Chapter 12

Virulence Factors and Pathogenicity of Mycobacterium

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Abstract

Virulence, is referred as the ability of a pathogen to cause disease, and for mycobacteria it depends on their ability to reside within host cells and evade the microbicidal mechanisms of macrophages. The outcome of tuberculosis (TB) infection is highly variable and it seems that the closest relationship between the *Mycobacterium* genre and humans has shaped the mycobacterial genome to encode bacterial factors that reflects a highly evolved and coordinated program of immune evasion strategies that interfere with both innate and adaptive immunity causing disease even in fully immunocompetent host. Although *Mycobacterium tuberculosis* (MTB) does not have classical virulence factors, it has described many virulence-associated genes and virulence lifestyle genes from *Mycobacterium tuberculosis* complex (MTBC). In this chapter, we describe the most important gene/molecule involved in the host defense modulation response, also the plethora of strategies to evade immune mechanisms of macrophage. We review the main genes whose inactivation in the mycobacterial genome leads to a measurable loss in virulence in the different validated TB models.

Keywords: virulence factor, pathogenicity, mycobacterium, cell wall, immune evasion, tuberculosis

1. Introduction

Tuberculosis (TB) is mostly caused by *Mycobacterium tuberculosis* (MTB) and *Mycobacterium africanum*, both members of the *Mycobacterium tuberculosis* complex (MTBC), a group of closely related species which are adapted to human and animals. The outcome of TB infection is highly variable and is determined by the response of the immune system and environmental variables, but a deeper knowledge of the global genomic diversity in the MTBC suggests that bacterial factors are also involved. To better understand the virulence mechanisms of the

MTBC, it is necessary to define what constitutes a virulence gene. There are a wide variety of conditions and parameters to define it, but undoubtedly a true virulence gene codifies for factors or enzymes producing factors that are involved in the interaction with the host, and are directly responsible for the pathological damage during infection and are absent in non-pathogenic bacteria. Perfect examples of virulence genes are the toxins produced by *Vibrio cholera* and *Escherichia coli* O157. MTB does not have classical virulence factors; however, many virulence-associated genes and virulence lifestyle genes from MTBC have been described [1]. Virulence-associated genes, are genes coding for factors that regulate expression of virulence genes and activate virulence factors by translational modifications, processing or secretion; whereas, virulence lifestyle genes codifies for factors that enable colonization of the host, enable evasion of the host immune system and enable intracellular survival. Besides, unlike other pathogens, MTB virulence is directly linked to its transmission. Thus its virulence can be measured through (1) the ability of the bacteria to survive the host immune response, (2) their capacity to cause lung damage and (3) to be successfully transmitted to infect a new host [2]. MTB pathogenicity, defined by its ability to cause disease in a host organism, has co-evolved with its physiology as specie. Initial infection is mainly through respiratory tract, here the alveolar macrophages are the most common cell type infected by MTB and the inflammatory signals arising from infection promote the influx of additional monocytes and macrophages, which become infected as well. Inflammation is required for initial control of infection but can also cause extensive tissue damage. Moreover, the bacteria exploit the host inflammatory signals to spread to other individuals. Because of that, it is believed that its pathogenicity is likely to have evolved from its specific adaptations to host immunity. In fact, Comas et al., showed that a large majority of the T cell epitopes, representatives of several linages of the MTBC, had high sequence conservation, indicating that there is a high strong selection pressure to keep those T cell epitopes unchanged, which suggest that MTBC might benefit from being recognized by T cells, because as referred above, MTB virulence depends on its transmission [3]. While a significant number of genes have been shown to be important for the progression of TB, we focus on only few examples. We place emphasis in those genes whose inactivation in the mycobacterial genome leads to a measurable loss in virulence in the different validated TB models. The mycobacterial cell wall and envelope are unique among bacteria and many of their components are known to play an important role in the TB pathogenesis. Taking these concepts into consideration, we analyzed separately their lipids, secreted proteins and systems that play a role in the synthesis of various cell surface molecules. Thereafter, we describe the main proteins that inhibit antimicrobial effectors of the macrophage.

2. Models for measuring MTB virulence

MTB virulence is studied in cell culture and animals models. Thus different parameters of pathogenicity according the TB models are used. A hallmark of MTB pathogenicity is the ability to infect and survive within macrophages; thus, primary macrophages and cell lines are used to analyze the virulence of MTB and mutants at the early stages of infection. Primary macrophages are more representative of the natural *in vivo* situation but they are difficult to propagate to

sufficient numbers for virulence experiments. Primary murine bone marrow-derived macrophages (BMDMs) and human macrophages from peripheral blood monocytes (HMDMs) are the most common primary macrophages used, whereas the immortalized cell lines THP-1, J774 and MH-S cells have been widely used to study MTB-macrophage interactions. Data from both cell type should be interpreted with care because the response of MTB to intracellular environment can vary greatly depending on the cell type used [4]. In addition to evaluate the survival, replication and the intracellular bacillary load of MTB in the macrophages models, it is possible to study some of its mechanisms to counteract the macrophage microbicide ability, such as (1) the resistance to reactive oxygen/nitrogen intermediates (ROI/RNI), (2) the phagosome arresting and (3) the inhibition of apoptosis [1]. On the other hand, in animal models it is possible to study all the stages of the TB infection, although their ability to replicate the different aspects of human TB pathology varies. The major models used are mice, guinea pigs and rabbits [5]. Mice is the most frequent *in vivo* model used because of their well-characterized genetics and the huge collection of immunological reagents as well as the existence of inbred strains, their susceptibility to MTB is low and their pathology is unlike that in humans [5]. Guinea pigs are very sensitive to MTB and develop a disease with multiple similarities to human disease, such as lung necrosis, lymphadenopathy and disease dissemination. And the rabbit model develops lung granulomas which closely resemble the histology of human TB when they are infected with *M. bovis*, due to their size, high cost and lack of immunological reagents that make the model less tractable than mice. The close similarity between MTB and *M. bovis* make the cattle model a very attractive way to study TB pathogenesis. The bovine TB disease pathology is very similar to human TB, with caseating granulomas in the lungs and a similar latent phase in the infection. The major advantage of the cattle model is the possibility to conduct field trials, which makes it very attractive for vaccine studies [6]. The non-human primate model are the only TB model that develops all the clinical disease states found in human TB, and although its use has been invaluable in TB research, it is limited by ethical concerns and high cost. The most important parameters associated with virulence in animal models, besides mortality and morbidity, are: the bacterial load, the numbers of bacteria found in the infected host after the initial infection, histopathological changes and inflammatory responses. Finally, it is important to mention that zebrafish model has also been shown to be very useful to elucidate the early events of the mycobacterial infection, particularly in the study of the mechanisms of granuloma formation and its role in controlling the infection [5]. Infection of zebrafish with *M. marinum*, a closest relative of MTBC, resembles many aspects of human tuberculosis; in fact, crucial virulence factors, host genes and immune cell types are conserved in the zebrafish-*M. marinum* model [7]. Using this model, the bacterial RD1 locus was found to be required for efficient granuloma formation and that the ESX-1 system was responsible for the cell death of infected macrophages [8].

3. Molecules involved in pathogenesis and virulence

3.1. Mycobacterial lipids

Mycobacterial cell wall is rich in lipids and has exceptional physico-chemical properties as a strong impermeability; and even it has peptidoglycan on it, mycobacteria are acid fastness

organisms due to the large amount of lipids. In this section, we describe mycobacterial cell wall components and their relation with pathogenicity and virulence, the second part of the chapter is dedicated to study the phenomenon produced by some of the mycobacterial molecules.

3.1.1. Lipoarabinomannan (LAM)

Lipoarabinomannan (LAM) is a glycolipoconjugate composed by an anchor mannosyl phosphate inositol (MPI), a polysaccharide backbone and diverse capping motifs species [9]. Correct translocation of LAM in to the cell wall constitutes an important feature for the mycobacterial stability, the lack of *O*-manosilation was associated with increased production of LAM and increased release of LAM/LprG protein and consequently with a reduction in virulence of MTB *O*-manosilated deficient strain [10]. Variation in the mannose-capped arabinan ManLAM motifs between LAM from different strains and clinical isolates, may be responsible of the production of interferon-γ (IFN-γ) in CD-1b-restricted cell lines; and also, in differences in adherence to macrophages [11]. LAM inhibits phagosome maturation characterized for the presence of immature phagosome marker rab5, and allows intracellular surviving. LAM is released in the macrophages and intercalates with endomembranes, phenomenon essential in order to block phagosome arresting and also, the delivery of lysosomal hydrolases via the molecule EEA1, which blocks impeding phagosomal acidification [12]. There are a large number of publications compiled in the review [13] that describe the cytokine expression activity trigger by LAM in macrophages and dendritic cells (DC). Although there are differences in methodology and results among the data; to compile, the 20 publications analyzed include: an increased expression of tumoral necrosis factor alpha (TNF-α) in most of the publications, increased of IL-10 in some of the data and results showed different IL-6 and IL-12 expression. ManLAM from MTB induce the expression of IL-12 and apoptosis in macrophages [14], whereas in T cells, lipid microdomains suffer insertion of PILAM with no apparent interaction with a specific receptor, this phenomenon trigger Th2 cytokine production and a decreased Th1 cytokine expression [15].

3.1.2. Lipomannan (LM)

Lipomannan (LM) is a multiglycosylated lipid or polymannosylated Phosphatidylinositol mannoside (PIM). LAM and LM coexist in the mycobacterial cell wall. LM has been considered an innate immunity antigen; tetra-acylated LM activates macrophages using TLR2/TLR4 in a dependent way of MyD88. Di-acylated molecules regulate and inhibit the production of NO secretion and cytokine in macrophages activated by lipopolysaccharide (LPS) [16]. LM purified from *M. kansasii* and *M. chelonae* in a CD14-TLR2-dependent mechanism, induce secretion and expression of mRNA of IL-8 and TNF-α in THP1 macrophages [17]. It had demonstrated that LM from *M. kansasii*, *M. bovis* bacillus Calmette-Guérin (BCG) and *M. chelonae* induced a dual function in macrophages: activation with surface expression of CD40, CD86; production of TNF and NO secretion in a TL2 MyD88 dependent way; and also, and inhibition of expression of TNF and IL-12p40 and NO in macrophages activated with lipopolysaccharide (LPS) in a TLR independent way [18]. LM from *M. kansasii*, *M. chelonae*

and MTB induced apoptosis and IL-12 in THP1 macrophages [14]. Tri-tetra-acylated forms of BCG LM were suggested as responsible of the pro-inflammatory response. Try-acyl LM response is dependent of TLR2/TLR1 and MyD88 TIRAP and produce IL12 and NO. In *M. smegmatis*, structural changes of LM and LAM unleash the loss of acid-fastness, faster killing by macrophages by THP1 macrophages and led to higher sensibility to antibiotics; whereas in MTB lead to an attenuated infectivity in mice and antibiotic sensibility [19].

3.1.3. Phosphatidylinositol mannosides (PIMs)

Phosphatidylinositol mannosides (PIMs) constitute a substantial component of the cell envelope, precursor of LAM and LM. PIM has a variable number of mannose units and acylation, virulent species have high order PIM (5 or 6 mannoses) that contribute to the uptake of macrophages by mannose receptor (MR); lower order PIM with few mannoses interact with DC-specific intercellular adhesion molecule-3-grabbing non-integrin DC-SIGN from DC [20]. The acylation state of PIM can induce granuloma formation and cell recruiting in BCG infection; specifically PIM_4, PIM_6 were used, and the acyl chain was responsible for NKT recruitment [21]. In contrast, glycolipids from MTB as PIM and ManLAM inhibited $CD4^+$ T cell activation by interfering in the phosphorylation and T cell receptor signaling [22]. Host inflammatory response such as TNF, IL-12p40 was inhibited by PIM in murine macrophages through CD14-dependent and CD14-independent mechanisms [23]. PIM induced an increased presence in culture supernatants of alveolar epithelial cells (AEC) of the anti-inflammatory cytokine transforming growth factor beta (TGF-β) and a significant production of reactive oxygen species (ROS) [24]. Diacyl-phosphatidylinositol dimannoside (Ac_2PIM_2), acylphosphatidylinositol hexamannoside ($AcPIM_6$) and diacylphosphatidylinositol hexamannoside (Ac_2PIM_6) from virulent MTB stimulate and drive proliferation in bovine PBMC from *M. bovis*-infected cattle; also the IFN-γ expression in PBMC was increased only during exposition to $AcPIM_6$ [25].

3.1.4. Trehalose-6,6′-dimycolate (TDM)

Trehalose-6,6′-dimycolate (TDM) also known as cord factor, is the most abundant and toxic lipid in the mycobacterial cell envelope. TDM is composed by two polar trehalose head group where two mycolic acids (MA) are esterified. MA variations constitute a strong determinant of the inflammatory response of TDM. TDM has biological functions, promoting angiogenesis [26], inhibits acidification of phagolysosome, prevents Ca^{2+} dependent phagosome-lysosome fusion and mycobacterial surface lipid removal, which increased trafficking of bacteria to the acidic compartments, causing 99% of killing in macrophages after 3 days of infection [27]. TDM coating beads produce a delayed maturation of phagosomes characterized by a non-acidified and hydrolytically restricted phagosome [28]. Cytokine production in macrophages exposed to TDM has been extensively described; there are a high diversity number of publications about it. The effect of these molecules has been correlated with the innate, early adaptive response (humoral and cellular immunity); to resume: cytokines as IFN-γ, TNF-α, IL-4, IL-6, IL-10; chemokines as MCP1, IL-8, are induced as response to exposition of TDM [29]. Reduction in the expression of MHCII, CD1d, CD80, CD40 and CD96 in the surface of

macrophages is induced by the exposure of the cells to TDM [30]. Microspheres coated with TDM showed an increased expression of enzymes and matrix metalloproteinases; molecules associated with tissue remodeling and tissue destruction during caseating granulomas [31]. The inflammatory profile induced by TDM has been related with granuloma development and maintenance, this phenomenon is related with TDM, and is dependent of TNF-α and IL6 expression; also C5a complement factor has been described as part of the granuloma maintenance microenvironment molecule [32].

3.1.5. Phthiocerol dimycocerosate (PDIM) and phenolic glycolipids (PGL)

Phthiocerol dimycocerosate (PDIM) and phenolic glycolipids (PGL) include a group of related cell wall lipids, non-covalently bounded to the mycobacterial surface. PDIM and PLG are major virulence factors of mycobacteria. PDIM and PGL are molecules required for bacterial duplication during the acute phase [33]. PDIM is involved in mycobacterial resistance to detergents, and also is linked with the permeability and envelope solidity [34]. PDIM is present in *M. marinum* and species from the MTBC, but is absent in *M. smegmatis*; it contributes to the intracellular bacterial surviving, protecting them against the action of reactive nitrogen intermediates species, and regulates TNF-α expression [35]. Phagosome acidification resistance is caused by exclusion of vacuolar proton-ATPase in the phagosomal membrane; PDIM deficient mutants can also provoke macrophages death [36]. PDIM is involved in the phagocytosis dependent of receptor through a macrophage plasma membrane reorganization mechanism [37]. Recent findings suggest that PDIM and ESAT-6 protein act together to induce phagosome membrane damage and apoptosis [38]. PGL is produced principally by fast growing mycobacteria; most of MTB isolates and H37Rv are unable to produce it, phenomenon caused by a mutation in the *phs15/1* gene. PLG1, an immunogenic glycolipid produced for all *M. leprae* isolates, contains a trisaccharide moiety, different to the MTB PLG; and could be obtained from *M. leprae* from infected tissues. PLG1 is responsible for the demyelination and damage in the axons, confers neurotoxic proprieties to the macrophages and increase the reactive nitrogen species (RNS) synthesis [39].

3.2. Secretion systems in mycobacteria

Molecular migration across the mycobacterial cell wall, constitute an important event related with the environment and host cells interaction. Mycobacterial waxy cell envelope controls the molecular movement and the secretion of substances across this structure is dependent of specialized proteins systems, some of these protein structures will be described below.

3.2.1. The twin-arginine transporter (TAT transporter)

The twin-arginine transporter (TAT transporter) is located in the cytoplasmic membrane and transport folded proteins. This system is composed by three membrane proteins named as TATA, TATB and TATC. *M. smegmatis* deletion mutants to *tatC* and *tatA* showed a growth defect on agar, defective exportation to active beta-lactamases and hypersensitivity to

sodium dodecyl sulfate (SDS), reason that suggests that TAT genes could be good candidates for vaccines and drug development [40]. Mycobacterial phospholipases, virulence-related molecules encoding by *plcA, plcB, plcC and plcD* genes, are secreted by the twin-arginine transporter [41]. TAT system is involved in secretion of relevant proteins, as example; the secreted protein encoded by the Rv2525c gene, which is involved in the cell wall biogenesis, this protein is conserved in *M. leprae* and present in MTB, it has been described as important for virulence and it is involved in the resistance to beta-lactam antibiotics [42]. The resuscitation-promoting factor, RpfB associated with the MTB reactivation stage, interacts with the virulence factor RipA, an endopeptidase protein secreted together with the chaperone MoxR1, which requires the TAT secretion system. Inhibition of this system increased the sensitivity to beta-lactam antibiotics and prevents the localization of the peptidoglycan hydrolase [43].

3.2.2. The ESX transporter

The ESX transporter has no counterpart in LPS bacteria; it is located in the cytoplasmic membrane and exports and secretes proteins across the mycobacterial cell envelope. ESX genes are encoded at the genome and plasmids [44] and codify to for ESX type proteins EspA, EspB, EspC, EspG, etc, the secreted proteins ESAT-6 y CFP-10; PE-PPE; and the conserved components EccB, EccC, EccD and MycP [45]. The ESX systems are named as ESX-1 to ESX-5, depending on the variation of the diverse systems and their components.

3.2.2.1. ESX-1

ESX-1 transporter system is important during mycobacterial infection in MTB and other pathogenic mycobacteria, in BCG the loss of the region of difference 1 (RD1) and the partial loss of the ESX-1 encoding region is related with the attenuation of the strain [46]. ESX-1 allows cytosolic contact and mediates vacuoles rupture [47]; the protein intervenes in host cell lysis in a contact dependent way, producing gross membrane disruptions [48]. DNA transfer through conjugation is also a function of ESX-1 system [49]; a phenomenon called "distributive conjugal transfer" that describes a genetic exchange between recipient and donor is dependent of ESX-1 in *M. smegmatis* [50]. *espB* gene located in the RD1 region has been related with cytotoxicity and their presence in the surface of apoptotic cells for clearance by macrophages through efferocytosis [51].

3.2.2.2. ESX-3

ESX-3 is involved in Zn and Fe uptake. ESX-3 proteins: EsxG and EsxH are associated with the (proline-glutamic acid, proline-proline-glutamic acid) PE and PPE secretion [52]. The EsxG and EsxH heterodimer, which harms macrophage phagosome maturation, is secreted by the ESX-3 system [53] and inhibits the endosomal-sorting complex required for transport (ESCRT) impairing MTB antigen-specific $CD4^+$ activation by macrophages and DC [54]. In *M. abscessus* ESX3 is composed by the genes *esxH, esxG, esx-3,* EsxG and EsxG proteins are related with enhancement of inflammatory cytokine generation in macrophages, *M. abcessus esx3* mutant resulted in less inflammatory response [55].

3.2.2.3. ESX-5

ESX-5 secretion system only present in slow-growing mycobacteria is linked to PPE and PE exportation and pathogenicity. The secretion mechanism of ESX-5 is activated in response to phosphate limitation through phosphate sensing of Pst/SenX3-RegX3 system [56]. In MTB, disruption of ESX-5 showed a strong attenuation, failure in the cell wall integrity and the loss of the secretion of the PPE protein [57]. ESX-5a region, from ESX5 is composed by duplicated genes and had been related with inflammasome activation [58]. Also, mutations in the ESX-5 system components as *esxC5* are related with ofloxacin resistance in MTB [59].

3.3. PE proteins: PE-PPE and PE-PGRS

The PE domain permits transportation of proteins, which share the domain. PE-PGRS and PE-PPE interacts with the TLR-2 on DC and macrophages, inducing: cytokine secretion, necrosis and apoptosis and enhance mycobacterial survival [60]. PE-PGRS33 interaction with TLR mediates macrophage entry [61]. PE-PGRS30 mutant showed an attenuated phenotype, specifically inhibits phagosome-lysosome fusion and showed decreased lung colonization and reduced tissue damage [62]. PE-PGRS32 gene is highly conserved in MTB strains, because it has been related with mycobacterial survival in macrophages, persistence and replication [63]. PPE10 has been described as an ESX-5 substrate in pathogenic mycobacteria; mutation of both of them reduced the envelope integrity and mycobacteria hydrophobicity [64]. Co-localization of PE-PGRS33 in the host cell mitochondria induces cell death: necrosis and apoptosis [65]. PE-PGRS47 disruption led to an *in vitro* and *in vivo* attenuated growth and autophagy inhibition in infected phagocytes [66].

3.4. Lipoproteins

MTB genome analysis showed around 90 putative lipoproteins, most of them are part of the mycobacterial cell envelope and the plasma membrane; their function is related with molecular exportation, cell wall homeostasis and nutrient uptake; their presence contribute to host-pathogen interaction.

3.4.1. LpqH (19 kDa protein)

This lipoglycoprotein *O*-glycosylated and acylated, is a major cell wall antigen. LpqH is recognized by the immune system and induces T cell proliferation *in vitro*, stimulates DC maturation and autophagy and activates TLR-2 [67]. The protein alters the expression and presentation of antigens by MHCII [68]. 19kDa protein, induces macrophage apoptosis by caspase-dependent and -independent mechanisms with activation of the initiator caspase 8 and executioner caspase 3 [69]. DC that phagocyte apoptotic macrophages induced by cell wall extract expressing LpqH, activates CD8 T cells through cross-presentation [70].

3.4.2. LppX

LppX is related with the release of complex lipids to the culture filtrate; LppX structure showed a large cavity that probably binds big motifs as the one present in PDIM. In a mice model, LppX-deficient mutant showed attenuation [71].

3.4.3. Mpt83

Mpt83 glycosylated lipoprotein related with host cell adhesion, is present in MTB and *M. bovis* and both proteins are identical, but with some glycosylation differences. Protein is recognized by the MR, and the native protein induces IL-6, IL-12 and TNF-α [72]. T cell proliferation and IFN-γ expression was detected in PBMCs from donors vaccinated with BCG or with latent tuberculosis after exposition to non-lypidated synthetic Mpt83 [73].

3.4.4. LprG

LprG also known as P27 lipoprotein, is a ligand of TLR2; inhibits antigen processing in macrophages MHC II [74]. It has a large cavity that binds triacylated agonist of TLR2: LM, LAM and PIM [75]; and determines LAM envelope localization and control phagosome-lysosome fusion [76]. Expressed in an operon with Rv1410c, binds triacylglyceride (TAG) in the cavity and regulates TAG levels, growth rate and virulence [77]. Involved in cell wall composition, MTB mutant deleted *lprG* showed a decreased amount of surface LAM, affecting interaction with the MR and host cells, phagosome-lysosome fusion disturbance, and in mice model showed a decreased number of bacteria in the lungs. [78]. *M. bovis* P27 is required for arresting phagosome maturation and replication in bovine macrophages [79].

3.4.5. RpfB

RpfB multidomain lipoprotein related with resuscitation after mycobacterial dormant state drives in DC Th1-type immunity through interaction with TLR-4 [80].

3.4.6. LpqS

LpqS MTB protein conserved in slow-growing pathogenic mycobacteria, is a protein related with survival during latency. *LpqS* mutant showed attenuated virulence in guinea pig models and provide better immunization against pulmonar tuberculosis in comparison with BCG [81]

3.4.7. LprN

LprN lipoprotein related with cellular entry and survival, is part of the *mce4* operon. Recombinant LprN expressed in *E. coli,* showed in *in vivo* assays on mice, an increased expression of IFN-γ and TNF-α, and also a higher T cell proliferation, but failed to protect mice against MTB challenge [82].

3.4.8. Lprl

Lprl lipoprotein present only in bacteria from MTBC, showed upregulation during mycobacterial macrophage infection. Lprl strongly attaches lysozyme, annulling completely their enzymatic activity. Lprl expression in *M. smegmatis* enhanced phagocytosis and survival in macrophages derivate from peritoneal monocytes; also protect the bacteria against lysozyme activity [83].

3.4.9. PstS

PstS phosphate transporter, glycolipoprotein, is recognized by MR, induces phagocytosis and reduces the production of ROS [84]. PstS-1, PstS-2, PstS-3 DNA vaccine was used in mice, only animals vaccinated with PstS-3 showed reduction of CFU on lungs and spleen [85].

4. Immune system evasion

Unlike other pathogens, MTB infects and resides within immune cells, this bacterium has the ability to live within the dynamic and heterogeneous environment of macrophage phagosome. Here, the bacilli use a plethora of strategies to evade the microbicidal mechanisms of macrophage, including: phagosome-lysosome fusion, recruitment of hydrolytic lysosomal enzymes, production of reactive oxygen/nitrogen species, antigen presentation and apoptosis. Disruption of those functions in turn disrupts the adaptive immune response. Phagocytosis is an active process that depends on the interaction with various surface receptors expressed on the macrophage such as complement receptor type 3 (CR3), FCγ receptors and lectin receptors and it can be opsonic or non-opsonic. However, non-opsonic phagocytosis of MTB results in higher intracellular survival, although it is difficult to assess if the engagement of specific receptor determines the course of infection [86]. MTB uses PDIM lipids to evade detection by TLRs, thereby preventing mycobacterial delivery into microbicide macrophages expressing iNOS [33]. Moreover, MTB actively blocks the phagosome maturation by their cell wall components or through the secretion of various macromolecules that interferes with this process, which enables bacterial survival in a non-acidified intracellular compartment [12].

4.1. Phagosome arresting

PtpA and SapM are two phosphatases that contribute with the phagosome arresting. PtpA binds to subunit H of the vacuolar V-ATPase in order to dephosphorylate its substrate, the vacuolar protein sorting 33B (VPS33B) resulting in the exclusion of V-ATPase from mycobacterial phagosome thus inhibiting phagosome acidification [87]. MTB mutant in PtpA was severely attenuated when infecting THP-1 cell line compared with wild type strain, these results show that PtpA is essential for mycobacteria survival within macrophage [88]. SapM is a secretory phosphatase that dephosphorylates phosphatidylinositol 3-phosphate (PI3P) on the phagosome membrane [87]. PI3P is essential for phagosomes to acquire lysosomal constituents; it is involved in the docking of rab effector proteins early endosomal autoantigen 1 (EEA1) and hepatocyte growth factor-regulated tyrosine kinase (HRS) substrate, which are important for phagosome maturation [89]. Disruption of *sapM* in MTB resulted in a highly attenuated strain with an impaired ability to grow in the THP-1 macrophages as well as in the guinea pig tissues [33].

Ndk is a nucleoside diphosphate kinase with ATP- and GTP-binding activity and it is widely conserved across all the three domains of life. This protein is autophosphorylated and secreted into the culture medium by MTB and possesses GAP activity towards Rho GTPases Rab5 and

Rab7, leading to reduced phagolysosome fusion [90]. Besides, Ndk also targets and inactivates the small GTPase Rac1, an essential component of the macrophage NADPH oxidase (NOX2) complex, inactivation of Rac1 was associated with reduced NOX2-mediated production of reactive oxygen species (ROS) and ROS-dependent apoptosis thus contributing significantly to mycobacterial virulence [91]. Another factor crucial for inhibition of phagolysosome fusion is the serine/threonine protein kinase G (PknG). In contrast to other mycobacterial kinases, autophosphorylation on Thr residues at the N terminus of PknG are not involved in the regulation of this kinase; however, it is essential for the capacity of PknG to block lysosomal delivery of mycobacteria and for the bacterial survival in murine BMDM [92].

The PE_PGRS protein family includes around 60 proteins but the role and function of these proteins remains elusive. Nonetheless, PE-PGRS30 was the first PE-PGRS protein with a certain role in the virulence of MTB. PE_PGRS30 mutant was impaired in its ability to colonize lung tissue and to cause tissue damage; and inactivation of PE_PGRS30 resulted in an attenuated phenotype in murine and human macrophages due to the inability of the MTB mutant to inhibit phagosome-lysosome fusion [62].

Several factors have been implicated in phagosome maturation arrest, such as LAM, TDM, LpdC, Zmpq and the Esx-1 secretion system [27, 93–95]. But more importantly it seems that the requirement for these processes in the different mycobacterial species may not necessarily be identical; for example, BCG is able to arrest phagosome maturation in spite of the absence of RD1 locus, thus phagosome arresting in the case of BCG can be without ESAT-6 and CFP-10 although these proteins are necessary for this arrest in *M. marinum* [94, 96]. Therefore it is noteworthy that phagosome maturation and its arrest are complex processes with multifactorial requirements.

4.2. Resistance to reactive oxygen and nitrogen species

Upon phagocytosis of mycobacteria, macrophages produce antimicrobial reactive oxygen and nitrogen species (ROS and RNS) via the enzymatic activity of NADPH oxidase (NOX2) and inducible nitric oxide synthase (iNOS), respectively. NOX2 is a multiprotein enzyme complex that assembles and activated in response to phagocytosis. This enzyme complex transfers electrons across the membrane from cytosolic NADPH to molecular oxygen, the reaction produce superoxide anions (O_2^-) which dismutates into hydrogen peroxide (H_2O_2) and generates toxic hydroxyl radicals [48]. iNOS is induced upon IFN-γ activation and produces nitrite and nitrate via nitric oxide (NO), this reacts with O_2^- and forms peroxynitrite ($OONO^-$) [97]. This reactive oxygen and nitrogen intermediates (ROI and RNI) react with a wide range of molecules, such as nucleic acids, proteins, lipids and carbohydrates, thus for intracellular pathogens like MTB survival upon exposure to oxidative stress is critical.

Among the factors that contribute to MTB success as a pathogen are: its ability to survive the redox stress manifested by the host and its capacity synchronize its metabolic pathways and expression of virulence factors. Two component proteins, namely DosS and DosT, are employed by MTB to sense changes in oxygen, nitric oxide and carbon monoxide levels, while WhiB3 and anti-sigma factor RsrA are used to monitor changes in intracellular redox

state [98, 99]. Using these and other unidentified redox sensors, mycobacterium orchestrates its metabolic pathways to survive in nutrient deficient, acidic, oxidative, nitrosative and hypoxic environments inside granulomas or infectious lesions [97]. MTB employs versatile machinery of the mycothiol and thioredoxin systems to ensure a reductive intracellular environment for optimal functioning of its proteins even upon exposure to oxidative stress [97]. Mycothiol is a low-molecular-weight thiol and functions like glutathione, the archetypal redox buffer, which is not produced by mycobacteria. Therefore it has antioxidant activity as well as the ability to detoxify a variety of toxic compounds. The thioredoxin (Trx) system is composed of NADPH, thioredoxin reductase (TrxR), and Trx is a small redox protein with two redox-active Cys residues in its active site [100]. Trx is responsible for maintaining a reducing intracellular environment, regenerating the reduced forms of methionine sulfoxide reductase and peroxiredoxins, as well as the redox regulation of enzymes and regulatory proteins by oxidoreduction and the detoxification of ROS [100]. MTB contains three types of Trx, although TrxB is the only one essential to fight against host defenses and for *in vitro* growth [101]. Additionally, mycobacterium employs a battery of protective enzymes, such as superoxide dismutase (SOD), catalase (KatG), alkyl hydroperoxidase (AhpC) and peroxiredoxins to detoxify and neutralize these redox stresses [97]. SOD is a metalloprotein produced by prokaryotes and eukaryotes to detoxify superoxide radicals. This enzyme dismutates O_2^- into H_2O_2 and molecular oxygen and has been shown to contribute to the virulence in a number of pathogens, including MTB, which have two genes, *sodA* and *sodC* [97]. Pathogenic mycobacterium species express and secrete higher levels of SodA compared to the non-pathogenic species. In fact, MTB mutants with reduced SodA expression displayed increased susceptibility to H_2O_2 and were markedly attenuated in mice [102]. Although SodA lacks a classical signal sequence for protein export, it is a protein dependent on SecA2 for secretion. MTB mutants in *secA2* are defective in the export of SodA and KatG, and are unable to grow in non-activated macrophages and showed reduced growth in mice [103]. SodC is a Cu/Zn superoxide dismutase anchored in the outer-membrane to protect MTB from reactive oxygen intermediates at the bacterial surface. Mutants in *sodC* show decreased survival in IFN-γ-activated murine peritoneal macrophages and have increased sensitivity to hydrogen peroxide [104].

Catalase peroxidases are enzymes that protect the bacterium from ROS damage and are used to detoxify H_2O_2, KatG from MTB degrades H_2O_2 and organic peroxides. Thus the major role of KatG in TB pathogenesis is to catabolize the peroxides generated by the phagocyte NADPH oxidase; although in the absence of this host antimicrobial mechanism, KatG is apparently dispensable [105]. Moreover, KatG also activates the anti-tuberculosis drug isoniazid (INH) converting it to several reactive species that inhibits a mycolic acid biosynthesis [105]. Although isoniazid resistance is multigenic, mutations in *katG* predominate among the INH-resistant strains, but the effect of these mutations on MTB virulence is variable. In general, *katG* mutations render MTB strains sensitive to endogenous or exogenous peroxides, generated during bacteria respiration or by phagocytes during infection [105], maybe because mutations also affect the peroxidase domain of KatG [106] though the activity of the alkyl hydroperoxidase C (AhpC) has been described as an important compensatory mechanism in INH-resistant strains [107].

AhpC is a member of the peroxiredoxin family that detoxifies organic peroxides into less reactive alcohol derivatives and confers protection against both oxidative and nitrosative stress. AhpC mutants show an essential role in the resistance to host oxidative agents in the early stages of infection [108]. Besides, phenotypic *M. bovis* mutants producing less AhpC were less virulent in guinea pig model than the wild type [109].

Peroxiredoxins (Prx) proteins are multifunctional antioxidant enzymes that reduce and thus detoxify hydroperoxides, organic hydroperoxides and peroxynitrite using electrons from Trx. Five Prx enzymes have been identified in MTB: AhpE, TPx, AhpC, Bcp and BcpB [97]. Tpx is the principal and most effective enzyme involved in the detoxification of H_2O_2 and peroxynitrite in mycobacteria. In macrophages, the *tpx* mutant shows impaired replication in activating and resting cells, and in mice the mutants were less lethal and persistent that the wild-type strain [110].

Deletion of genes that encode methionine sulfoxide reductase (msrA), Mtb proteasome (prcBA), nucleotide excision repair (uvrB) and F-420 biosynthesis (fbiC) are also hyper-susceptible to RNS [111]. Likewise, α-crystalline (HspX), bacterioferritin (bfrB) and the DosR regulon are upregulated by conditions that inhibit aerobic respiration; however, their role in MTB virulence is little understood [1].

4.3. Inhibition of apoptosis

During MTB infection, several forms of cellular fates have been observed such as necroptosis, apoptosis and autophagy, among those, apoptosis and autophagy have been recognized as innate macrophage defense mechanisms. Apoptosis is highly regulated process where the cytoplasm and other cellular organelles of dying cell are enclosed in membrane bound vesicles called apoptotic bodies. The apoptotic bodies are taken up by macrophages via receptor-mediated phagocytosis in a process defined as efferocytosis without elicit any inflammatory response [112].

Apoptosis reduces the viability of different mycobacterial species, including MTB; in fact many attenuated strains of mycobacteria induce more apoptosis than their wild type counterparts and exists a reciprocal relationship between virulence and apoptosis [113]. MTB infection mainly results in necrosis, while attenuated mutant strains including BCG and H37Ra primarily induce apoptosis [113]. Most of the factors that have been described as anti-apoptotic molecules play roles in the bacterial redox homeostasis (*katG*, *sodA*, *secA2* and *pknE*) because phagosome ROS promotes the apoptosis.

The *nuoG* gene of MTB encodes the NuoG subunit of the type I NADH dehydrogenase (NDH-1) that is important in the inhibition of host macrophage apoptosis, since MTB mutant-induced apoptosis in human THP-1 cells. Moreover, BALB/c and SCID mice infected with this mutant survived longer and the bacterial load in lungs were smaller than of the wild-type strain [114]. In addition, MTB can neutralize ROS in order to inhibit TNF-mediated host cell apoptosis via a NuoG-dependent mechanism [115]. Similar function can also be observed for SecA2, which is the ATPase of the canonical bacterial Sec secretion system, and as stated above SecA2 is required for intracellular growth of MTB in macrophages preventing phagosome maturation

[116]. Mutant of MTB in *secA2* induces more apoptosis than wild type in infected macrophages, but more importantly the author shows that SodA secretion was the major SecA2 process involved in the inhibition of apoptosis [117]. Therefore, it can assume that the SecA2 secretion system, most likely through SodA, inhibits apoptosis in a mechanism probable independent of oxidative burst [118].

The serine/threonine kinase E, pknE contributes to the survival response of MTB by regulating the bacilli machinery to resist apoptosis during nitrate stress. Deletion of *pknE* results in a mutant that was more susceptible to NO exposure, inducing higher level of apoptosis than wild-type strain and less production of TNF-α and IL-6. However, further *in vivo* studies are needed to establish the role of PknE in the virulence of MTB [119].

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