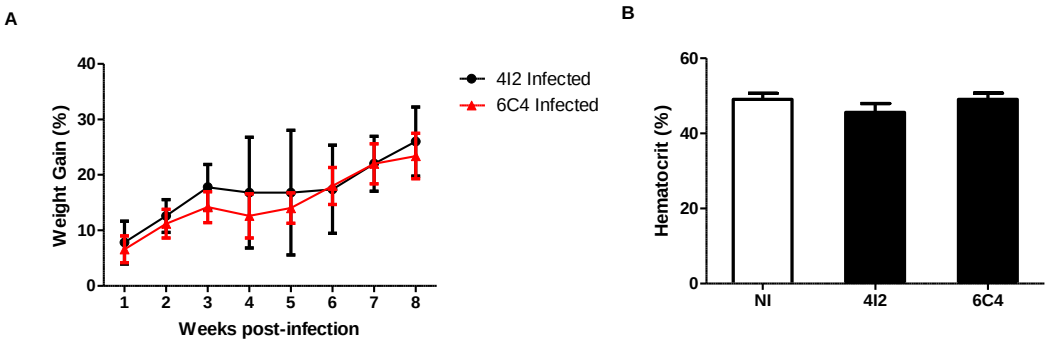

Figure 17. Infected animals did not display different signs of disease. (A) During the course of infection animals were weighted once a week. Each point of the lines represents Mean ± SD of 5 animals. (B) Thirty days after infection, blood from infected animal with either clinical isolate 4I2 or 6C4 was collected and the percentage of hematocrit was determined. Each bar represents Mean ± SD of infected animals of each group of infection and 6 non-infected animals. A statistical analysis was performed by one-way ANOVA, and no statistical differences were observed between the three groups of animals.

4.7. Differences between the clinical isolates are more likely related to functional rather than structural differences within the isolates

To investigate the bacterial determinants for the differences in inflammatory response induced by either clinical isolate, whole genome sequencing and analysis were performed, in collaboration with Dr. Nuno Osório, at Instituto de Investigação em Ciências da vida e da Saúde (ICVS). The genomes of isolates 4I2 and 6C4 were compared and only 338 single-nucleotide polymorphisms (SNPs) were found to distinguish the two bacteria. This close proximity between the 2 isolates is in line with their common phylogeny. Then, the 338 SNPs were analysed in a pathway-enriched manner, meaning that common functions linking the genes they were in were looked for. This analysis revealed that the genetic differences between the 2 Mtb isolates were mostly related to two major features of the bacteria: ATP binding and catabolic activity, and cellular compartment. The enrichment in SNPs in loci related to catabolic activity was slightly higher than that related to cellular compartment. This analysis thus suggests that differences both at the metabolic and structural levels could be linked to the distinct phenotype of the bacteria, namely to the differential activation of macrophages.

Taking the *in silico* data into account, we started to assess if the bacterial determinants for the differences in cytokine response by infected macrophages was of structural or metabolic origin. To do so, we heat-killed the two clinical isolates, by submitting them to a temperature of 80º C for 20 minutes. The inactivation of the inocula

was confirmed by plating the killed bacteria on 7H11 agar medium and confirming that no growth was visible after 1 month of incubation at 37ºC. We then used the heat-killed bacteria to stimulate BMDM and measured cytokine production 24h post infection. Interestingly, we observed that the differences in IL-1β production were completely abolished when BMDM were stimulated with heat-killed isolates (Figure 18). This result highly suggests that the differences in inflammasome activation and cytokine production require live bacteria and may therefore be due to differences induced on the cell state by the different isolates, rather than structural differences between them.

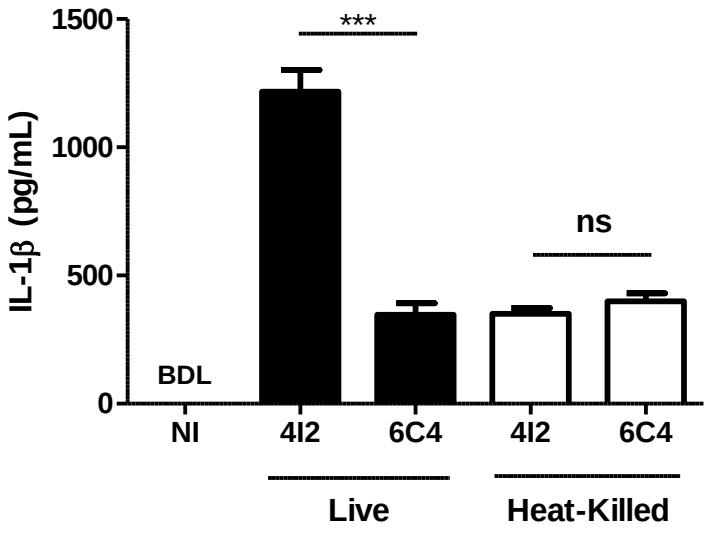

Figure 18. Heat-killing Mtb isolates 4I2 and 6C4 abolishes the differences in IL-1□ production induced by the two isolates. WT mouse BMDMs were infected with either Mtb isolate 4I2 or 6C4, or with their heat-killed counterpart at moi of 2. Twenty-four hours post-infection the supernatants were collected and the IL-1β production was assessed by ELISA. Bars represent Mean ± SD of triplicate wells. Statistical analysis, performed by Student's t-test, refers to differences within the live-infection group and within the heat-killed infection group. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$, ns = non-significant.

Chapter V: Discussion

Discussion

TB remains a major health concern worldwide, being the number one killing disease caused by a single infectious agent[6]. Every year millions of new cases of TB are reported, as well as millions of deaths as a result[7, 8]. Even more concerning is the fact that about 2 billion people are latently infected with the causative agent, Mtb, and it is estimated that 5-10% of these individuals can potentially develop the disease at any point of their lives, making them a very concerning reservoir[10]. In fact, this reservoir makes TB a theoretically impossible to eradicate disease.

Mtb, the infectious agent responsible for TB, belongs to a complex of bacteria of the same genus that affect other mammalian species[17]. The MTBC is highly stratified and the species of the complex are highly adapted to their respective hosts[33]. For Mtb specifically, there are numerous evidences of a coevolution with the Human populations for about 70 000 years. This coevolution and constant adaptation to the human host has led to the arise of 7 different phylogeographically distributed lineages [24]. The differences within lineages have been gaining a lot of focus on the last years, due to their links with the outcome of disease and the triggered immune response. TB is highly recognized as a very heterogenous disease[129], inducing very aggravating symptoms in some individuals[41], and causing only mild features of disease in others[42]. For many years, this heterogeneity was solely attributed to the host genetic variability, lifestyle and immune state[38, 39]. However, from several recent studies on the bacterial variability and its effects on the immune response, it became clear that the pathogen also plays a key role in determining TB heterogeneity[44, 45]. This intricate host-pathogen relationship calls for an integrative approach in which both factors from the host and the bacteria contributing to the outcome of disease are unveiled as whole.

In this context, our group has taken a phylogeny-based approach to try and take one step further in the understanding of the disease and in predicting the best response when someone is confronted with the pathogen. To do so, we retrospectively studied a cohort of patients from HSJ, their clinical files and the associated Mtb isolates. This has allowed us to grab a detailed picture of the phylogenetic distribution of the Mtb isolates in the geographic area of the study (B. Cá, in preparation). These clinical isolates were then used to infect PBMCs from healthy donors of the same geographic area. In this way, the phylogeographic adaptation was respected. The results showed a high heterogeneity in the cytokine response induced by the different clinical isolates, with some isolates being strong cytokine response inducers, and other isolates only leading

to a poor cytokine production (J. Sousa, on-going). The fact that the cytokine induction results were independent from the host suggested that there was a significant role of the bacteria in these differences.

This study aimed at investigating the bacteria molecular determinants responsible for shaping the host immune response, ultimately impacting on the outcome of disease. For that, we selected a high cytokine inducer clinical isolate, 4I2, and a poor cytokine inducer Mtb isolate, 6C4. We resorted to mouse BMDMs, to eliminate the host variability and consider only the bacteria intrinsic characteristics. We started by analysing the cytokine production by BMDMs 24 hours post-infection with either isolate. Our results showed the same pattern observed upon infection of human PBMCs. Mtb isolate 4I2 was a much stronger inducer of cytokine production in mouse BMDMs than isolate 6C4 (Figure 8), a difference that was most pronounced for IL-1β and IL-1α (Figures 8A and B). The essential role of these two cytokines upon Mtb infection has been increasingly reported on the past years, with double KO mice for these cytokines[71, 72], or MyD88 (the TLR and IL-1R adaptor molecule) KO mice displaying a severely decreased survivability when challenged with Mtb infection[66, 67, 117]. Therefore, not only the results from mouse BMDMs were in line with the results from human PBMCs, further proving that the bacteria play a central role on the intensity of the triggered cytokine response, but also highlighted a differential production of both cytokines from the IL-1 family.

Then, we proceeded our experiments resorting to mouse BMDMs and tried to find an explanation for the severe differences observed in IL-1 production. IL-1α and IL-1β are localized at the same genetic locus and their expression is regulated at the transcriptional level[84]. Thus, we sought to investigate if the clinical isolates could be inducing distinct levels of transcription of the IL-1 locus. In fact, we observed significant differences in transcription of IL-1α and IL-1β at 1 hour, and of IL-1β 6 hours post-infection with either clinical isolate (Figures 9A and B). We also assessed TNF-α as a control, and although a tendency for the same differences between infection with either clinical isolate was observed, there was no statistical significance (Figure 9C). Although the two clinical isolates induced different transcriptional levels, these differences are unlikely enough to explain a 10-time difference in IL-1α and IL-1β production.

Besides being regulated at the transcriptional level, IL-1 is also regulated post-translationally. IL-1β is produced in a biologically inactive form (pro-IL-1β), which needs to be cleaved by caspase-1 to be secreted and exert its functions. The complex that leads to caspase-1 activation and consequent IL-1β cleavage is the inflammasome[86].

We hypothesized that the differences observed upon infection with either clinical isolate could be due to a differential inflammasome activation by either isolate. To test this hypothesis, we inhibited the NLRP3 inflammasome with isoliquiritigenin and assessed the IL-1β production 24 hours after infection. Interestingly, by inhibiting the inflammasome, the IL-1β production upon infection with isolate 4I2 drastically decreased, and went below detection levels upon infection with isolate 6C4 (Figure 10A). Further supporting the role of the inflammasome in this setting, we were able to rescue the production of IL-1β upon infection with isolate 6C4 by providing an inflammasome activator during the infection (Figure 10B). We will now further explore the differential activation of the inflammasome by these two Mtb isolates. For that, and since the efflux of K^+ is a necessary condition for the NLRP3 inflammasome activation[102, 106-108], we will block the NLRP3 inflammasome with a gradient of KCl, specifically inhibiting NLRP3 and further proving the differential activation of this inflammasome by either clinical isolate. Additionally, and since the AIM2 inflammasome was also implicated in the context of Mtb infection[91], we shall assess the implications of specifically inhibiting this complex upon infection with Mtb isolates 4I2 and 6C4. Furthermore, we will try to figure out what are the exact components of the inflammasome pathway that are related to the differences observed, by inhibiting each of the steps of the pathway with small interfering RNA (siRNA).

Additionally, a difference on the gene Rv0198c, also known as *zmp1*, encodes a zinc metalloprotease that can inhibit the inflammasome activation and, consequently, inhibit IL-1β production[130] could be playing a role on the differences we observed. This could be the reason for the different IL-1 production. However, an analysis of the coding and regulatory differences of this gene in the two clinical isolates performed by our collaborator Dr. Nuno Osório at ICVS showed no differences between the isolates, suggesting this is not the mechanism by which Mtb differentially shapes IL-1 production.

On the other hand, the differential production of IL-1 could be linked to differential type-I IFN production. There are evidences that on the context of Mtb infection there is a cross talk between IL-1 and type-I IFNs, with type-I IFNs inhibiting IL-1α and IL-1β production, through induction of IL-10 production[68, 131]. We are going to measure type-I IFN production upon infection by either isolate 4I2 or 6C4. However, we think this hypothesis is unlikely, because, as we can see by Figure 8D, the production of IL-10 was higher upon infection by isolate 4I2.

IL-1β has been described as having an autocrine action in macrophages, and causing a feed-forward loop on other proinflammatory cytokines[132]. In line with this, we observed that the inhibition of the inflammasome influenced the production of IL-1α and TNF-α 24 hours after infection (Figures 10C and D). As neither IL-1α nor TNF-α secretion depends on inflammasome activation, we will investigate if our findings do relate to an autocrine effect of IL-1β. For that, we will assess the production of these cytokines upon inflammasome inhibition during infection with isolate 4I2, or upon inflammasome activation upon infection with isolate 6C4. Furthermore, we will infect BMDM generated from IL-1R KO with 4I2 and assess the production of the different cytokines. We will also infect BMDM with isolate 6C4 in the presence of exogenous recombinant IL-1β and again assess the production of the different cytokines. All these experiments should lead us to conclude on any feedback loops provided by IL-1β in infected BMDM.

Following the observation that differential production of IL-1β was triggered upon infection of BMDM by either clinical isolate, we questioned what was the effect of these differences for the outcome of infection. We started by assessing if there were any differences in the intramacrophage growth of the two clinical isolates. For that we performed a 3-day BMDM infection with either clinical isolate. Surprisingly, there was a significantly better control of bacterial burden upon infection by isolate 6C4 (Figure 11), indicating that a more efficient microbicidal response may be triggered in BMDMs in this case. This was surprising, considering that a lower IL-1 was detected upon infection with 6C4 and that IL-1 has been reported to be involved in microbicidal mechanisms induction[127]. To gain mechanistic insights on this matter, we investigated some microbicidal mechanisms known to be in place in macrophages, namely the production of NO, superoxide and hydrogen peroxide species. Surprisingly, we observed a higher production of NO 24 hours after infection with clinical isolate 4I2 (Figure 12A), which may seem contradictory to a lesser control of this isolate. However, it is possible that this higher NO production might be a result of a feedback loop induced to control the activation of inflammasome and the production of IL-1β. As mentioned earlier, the NLRP3 is negatively regulated by IFN-γ, through the induction of iNOS to produce NO that on its turn nitrosylates NLRP3, inhibiting the inflammasome[75]. Thus, the NO production by 4I2-infected BMDM might be a result of a negative feedback to control an early potent production of IL-1β in response to infection by this clinical isolate. Measures of NO production at earlier and later timepoints during infection of BMDMs should give a better picture of the control of this microbicidal mechanism during the 3-day infection of

BMDMs. We also assessed production of ROS. Unfortunately, due to technical issues, we failed to detect hydrogen peroxide species and could only measure superoxide production. Interestingly, and in line with a better control of infection, the production of superoxide by BMDM was higher upon infection with clinical isolate 6C4 (Figure 12B). Since we have seen that isolate 6C4 is better controlled, these results might indicate that the production and microbicidal action of superoxide might overcome the action of NO, however, further replicates of the experiment will have to be performed, to try to assess the production of hydrogen peroxide, but also to gain statistical power. If these findings hold, we will reverse the effect of superoxide by blocking NADPH, the electron donor in the reaction that leads to superoxide production, catalysed by NAPDH oxidase[133], in order to observe if in the absence of superoxide the two isolates are controlled in similar extent. We will also assess if the control of intramacrophage growth is really related to IL-1. To do so, we will make several approaches, including providing nigericin or recombinant IL-1β upon infection with 6C4, or, on the other hand, administrating isoliquiritigenin upon 4I2 infection. Should IL-1 play a role in controlling the intramacrophage growth of the isolates, the differences in control of bacterial growth between infection with the two isolates will be abolished by these treatments.

Once we observed all these differences in the early stages of the immune response, we were interested in understanding if these differences were reflected in the outcome of infection and disease in a whole organism. To do so, we resorted to a mouse model of infection and are following mice infected with either isolate for a period of 90 days. For the purpose of this thesis, only data on day 30 was included. Interestingly, 30 days post-infection, there was a clear difference in the bacterial burden observed for either isolate. Although Mtb isolate 4I2-infected mice presented a much higher bacterial burden in the lungs, isolate 6C4 could disseminate a lot better than isolate 4I2, even afflicting the liver, a non-lymphoid organ (Figures 14A, B and C). This observation is interesting because isolate 6C4 was collected from a patient with a severe case of TB, highly symptomatic, with high c-reactive protein (CRP) and an advanced X-ray. Isolate 4I2 on the other hand came from a low symptomatic patient, with a mild X-ray and, in general, a mild TB. So, it is tempting to speculate that although the early local control of isolate 6C4 is more efficient (which is in line with our BMDM data), this isolate may be evading the immune system at a different level, which culminates with dissemination of the bacteria and a more severe disease. Further experiments are needed to follow up this hypothesis. We are currently starting another 90 day infection protocol similar to the one presented in this study, but along with BL6 mice, we are also using C3HeB/FeJ mice,

a mouse model of susceptibility to Mtb infection[134], and Rag KO mice. C3H mice will allow us to make a better parallel with the human infection, since these mice develop necrotic granulomas[134], and thus, allow a better comparison with the human scenario, providing us information about the stages of development and establishment of disease in a more comparable way than BL6 mice. Furthermore, they provide us information of how drastic the infection is when the acquired immunity is not adequate. The Rag KO mice, which are deprived of adaptive immunity[135, 136], will help us clarify the role and importance of the innate immunity mechanisms and how differently they triggered by either clinical isolate.

To gain further insights on the disease outcome, we are assessing other disease metrics than bacterial burden. We performed a histological analysis of the lungs of mice infected with either isolate, and non-infected mice in order to assess differences in tissue damage caused by the infection. We observed that lungs from mice infected with either clinical isolate, 4I2 or 6C4, presented a reasonable amount of inflammatory infiltrates concomitant with the recruitment of myeloid and lymphoid immune cells (Figures 16 A and B). Although we have seen higher bacterial burden upon infection with isolate 4I2, there were no significant differences observed in the extent of the immunopathology induced by either isolate. As we have detected a higher dissemination upon infection with clinical isolate 6C4, it is possible that isolate 4I2 is more easily contained in small niches in the lung, or, on the other hand, that isolate 6C4 has a better capacity to spread not only through the organism, but through the lung itself, creating more foci of infection in the whole organ. Analysis of histology at day 90 post-infection should provide us more information and allow us to better assess if there are significant differences in tissue damage induced by infection with either isolate. We also assessed weight gain/loss and anaemia as metrics of disease. As figures 17 A and B show, all the animals had a general gain of weight, and none of them showed signs of anaemia, indicating that although showing progression of infection at the site of infection and two other organs, the mice were in general able to control and survive the infection. This fact is in line with BL6 being a TB resistant strain[137].

Another aspect that we tackled during the *in vivo* infection was the recruitment of immune cells for the lung of infected mice. For the analysed time point, we failed to analyse the populations of $CD4^+$, which would give us a better picture of the differences in cell-mediated immunity given their critical role in controlling TB[138] and key participation in granuloma formation[139]. Thus, for the purpose of this thesis, only differences in B cell recruitment will be discussed. Interestingly, upon infection of mice

with isolate 4I2, there was a significantly higher recruitment of B-cells to the lungs compared with infection by isolate 6C4 (Figures 15A, B and C). This observation may be a consequence of differential bacterial load in the mice infected with either isolate, something that we will assess at later time points. However, this observation is still quite striking, as B-cells are mostly recognized in adaptive immunity by their capability to produce Abs, which, due to their extracellular localization, are often seen as not having an importance in the combat of intracellular pathogens like Mtb[140]. However, a recent row of evidences has pointed for a role of both B-cells and Abs in the protection against TB. For instance, a study from Pethe *et al* has shown that passive Ab transfer can lead to a reduction of dissemination of Mtb[141]. Further supporting this, several studies found lower levels of Abs against Mtb antigens in children and adults with disseminated TB compared to individuals with focused pulmonary TB[142, 143]. B-cells on their turn can also modulate the immune response against Mtb, by producing a wide variety of cytokines and by their ability to present antigens[144]. Therefore, perhaps the higher presence of B-cells in the lungs of mice infected with the 4I2 isolate is preventing, at least in part, the dissemination of the bacteria, thus having a protective role on the disease. This protective role of B-cells or Abs could be further investigated by neutralizing this cell population *in vivo*, by for instance administration of Abs against CD19, or Abs against Fc receptors present on other cells, and observing if mice display a higher dissemination of the 4I2 isolate.

The final question addressed in this thesis was: what were the bacterial determinants for the differences we observed in inflammasome activation? As aforementioned inflammasome triggering by pathogens can be performed both by structural features of the microorganism, but also by signals of microbial origin or damage induced by the pathogen[104, 105]. To gain a first general insight on the differences between the two clinical isolates, we resorted to bioinformatic tools to analyse them. From the WGS analysis, we concluded that the two isolates are only distinguishable by as little as 338 SNPs, which is quite a small fraction of variation, considering that the genome of the standard Mtb strain, H37Rv, comprises as much as 4,411,529 base pairs[145]. This further evidences that very little variability within the Mtb isolates can lead to dramatic changes in the phenotype. Of these 338 SNPs, there was an especially high enrichment on polymorphisms in regions predicted to be related with ATP binding and catalytic metabolism. This is quite striking, considering that NLRP3 assembly can be triggered by changes in ATP production or ATP influx via the ATP receptor P2X purinoceptor 7 (P2X7)[146, 147]. It may be that these polymorphisms in the regions related

to ATP binding and catalytic metabolism might have a phenotypic impact leading to a differential inflammasome activation by either isolate. Also, although to a smaller extent, there was an enrichment in regions related to cell wall components. Altogether, these data suggested that both functional and structural features of the isolates could be related to the differential inflammasome activation, with perhaps a slightly higher indication for functional features. Our first approach to disentangle this problem was to study the response of BMDMs to live or heat-killed bacteria, in what regards IL-1β production. Quite strikingly, the differences in IL-1β production observed upon infection with the live isolates were completely abolished when we heat-killed them (Figure 18), indicating that the differential inflammasome activation by different isolates is related to functional differences within the isolates rather than structural differences. We are currently looking forward to further prove this finding by metabolically inactivating the isolates, by submitting them to high concentrations of rifampicin. This is important as it would allow for the maintenance of the bacterial wall, which is not guaranteed with the heat-killing method. Furthermore, as we cannot eliminate the structural hypothesis, we are also going to perform a cell wall analysis on the two isolates, to assess if there is any interesting feature that might have a role on the results we observed so far.

In summary, we here show that small variability within highly related Mtb isolates plays a major role on the outcome of infection and disease. We also show that bacteria-manipulated differential activation of the inflammasome and consequently of IL-1β production by host cells likely impacts the outcome of infection at the macrophage, but also the whole organism level. Since our findings suggest that different functional phenotypes may be derived from small genomic polymorphisms, this work opens new research avenues in which specific bacterial pathways may be targeted to ameliorate TB outcome and also to develop novel molecular tests for disease outcome prognosis, based on bacterial factors. Finally, understanding how different thresholds of the immune response can balance protection and pathology is critical to understand the protective bases of immunity during TB, which in turn will guide novel vaccines and therapies to this devastating disease.

Chapter VI: References

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