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## **Regulation of the Immune Response by *Mycobacterium tuberculosis* Beijing Genotype**

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### **1. Introduction**

Tuberculosis (TB) is an old infectious disease that has affected humans for millennia [1]. This disease is caused by infection with bacilli belonging to the *Mycobacterium tuberculosis* (Mtb) complex and is responsible for 1.3 million deaths around the world, making TB the second leading cause of death by infectious disease caused by a single pathogen. In addition to this high number of deaths, a large proportion of individuals present latent TB infection (LTBI): it is estimated that at least one third of the human population could have LTBI [2]. Why more than 90% of the individuals infected with Mtb do not develop the active form of the disease is unclear, although the immune status of the individual seems to play a crucial role for Mtb containment [3]. This is evident in individuals with Human Immunodeficiency Virus co-infection, who are more prone to develop active TB: a quarter of TB deaths occur in individuals co-infected with HIV [2].

However, other factors are associated with TB development, such as the host environment and the genetic diversity of Mtb. This genetic diversity has been organized into genotypes and lineages, according to the genetic profiles obtained by techniques such as IS6110 fingerprinting, spoligotyping and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR). Interestingly, these genotypes show a differential distribution around the world and exhibit different levels of virulence [4]. One of the genotypes that has attracted more attention in recent years is the Beijing family, because it is highly prevalent among TB

patients, it favours the spread of multidrug-resistant tuberculosis, and it has been suggested that BCG vaccination confers poor protection against it [5].

It is not clear why the Beijing genotype prevails in infected individuals. One factor that could be responsible for this phenomenon is the interaction that Mtb Beijing establishes with the immune response. In this chapter, we analyze and discuss current knowledge regarding the cellular and molecular mechanisms of the immune response to Mtb, and how these mechanisms are affected by the Beijing genotype in relation to human infections.

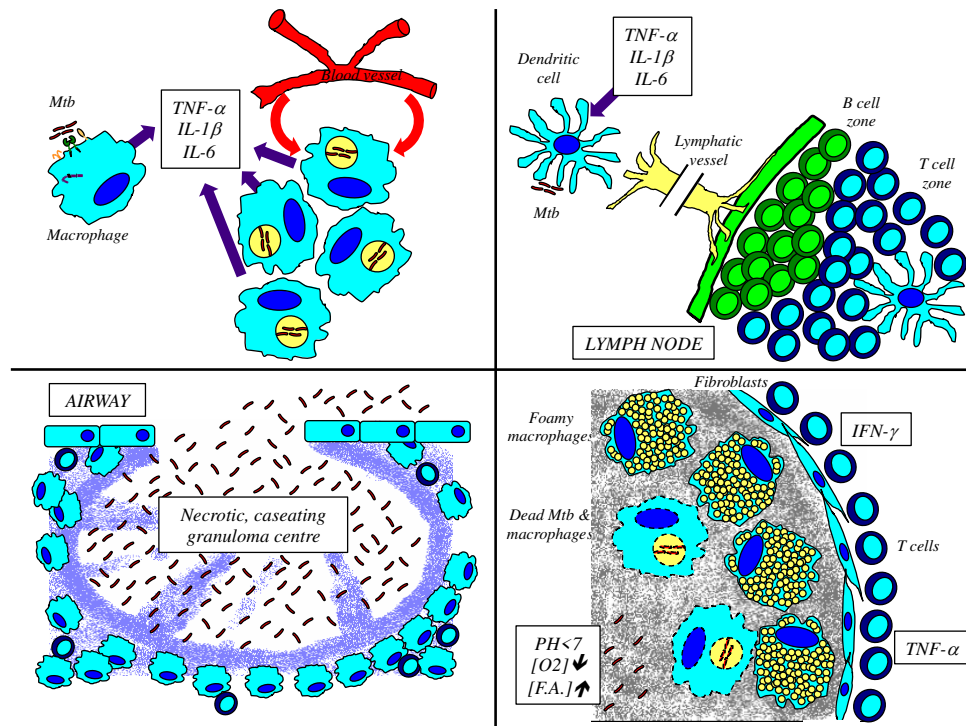
## 2. Immune response to *Mycobacterium tuberculosis*

Mtb can enter the body through several routes, but the most usual one is through the airways [6]. This process is initiated by the release of small saliva droplets containing Mtb bacilli that are expelled by active TB patients [7]. Some droplets are small enough to cross the mucociliary barrier and reach the alveoli, where they interact with the first cellular element of the immune response, the alveolar macrophage [8]. Macrophages are armed with a vast array of innate immune receptors that allow direct recognition of Mtb; these receptors include Toll-like receptors (TLR), Nod-like receptors (NLR), C-type lectin receptors (CLR) and scavenger receptors [9]. After recognition, macrophages ingest bacilli by phagocytosis, but they are unable to kill them due to the evolutionary mechanisms acquired by Mtb that actively inhibit phagosome maturation and protect Mtb from destruction by toxic compounds, such as reactive nitrogen and oxygen intermediates and hydrolytic enzymes from lysosomes; these mechanisms allow intracellular Mtb survival and proliferation [10, 11].

In addition to this intracellular proliferation, Mtb also induces a strong inflammatory response, characterized by macrophage production of cytokines like TNF- $\alpha$ , IL-1 $\beta$  and IL-6, which promote the recruitment of more macrophages from the blood [12]. The recruited macrophages are able to ingest bacilli but, like alveolar macrophages, they are unable to eliminate the ingested Mtb. The continuous recruitment of macrophages during these early stages provides a constant supply of cells susceptible to infection, as well as an increase of inflammatory signals that induces the recruitment of more susceptible cells from the blood, without causing significant tissue damage. At the same time, lung resident dendritic cells (DCs) engulf bacilli or their derived products; the inflammatory environment promotes DCs migration from the lungs to the draining lymph nodes, where they activate Mtb antigen-specific naïve T cells and induce a strong T cell response characterized by proliferation and activation of Mtb antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells [13]. These T cells travel through the blood and are recruited to the infection site by the ongoing inflammatory process.

Upon recognition of the infected macrophages, T cells produce significant amounts of TNF- $\alpha$  and IFN- $\gamma$ , the cytokines that characterize a Th1 response. TNF- $\alpha$  and IFN- $\gamma$  increase the microbicidal mechanisms of the infected macrophages, which eventually leads to the intracellular killing of Mtb. Macrophage activation can be limited by the secretion of IL-4, IL-10 and TGF- $\beta$  produced by Th2 and T regulatory (Treg) cells [14-16]. The Th1 response, however, does not lead to a complete elimination of Mtb, but to the formation of an organized cellular

structure, the granuloma, where live mycobacteria persist in a dormant state known as latent infection [17]. The structure of the granuloma varies, but usually its center is formed by caseum, a structure that contains abundant remains of dead cells and forms a hypoxic, acidic and lipid-rich environment that limits extracellular Mtb proliferation. The caseum is limited by highly activated macrophages (multinucleated Langhans cells and foamy macrophages) surrounded by CD4+ and CD8+ T cells [18]. While the host immune response is unaffected, the granuloma structure is preserved and Mtb is contained within; however, the alteration of crucial elements of the immune response, such as the decrease of CD4+ T cells during HIV infection or the blockade of TNF- $\alpha$  by therapeutic antibodies, causes disorganization of the granuloma structure, caseum liquefaction and oxygen access, which promotes Mtb extracellular proliferation and dissemination into the airways, from where Mtb can reach susceptible hosts [19-21] (Figure 1).



**Figure 1. Development of the immune response to *Mycobacterium tuberculosis*.** The main four stages of the immune response to *Mycobacterium tuberculosis* (Mtb) are depicted. Macrophages are the first component of the cellular immune response that is encountered by Mtb (top left); they are used by Mtb to replicate and to induce an inflammatory response that allows the recruitment of more macrophages susceptible to infection. Dendritic cells (top right) carry Mtb antigens from the infection site to the draining lymph nodes, to activate antigen-specific T cells. The mycobacterial granuloma (bottom right) plays a fundamental role in Mtb containment. Alterations in the dynamic equilibrium of the granuloma (bottom left) promote disorganization and access of extracellular Mtb to oxygen, which allows its replication and dissemination through the airways.

### 3. *Mycobacterium tuberculosis* Beijing genotype

Because Mtb strains present over 99 % of similarity at their nucleotide sequence level and have identical sequences of their 16S RNAs [22, 23], for several years it was thought that they were genetically homogeneous and lacked genetic diversity. However, the development and use of different genetic tools for the study of Mtb revealed greater diversity. Studies that analyzed repetitive DNA elements in Mtb led to the discovery of high polymorphism in several strains. The study of restriction fragment length polymorphism (RFLP) in IS6110 allowed van Soolingen *et al* to identify a group of strains, isolated in Beijing, Mongolia, South Korea and Thailand, which shared a unique RFLP pattern. Since the majority of these strains came from Beijing, they became known as the “Beijing family”. Further genetic characterization showed that all these strains shared an identical spoligotyping of the direct repeat (DR) region, and similar patterns of polymorphic GC-rich sequence (PGRS) as well as an IS1081 restriction fragments [24]. Mtb strains with these genetic characteristics were later found outside the Southeast Asian region, in Russia, Spain, the United States, Cuba, Peru, Colombia, Iran, Israel and South Africa [5, 25].

Beijing strains were classified as “typical”, if they presented one or more IS6110 insertions in the noise transfer function (NTF) region, or “atypical”, if they lacked this insertion [26, 27]. Analysis of large sequence polymorphisms (LSP) indicated that Beijing strains could be divided into four different lineages, depending on the presence of RD105, RD142, RD150 and RD181 [28]. Further studies, using PCR to analyze 40 different loci in Beijing strains, showed that this group could also be divided into seven different sub-lineages, thus revealing great genetic diversity in this group [29].

### 4. Clinical importance of *Mycobacterium tuberculosis* Beijing genotype

In addition to the high prevalence of Beijing strains in the Southeast Asian region, Beijing strains have been associated with outbreaks in different parts of the world. The strains that were responsible for a TB outbreak in New York in the 1990's were from the Beijing genotype, and they were more frequent in patients co-infected with HIV, reaching a mortality rate of more than 80%. Interestingly, strains from this outbreak had multidrug resistance to isoniazid, rifampicin, streptomycin, ethambutol, ethionamide, rifabutin and kanamycin, favouring treatment failure [30-32]. Later studies showed the global emergence of this genotype in different parts of the world [25, 33, 34].

Several epidemiological studies have documented the hyper-virulence of the Beijing genotype. For example, patients infected with Beijing strains have increased rates of treatment failure and relapse [35-39]. In line with these studies, Kong *et al* showed that patients infected with Beijing strains developed more extra-thoracic TB than patients infected with other genotypes [40]. Other studies demonstrated that individuals infected with strains from the Beijing genotype were more likely to progress to active TB [41, 42] and had increased drug resistance [43, 44]. Furthermore, patients infected with these strains were younger than patients infected



with other genotypes [45], and they developed febrile responses in earlier stages of treatment [46]. Moreover, infection with Beijing strains was associated with HIV co-infection [47] and BCG vaccination [48]. However, others have been unable to find an association between infection with Beijing strains and the aforementioned clinical manifestations of the disease [38, 45, 49-54].

Few studies focused on the immune response have been conducted in patients infected with Beijing strains. Sun *et al* found that the sera of patients infected with Beijing and non-Beijing strains had similar levels of IFN- $\gamma$  and IL-18, while peripheral blood mononuclear cells (PBMCs) expressed similar mRNA levels for IFN- $\gamma$ , IL-2, IL-18. However, patients infected with Beijing strains had decreased levels of IL-4 [55]. In contrast with these results, Rakotosamimanana *et al* observed that PBMCs from patients infected with the “modern” Mtb strains from Beijing and Central Asia produced less IFN- $\gamma$  in response to ESAT-6 than PBMCs from patients infected with other genotypes [56]. These results suggest that Mtb Beijing genotype could induce an altered immune response that could favor infection by these strains.

## 5. Effects of *Mycobacterium tuberculosis* Beijing genotype on the immune response: *in vivo* models

The first evidence of the hyper-virulence of the Mtb Beijing genotype came from studies with different mouse models of infection. Manca *et al* showed that the Beijing strain HN878, isolated from cases from an outbreak in Texas, led to higher bacterial loads in the lungs and to accelerated death in immune-competent mice, compared to a non-Beijing strain. Moreover, the lymph node and spleen cells from HN878-infected mice showed decreased proliferation and IFN- $\gamma$  production after *in vitro* re-stimulation, suggesting a defective induction of a Th1 response because of the early induction of type I IFN by HN878 [57, 58]. Later studies showed that the decrease in the Th1 response was associated with the induction of Treg cells in mice and also in guinea pigs, an animal model that resembles human tuberculosis [59, 60]. Intracisternal infection of rabbits with Mtb leads to the development of tuberculous meningitis. In this model of infection, the Beijing strains HN878 and W4 led to higher bacterial loads in the cerebrospinal fluid and brain, increased dissemination to the lungs and liver, earlier leukocytosis, increased levels of TNF- $\alpha$  during the later stages of the infection, and severe clinical manifestations, compared to the low-virulence strain CDC1551 [61].

López *et al* reported that three Beijing strains, representing the predominant genotypes in Asia, were hyper-virulent in a mouse model of progressive pulmonary tuberculosis, compared to other genotypes (Somali, Haarlem, Canetti and H37Rv). This hyper-virulence was reflected as an increased number of bacteria in the lungs and a rapid death of the mice infected with the Beijing strains. The mice infected with the Beijing strains had altered immune responses: they expressed TNF- $\alpha$  and iNOS in their lungs during the early stages of the disease, but had a defective production of iNOS and IFN- $\gamma$  during the later stages of the infection [62]. Further studies associated this phenomenon with a decrease in Th1 cells caused by apoptosis and with a defective cytotoxicity, indicative of a deficient CD8+T cell response [63, 64]. A more recent

report with the Beijing strain K1, isolated in Korea, showed that this strain is hyper-virulent, and the lungs of infected mice had a decreased production of Th1 and Th2 cytokines [65]. The hyper-virulence of the Beijing strains was also observed when they were compared to East African-Indian strains in mice [66].

However, Dormans *et al* showed that hyper-virulence is not associated with all the Beijing strains. He observed that strain 9402008, isolated from Asia, caused little lung pathology, bacterial load and mortality in mice [67]. Furthermore, Aguilar *et al* showed that Beijing strains isolated in South Africa, which presented low transmissibility in humans, were less virulent than the Beijing strains that were highly transmissible [68]. The highly transmissible Beijing strains caused an early induction of TNF- $\alpha$  and delayed IL-4 production, as well as defective induction of IFN- $\gamma$  and iNOS in the lungs during the later stages of the disease, compared to the low-virulence Beijing strains [68]. This difference in virulence among Beijing strains was also observed in a mouse model of infection with seven Beijing strains isolated in Vietnam [69], and in guinea pigs infected with ten Beijing strains isolated in the USA. Interestingly, the lungs of these guinea pigs also showed differential patterns of T cell infiltration and cytokine production: virulent strains induced less IFN- $\gamma$  during the later stages of the infection, compared to low-virulence strains [70]. A more recent study showed that heterogeneity in virulence was also observed in mice infected with Beijing strains isolated from Brazil and Mozambique [71].

From these *in vivo* experiments, it is clear that hyper-virulence is not a unique characteristic that applies to all the strains from the Beijing genotype. However, all the hyper-virulent strains from the Beijing group share a common characteristic: they are able to interfere with the Th1 response that is crucial for infection containment, favoring the dissemination of bacilli in the lung and an increased pathology.

## 6. Effects of *Mycobacterium tuberculosis* Beijing genotype on the immune response: *in vitro* models

### 6.1. Macrophages

Several studies have analyzed the ability of the Beijing strains to replicate inside macrophages and to induce cytokine production by these cells. One of the first studies that showed a differential response of macrophages to the Beijing strains was performed in human macrophages. The Beijing strain 210, isolated in the USA, grew faster in human macrophages than other Mtb strains tested, but there were no differences among the strains regarding the induction of TNF- $\alpha$ , IL-6, IL-10 or IL-12 [72]. A faster intracellular replication of the bacilli in human macrophages was also observed with four different Beijing strains [73]. In contrast with these results, Manca *et al* showed that human monocytes infected with the Beijing strain HN878 produced increased mRNA levels for IL-4, IL-11, IL-13, type I IFN, TRAIL and VEGF, compared to monocytes infected with the low-virulence strain CDC1551 [74], and our group reported that the hyper-virulent Beijing strain 9501000 induced higher expression of IL-1 $\beta$ ,

TNF- $\alpha$  and IL-12 in mouse bone marrow-derived macrophages (mBMDM), in comparison to a low-virulence strain of the Canetti genotype [75].

Theus *et al* used fifteen different strains of the Beijing genotype and infected IFN- $\gamma$ -activated THP-1 cells (a human monocyte-like cell line); interestingly, they observed a spectrum of growth and TNF- $\alpha$  induction among these Beijing strains [76]. Tanveer *et al* observed that ten Beijing strains induced similar levels of TNF- $\alpha$  in PMA-stimulated THP-1 cells than Central Asian strains, but lower levels of TNF- $\alpha$  than H37Rv [77]. A low production of TNF- $\alpha$ , IL-6, IL-10 and CXCL1 was also observed in monocyte-derived human macrophages infected with nine different Beijing strains, compared to macrophages infected with H37Rv [78]. In line with this study, Reiling *et al* reported that human macrophages stimulated with four Beijing strains produced lower levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12 and CCL5 than Haarlem strains [66], and Portevin *et al* extended this observation to “modern” Mtb lineages, including Beijing genotype strains [79]. However, Krishnan *et al* observed that seven Beijing strains induced higher IL-1 $\beta$  and TNF- $\alpha$  production than Euro-American strains, but there was considerable heterogeneity in the amount of cytokines induced by each Beijing strain [69]. Heterogeneity in the amount of IL-1 $\beta$  produced was also observed in mBMDM infected with seven Beijing strains [80].

Altogether, these results point to a heterogenic response of macrophages to different isolates of the Beijing genotype. Besides the obvious differences in the biological systems used in the studies summarized in the previous paragraphs, which could explain the differing results, experiments that have used the same *in vitro* model show that the induction of heterogenic cytokine responses is a common characteristic of the Beijing genotype. Two patterns are observed: one is characterized by low induction of pro-inflammatory cytokines, which is associated with a poor regulation of intracellular bacterial replication. The second pattern is characterized by a higher production of pro-inflammatory cytokines, and *in vivo* evidence indicates that, during the early stages of an infection with these strains, there is an inflammatory response of equal or higher magnitude than the inflammatory response induced by other genotypes. This, however, raises a question: how could high levels of pro-inflammatory cytokines be favorable to the pathogen? The paradox may be explained by the function that these cytokines play during Mtb infection. For instance, macrophage-produced TNF- $\alpha$  is associated with macrophage self-activation and control of the intracellular replication of Mtb during the early stages of the infection but, according to a mouse model of infection, it is the T cell-produced TNF- $\alpha$  that is essential for mycobacterial containment during the chronic infection [81]. The recruitment of macrophages with low anti-mycobacterial capacity during the early stages actually favors infection dissemination [82], so the induction of an inflammatory environment that allows the recruitment of such cells could be actively pursued by some virulent Mtb strains.

## 6.2. Dendritic cells

Although DCs play a crucial role for the mounting of T cell responses to Mtb, few studies have analyzed how Beijing strains affect DC functions. The first studies on this subject analyzed cytokine production by DCs. Wang *et al* observed that human derived DCs stimulated with



Beijing strains produced low levels of TNF- $\alpha$ , IL-6 and CCL3, compared to DCs stimulated with H37Rv [78]. Portevin *et al* observed that human DCs stimulated with “modern” Mtb lineages, which include Beijing genotype strains, produced less pro-inflammatory cytokines than DCs stimulated with “ancient” Mtb lineages [79], while Krishnan *et al* did not observe any difference in cytokine production by mouse bone marrow derived DCs (mBMDDCs) stimulated with Beijing strains, H37Rv or other genotypes [69]. Recently, our group reported that a hyper-virulent Beijing strain failed to activate mBMDDCs (assessed by MHC class II expression) but did not affect the induction of CCR7 expression, which is essential to drive DC migration from the tissues to the lymph nodes. Moreover, these DCs had higher levels of PDL-1 and IL-10 than DCs stimulated with low-virulence strains. Interestingly, the phenotypic changes correlated with alterations in DC function, as revealed by their inability to induce a Th1 response [83]; a phenomenon usually observed during *in vivo* infections with hyper-virulent Beijing strains.

### 6.3. Human peripheral blood mononuclear cells

Human PBMCs consist mainly of monocytes, T cells, B cells and NK cells. Assays where PBMCs are stimulated with pathogens or their antigens can be used to evince T cell-mediated immune responses [84]. In one of the first studies that used PBMCs to assess granuloma formation *in vitro*, the authors analyzed two Beijing strains and did not observe a difference in the number or size of the granulomas induced by these strains, compared to the granulomas induced by Haarlem, Latin American-Mediterranean or East African-Indian strains [78]. Our group reported that the PBMCs from healthy BCG-vaccinated individuals stimulated with Beijing strains presented CD4+ and CD8+ T cell proliferation responses similar to those induced by the less virulent Canetti and H37Rv strains, and that the Beijing strains induced higher production of IL-10 and IL-12, but similar levels of IFN- $\gamma$  [85]. Recently, van Laarhoven *et al* analyzed the effect of fourteen Beijing strains on cytokine production by PBMCs from non-BCG-vaccinated healthy individuals; “modern” Beijing strains induced lower levels of IL-1 $\beta$ , IFN- $\gamma$  and IL-22 than “ancient” Beijing strains [86]. In contrast, Portevin *et al* did not observe a difference in cytokine production by PBMCs stimulated with several Beijing strains [79]. One explanation for the different response of PBMCs, beyond the genetic diversity of Beijing strains, is the immune status of the donors: only one study analyzed the response of BCG vaccinated individuals, and BCG vaccination has been associated with increased susceptibility to infection with Beijing strains [48].

The studies described above show that Beijing strains modulate the response of different cell populations that participate during the immune response to this pathogen. However, most of these studies are focused on macrophages, leaving aside other cell populations that are known to participate in the immune response to mycobacteria, such as pneumocytes, neutrophils, mast cells, NK, NKT cells,  $\gamma\delta$  T cells, B cells, etc. Studying how these cells respond to Beijing strains will help to understand the mechanism used by this Mtb genotype to override the immune response.

## 7. Components of *Mycobacterium tuberculosis* Beijing genotype that modulate the immune response

### 7.1. Lipids

Several Mtb components are associated with evasion of the immune response. Among them, lipids have been widely studied, mainly because of the atypical structure of the mycobacterial cell wall and the predominance of some types of lipids in this cell wall. Lipid extracts from the Beijing strain HN878 induced higher expression of IL-13, G-CSF and VEGF in human macrophages, compared to the expression induced by lipids from the CDC1551 strain [74]. The cell wall lipids from hyper-virulent Beijing strain 9501000 also induced higher expression of TNF- $\alpha$  and IL-10 than the cell wall lipids from H37Rv and Canetti genotype [87].

Reed *et al* showed that three hyper-virulent Beijing strains expressed a phenolic glycolipid (PGL) that was not expressed by the lower-virulence strains H37Rv and CDC1551. When the HN878 strain lost the expression of this PGL, its hyper-virulence in mice and rabbits was diminished, and the cytokine pattern that it induced in macrophages became similar to that induced by H37Rv [88]. Later studies showed that 102 Beijing strains had unaltered *pks15/1*, the open reading frame (ORF) coding for the enzyme responsible for PGL production [28]. However, the presence of intact *pks15/1* is not exclusive of the Beijing genotype [89], and some Beijing strains that express this ORF do not produce PGL [90]. In addition, a study reported that induction of PGL production by non-Beijing strains did not increase their virulence [91]. Regarding other lipids, Reed *et al* observed that 36 Beijing strains, but not Indo-Oceanic or Euro-American strains, uniformly expressed triacylglycerides [90]. Huet *et al* showed that Beijing strains presented a pattern of phthiocerol dimycocoserates (PDIM) different to that of other Mtb strains; this difference was associated with the mutation *Rv2952<sup>G526A</sup>* in the Beijing strains, but it was not associated with the virulence of these Beijing strains [92]. Another study reported that total lipids extracts from Beijing strains induced higher TNF- $\alpha$  production by mBMDM than total lipids extracts from Euro-American strains. These Beijing strains had a characteristic pattern of PDIM, which was different to that of other Mtb strains [69].

These studies indicate that lipids from Mtb Beijing play an important role in the regulation of macrophage activation and in the induction of cytokine production. However, further studies are needed to clarify how these lipids impact other arms of the immune response that are crucial for infection containment, such as DCs and T cells specific for Mtb lipid antigens [93]. Another potential role for the lipids of the Beijing strains could be as carbon and energy reservoirs, which would be valuable in the harsh conditions that the bacilli find inside macrophages or in the anoxic granulomas [90].

### 7.2. Proteins

One of the first studies that analyzed proteins identified that a Beijing strain, but not Canetti or H37Rv strains, expressed mRNA for PE\_PGRS Rv3652 [94]. Wu *et al* observed that the Beijing strain 210 had higher SigA mRNA levels than H37Rv, and that blockade of SigA expression attenuated bacterial growth in human macrophages and in the lungs of infected

mice, a phenomenon that was associated with increased Mtb susceptibility to reactive oxygen intermediates [95]. Later studies showed that SigA induced up-regulation of Eis, which in turn promoted TNF- $\alpha$  and IL-10 production by macrophages [96, 97].

Pheiffer *et al*, using proteomic techniques, found that a Beijing strain expressed higher levels of  $\alpha$ -crystallin and lower levels of Hsp65, PstS1 and 47kDa protein than H37Rv [98]. Interestingly, Reed *et al* found that Beijing strains constitutively expressed high mRNA levels of genes in the DosR regulon, including *dosR*, *Rv3130c*, *hspX*, *fdxA* and *narX*; the levels of these mRNAs were up to 50 times higher than the levels found in non-Beijing strains [90]. Usually, Mtb induces the expression of the DosR regulon under two conditions: 1) in the lungs of infected mice and in artificial granulomas [99], and 2) inside macrophages that are activated with IFN- $\gamma$  [100]. In consequence, the authors hypothesized that a constitutive high expression of the genes in the DosR regulon would allow a fast adaptation of Mtb to this harsh conditions leading to Mtb survival. The mechanisms that maintain this high expression of the DosR regulon are unknown, but mutations in DosT sensor kinase and chromosomal duplication have been implicated in this phenomenon [101, 102].

Rindi *et al* found that Beijing strains expressed high levels of PPE44, a member of the Mtb PPE proteins that are related with antigenic variation [103]. Another approach, using proteomic analysis among several Beijing strains with different virulence, showed that the virulent strains over-expressed 53 proteins involved in regulation, cell wall synthesis and cell processes [104]. Recent reports indicate that the virulent Beijing strain K1 expressed higher levels of ESAT-6, HSP-X and CFP-10 than strains from other genotypes, and it also expressed InsB, a protein which shares homology with ESAT-6 [105, 106].

Altogether, these results indicate that Beijing strains express a different set of proteins that could participate in many stages of the infection. First, they could modify the innate immune response through a differential engagement of pattern recognition receptors (PRRs), inducing a response that would favor infection [107]. Second, they could facilitate a rapid adaptation of the bacilli to the adverse conditions found during infection (hypoxic, or rich in reactive nitrogen and oxygen intermediates, for example) [90]. Third, the regulation of the expression of proteins that are recognized by the adaptive immune response could facilitate immune evasion and fostering survival of the bacteria [98].

### 7.3. Other molecules

Carbohydrates from Beijing strains have been poorly analyzed. Usually, carbohydrates are associated with lipids or proteins, and they are abundant on bacterial cell walls. Carbohydrates play a crucial role in the recognition of microbes by innate immune cells through PRRs, and consequently, they could modulate the whole immune response to the bacilli [108, 109].

As we have seen, the immune response is crucial role for infection containment, but other non-immune factors contribute to Mtb virulence, and a conjunction of all these factors defines the outcome of the infection. Metabolomic studies of hyper-virulent and hypo-virulent Beijing strains have reported a difference in the presence of different metabolites, including amino

acids, carbohydrates and lipids [110]. How this difference impacts bacterial phenotype needs further clarification.

## 8. Evolution as a driving force in the success of *Mycobacterium tuberculosis* Beijing genotype

The studies cited above highlight a great variation in the responses to infection with Beijing strains, both *in vivo* and *in vitro*. One explanation for this variation could be the genetic diversity of the Beijing strains, but some studies have been unable to correlate the different responses with the genetic diversity of the Beijing genotype [111, 112]. So, how can we understand this variation? Evolution has played a fundamental role in shaping the actual scenario of TB on earth. Humans have co-existed with Mtb since our appearance as a species on this planet, and Mtb accompanied humans since the first migrations from Africa to other parts of the world [1, 113]. These migrations and settlings allowed a co-evolution of each human population with its pathogens, including Mtb, and this is reflected in the distribution of Mtb diversity: six different Mtb lineages are stably associated with specific human populations, according to their geographic distribution [4]. Mtb lineages are so well adapted to their hosts that even in cosmopolitan areas, where diverse human populations from different geographic origins coexist, a specific Mtb lineage preferentially infects individuals from a specific geographic origin (sympatric transmission) [114]. Allopatric human transmission of Mtb occurs, but infection of individuals from one geographic origin with Mtb from a different lineage is usually associated with other factors, such as HIV co-infection. Patients with allopatric infections have increased pulmonary impairment, compared to patients with sympatric infections [114, 115].

Mtb Beijing genotype belongs to the East-Asian lineage, and it is strongly associated with hosts from this geographic area. When Mtb Beijing genotype is introduced to new areas, where it is uncommon, it preferentially infects hosts that also come from its original geographic area. Studies in Italy and Sweden indicate that infection with Mtb Beijing genotype is uncommon among natives and more frequent among migrants from areas commonly infected by this genotype, which suggests a poor adaptation of Mtb Beijing genotype to the native hosts [116, 117]. In fact, the genetic diversification of Mtb Beijing genotype has been associated with adaptation to particular human populations [118].

Which host factors predispose to infection with Mtb Beijing genotype? Some studies indicate that infection with Beijing strains occurs preferentially in hosts that have particular polymorphisms in immune response genes. In Vietnamese patients, infection with Beijing strains was associated with the presence of the TLR-2 allele T597C [119], and recent reports indicate that TLR-2 polymorphisms are differentially distributed in human populations according to their geographic localization and ethnicity [120]. Whether these polymorphisms affect the immune response to Beijing strains still needs to be analyzed, but differential activation of TLR-2 and TLR-4 by Mtb strains from different genotypes has an impact on the immune response to the bacilli [121].



Crevel *et al* found that, in Indonesian patients, the polymorphism D453N G and an insertion in the 3'untranslated region (UTR) of the *slc11a1* gene (formerly known as *nramp1*) were strongly associated with Beijing strains infection [122]; polymorphisms in this gene (which codes for an iron transporter in macrophages) have been associated with TB susceptibility [123]. Salie *et al* documented an association between MHC class I alleles and infection with Beijing strains in South Africans, where the allele B\*07:05 increased the odds of infection with Beijing strains, while the allele B\*35:01 had the opposite effect. Interestingly, B\*07:05 is a frequent allele in East Asian populations [124]. MHC class I molecules are essential for antigen presentation to CD8+T cells, which are needed for the containment of Mtb infection [125]. These studies provide evidence of a co-evolution between Beijing strains and their hosts.

## 9. Conclusion

Mtb Beijing genotype has attracted attention because of its high prevalence among TB patients, but despite multiple studies, the causes of this success remain obscure. An interesting aspect of Mtb Beijing genotype infection is the diversity of the immune responses that are induced in humans and in experimental models (*in vivo* and *in vitro*). It is possible that this diversity results from the adaptation of Mtb Beijing genotype to the genetic background of its hosts and to other evolutionary pressures, such as drug treatment, BCG vaccination and HIV co-infection. These factors, and others still unknown, will shape the strategies used by Beijing strains to override the immune response and to establish successful infections in the host.

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