REVIEW

PROTEASES ASSOCIATED WITH *Mycobacterium tuberculosis* INFECTION

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ABSTRACT

Tuberculosis is a contagious infectious disease caused by *Mycobacterium tuberculosis*, an obligate intracellular bacterium that relies on infection and host-to-host transmission to survive. In a co-evolutionary process, the pathogen developed virulence mechanisms to evade the host's immune system and endure a number of factors, such as cellular stress. One of the strategies used by pathogens to succeed in causing infection is the production of proteases, which are enzymes that cleave peptide bonds between the amino acids in a protein. Proteases are widely distributed in nature and have different roles considered important to the bacteria's biological cycle. *M. tuberculosis* has several protease coding genes in its genome, many of which with unknown functions, but several with recognized roles in the infection process. This review presents the literature researched from 2014 to 2018 that addressed the roles of the proteases involved in *M. tuberculosis* infection.

KEY WORDS: *Mycobacterium tuberculosis*; virulence; infection; protease.

INTRODUCTION

Tuberculosis (TB) is a contagious infectious disease that has smitten humanity for thousands of years. Therefore, still a global public health concern with 10 million new cases of TB registered worldwide in 2017, the equivalent of 140 cases per 100,000 inhabitants (Frieden et al., 2018). In Brazil, in 2017, 69,569 new cases of tuberculosis were reported. The incidence coefficient was equal to 33.5 cases/100 thousand inhabitants (Brasil, 2018). Its etiological agent, *M. tuberculosis*, is a primarily intracellular pathogen that is capable of successfully maintaining its viability within the host, involving a delicate interplay between the immune system and the pathogen's defenses, acquired and developed throughout thousands of years of host-pathogen interaction (Dorhoi & Kaufmann, 2016).

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M. tuberculosis is a bacterial pathogen that has been adapting and creating strategies to evade host defenses by means of various mechanisms to establish an infectious process (Jurkoshek et al., 2016). The hallmark of tuberculosis pathology involves granuloma formation, a complex structure that involves the activation of Th1, Th17 and Tc CD8 lymphocytes that culminate with the activation of macrophages and neutrophils, among other cells, which in turn are modulated by the bacilli (Cadena et al., 2017). *M. tuberculosis* depends on the action of a host and its own proteases to be able to begin infection. For example, upon reaching the lung, the pathogen induces the secretion of matrix metalloproteinases (MMPs) by the host cells that are capable of disrupting the extracellular pulmonary matrix causing inflammatory cellular infiltrations and the formation of granuloma contributing to the pathology of tuberculosis (Brilha et al., 2016).

M. tuberculosis has approximately 4,411,529 base pairs and 4,056 open reading frames (ORFS) (Cole et al.,1988). In its genome, 101 potential genes encode for proteases or peptidases as described in the MEROPS database (Rawlings et al., 2018). Studies have shown that these molecules indicate a promising future for the development of new therapeutic targets as well as new vaccines, including proteases involved in cellular processes such as secretion and turnover and those involved in the virulence process (Roberts et al., 2013). Table 1 sumarizes the list and main functions of *M. tuberculosis* proteases presented in this review.

Proteases are able to selectively modify proteins by cleavage, and consequently activate molecules such as zymogenic enzyme forms and blood coagulation proteins or enable proteins to be secreted during protein transportation through membranes. In addition to their enzymatic function, proteases play several roles, they can accelerate the disease process and host impairment during infection, such as systemic activation of the protease cascade, accompanied by the degradation of several inhibitors, which will lead to sepsis / septicemia, hypotension, disseminated intravascular coagulation (DICV), and systemic inflammatory response syndrome (SIRS). These events are the most common causes of death in the late stages of infection (Forrellad et al., 2013).

According to the hydrolysis site, the proteases are classified as: endopeptidases, when they cleave the internal peptide bonds, which differ from the exopeptidases that cleave the terminal bonds. The latter are subdivided into amino peptidases and carboxypeptidases, depending on which termination they act on. In addition to being classified by the site of action, the proteases are also classified according to the cofactor present in the active site involved in the hydrolysis reaction. Thus, those having aspartic acid residues in the catalysis site are called aspartic proteases, such as HIV proteases. In contrast, metalloproteases require a bivalent metal ion at their active site, zinc being the most common, but cobalt, iron and manganese may also be found. Matrix metalloproteases and methionine metalloprotease are examples of metalloproteases. Finally, serine proteases require a serine residue to carry out hydrolysis and are exemplified by chymotrypsin, trypsin (digestive enzymes of the intestine) and proteases involved in the coagulation cascades (Hooper, 2002). Table 2 shows selected metalloproteases involved in *M. tuberculosis* infection which are presented in this Review.

Table 1. Mycobacterium tuberculosis proteases cited in this review involved in the tuberculosis pathogenesis.

Proteases	Function	Action locality	Response Type/Essentiality
LepB	Secretion: Type I signal peptidase	Cytoplasmic membrane	SPase I that is essential for bacterial growth; Inhibition of the *M. tuberculosis* SPase I activity using MD-3, a known SPase I inhibitor, leads to the death of both replicating and non-replicating bacteria.
Mycosins: MycP1	Virulence	Secreted or cell wall associated, but also present in the cytoplasmic membrane	Increased macrophage response and decreased virulence in the chronic stage of infection. Inhibitors targeting this pathway could be of interest for disrupting latent infection;
MycP3			MycP3 is essential for the growth of *M. tuberculosis.*
HtrA proteases	Virulence and other functions	Membrane-bound	Serine proteases family; HtrA1 (Rv1223), HtrA2 (Rv0983) and HtrA3 (Rv0125). This large family of proteases is responsible for maintaining the proteome of a cell by degrading misfolded proteins.
POPMt (Rv0457c)	Induce proinflammatory responses.	Immunomodulatory properties	Recruitment of inflammatory cells to the lungs, followed by dissemination to the lymph nodes during the adaptive immune response
MtLAP (Rv2213)	Unknown	Cytosol	Exhibited strong sensitivity to bestatin, a strong aminopeptidase inhibitor. Possible drug target.
Zmp1 (Rv0198c)	Virulence	Cytosol	The deletion of ET-1 during infection resulted in an increase in bacterial load and lung lesions.
RIP-1(Rv2869c)	Signal transduction	Membrane-bound	RIP-1 cleaves substrates of anti-sigma factors to control downstream pathways involved in lipid biosynthesis and protects against stress

Because *M. tuberculosis* infection presents a spectrum of clinical outcomes among the affected individuals, and proteases from both the host and the pathogen have been shown to play biological and physiological roles that allow the microorganism to succeed in infection, understanding the roles and functions of these enzymes, through a current literature review, will provide tools for new therapeutic strategies or options for new vaccines to improve disease control.

Table 2. Host metalloproteases involved in degradation of the cell matrix induced by *Mycobacterium tuberculosis* and cited in this review

Proteases	Action locality	Response Type/Essentiality
MMP-1	Cell matrix	Expression of the MMP-1 gene involves the activation of activating protein 1 (AP-1), an expression factor that regulates gene expression in response to a variety of stimuli such as stress and bacterial infection.
MMP-2	Central Nervous System	In active TB, excess MMP-2 is probably due to secretion by microglial cells, since these are produced by monocytes and macrophages infected with *M. tuberculosis*.
MMP-9	Leptomeninges	The level of MMP-9 increased according to the evolution of tuberculosis raising the possibility of targeting MMP-9 as a therapeutic strategy.

METHODOLOGY

This review researched articles published over the last five years (2014 to 2018) using the keywords: *M. tuberculosis*, proteases, virulence and infection found in the electronic database of PubMed. Articles focusing on the *M. tuberculosis* proteases involved in the mechanisms of host survival/virulence were included. Of the 30 articles found, 14 articles were excluded, since these articles were not specifically concerned with proteases involved in the infection or virulence of *M. tuberculosis*. Articles published prior to 2014 of seminal importance were also included.

RESULTS AND DISCUSSION

Proteases induced by M. tuberculosis *involved in degradation of the cell matrix*

The lung is composed of important fibrillar collagens as they provide protection against possible pathogen invasions and are highly resistant to enzymatic degradation (Elkington et al., 2011). However, MMPs are the only known enzymes capable of cleaving the fibrillar collagen of the lung and may also be involved in remodeling and repairing various tissues. *M. tuberculosis* subverts the immune response of the host in its favor, leading to proteolytic destruction of the extracellular matrix. This process of degradation occurs through the regulation of the secretion of MMPs by host cells, contributing to the pathogenesis of *M. tuberculosis* facilitating bacillary dissemination (Ulrichs & Kaufmann., 2006).

Among the different MMPs, MMP-1 (interstitial collagenase) plays a critical role in conducting immunopathology in active pulmonary TB during infection (Elkington et al., 2005). An expression of the MMP-1 gene involves the activation of activating protein 1 (AP-1), an expression factor that regulates gene expression in response to a variety of stimuli such as stress and bacterial infection. The c-Jun N-terminal kinases (JNKs) play a role in stress signaling pathways. These factors are described as being induced in response to oxidative stress (Andrade et al., 2015). Hemeoxygenase is an important antioxidant highly expressed in the lungs and its role was potentially associated with cell protection that led Andrade et al. to a hypothesis that *M. tuberculosis* infection may be influenced by stress-induced HO-1 that acts on lung damage / remodeling by MMPs. It was observed that the levels of HO-1 and MMP-1 had increased in patients with active pulmonary TB possibly as a result of the interaction between host and pathogen (Andrade et al., 2015).

Tuberculous meningitis is an infection of the leptomeninges (structures that form the meninges) basally affecting the brain. When bacilli cross the blood-brain barrier they result in tuberculomas, which rupture in the cerebral

spinal fluid leading to adjacent brain structures becoming infected (Spanos et al., 2015). During this process, the microglia cells present in the host are activated and secrete proteases capable of degrading the extracellular matrix causing tissue destruction. Among these proteases, MMPs appear to play a prominent role in tissue destruction. The level of MMP-9 increased according to the evolution of the tuberculosis raising the possibility of targeting MMP-9 as a therapeutic strategy to prevent the progression of the disease in more advanced stages. When SB-3CT or dexamethasone inhibitor drugs were used, MMP-9 levels were reduced to almost undetectable levels and the CFU count was significantly reduced by treatment with anti-tuberculosis drugs. Thus, the importance of MMP-9 in tissue destruction during tuberculous meningitis. Its inhibition may also be beneficial in the treatment of the disease (Cui et al., 2012; Majeed et al., 2016).

In the Central Nervous System (CNS), MMP-2 degrades the basement membrane, type IV collagen and laminin. MMP-2 is constitutively expressed at high levels by many cells and is regulated by pro-peptide activation (Be et al., 2009). In active TB, excess MMP-2 is probably due to secretion by microglial cells, since monocytes and macrophages infected with *M. tuberculosis* do not secrete MMP-2 and astrocytes do not alter its MMP-2 secretion in response to *M. tuberculosis* infection (Majeed et al., 2016; Harris et al., 2007).

One of the host's key responses to contain the bacillus during infection is the formation of a granuloma, which when intact becomes beneficial as it keeps the pathogen under control (Ulrichs & Kaufmann, 2006). Ramos-Martínez et al., (2015) analyzed the expression of MMPs associated with inflammation during experimental tuberculosis infection. In this work, BALB/c mice were infected intratracheally with *M. tuberculosis* H37Rv, numerous granulomas, composed of activated macrophages producing TNFα and iNOS, and T helper cells expressing IFN-γ and IL-2 developed, temporarily controlling bacterial growth. Bacterial proliferation was accompanied by the increasing expression of anti-inflammatory cytokines such as TGF-β, IL-10 and IL-4, and decreased IFN-γ, TNF-α and iNOS, as well as extensive tissue damage (progressive pneumonia, interstitial fibrosis) and death of the animals. However, the expression of MMPs increased and reached its highest expression on day 21 post infection, when, the cell-mediated immunity reached the highest level of protection. These results suggest that the extracellular matrix breakdown seems necessary for the initial immune events (Ramos-Martínez et al., 2015).

M. tuberculosis *proteases involved in immune response modulation*

Proteases secreted by the pathogen are also involved in the modulation of the immune response by *M. tuberculosis*. The first response generated by the host against infection is the recruitment of inflammatory cells to the lungs, followed by dissemination to the lymph nodes during the adaptive immune

response (Wolf et al., 2008). However, proteases have the ability to modulate these responses. Portugal and colleagues reported the immunomodulatory properties of a putative prolyoligopetidase POPMt (Rv0457c), classified as serine peptidase of the S9 family. This led Portugal and colleagues to evaluate the immunomodulatory properties in triggering immune responses. Peritoneal macrophages, when stimulated with POPMt for 24 hours, produced proinflammatory cytokines such as TNF, IL-12p70, IL-6, IL-23 and IL-1b, as well as chemokines such as (MCP-1) after 24h, confirming its importance during the response generated by the host (Portugal et al., 2017).

M. tuberculosis *proteases with diverse function*

Several *M. tuberculosis* proteases that aid in the pathological process have already been described. The bacterium requires the use of lipids and host fatty acid for survival (Lee et al., 2013). Research has shown that *M. tuberculosis* accumulates triacylglycerol (GAG) in hypoxic environments. These are synthesized by bacterial TAG synthase using the host-derived fatty acids (Daniel et al., 2011). Based on in silico, in vitro and ex vivo studies, Singh et al., (2017) showed a mycobacterial gene, Rv2672 that encodes for a protein with lipase and hydrolase activity, as it catalyzes the lipid hydrolysis of the host. In an in vitro model of infection by *M. tuberculosis*, Rv2467 was characterized as a Mycobacterial Secreted Hydrolase 1 (Msh1). This enzyme with possible protease and lipase domains showed its importance for the growth of *M. tuberculosis* in lipid-rich hypoxic environments such as granulomas (Singh et al., 2017).

There is evidence that in protozoa, parasites and helminths the leucyl aminopeptidases (LAPs) belonging to the M1 or M17 family of peptidases, which constitute a group of Zn-dependent metallopeptidases, play an important role in infection. An endopeptidase was described as being required for *M. tuberculosis* virulence as well as for its survival in macrophages. The enzyme designated as Zmp1 has homology with the human endothelin-converting enzyme. Endothelins are important peptides for constricting blood vessels and increasing blood pressure, which are considered important activities in the control of granulomas formed during TB infection. According to Correa et al. (2014), Zmp1 (Rv0198c) showed a proteolytic activity that did not cleave the endothelin precursor (Big ET-1) to release the ET-1 peptide but was able to degrade it. The results showed that the deletion of ET-1 during infection resulted in an increase in the bacterial load and lung lesions. However, *M. tuberculosis* produces Zimp 1 capable of cleaving ET-1 and consequently interferes with ETA or ETB receptor signaling. Thus, a balance between ET-1 levels controlled by Zmp1 and ETA/ETB signaling may allow the adaptation and survival of *M. tuberculosis* in lung tissue (Singh et al., 2017).

M. tuberculosis has to respond to a variety of stress conditions inflicted by the host, including iron limitation, reactive nitrogen and oxygen intermediates and starvation (Urban, 2009). These stress characteristics require signal transduction systems that transmit information within the cell. The signal transduction mechanism controls for example the availability of alternative sigma factors that direct RNA polymerase to specific promoters (Sklar et al., 2010). Regulated intramembrane protease (RIP1) is a metalloprotease (Rv2869c) that can cleave three anti-sigma factors (i.e. anti-Sigk, anti-SigL and anti-SigM) which are negative regulators of sigma K, L, M factors, respectively. RIP-1 cleaves substrates of anti-sigma factors to control downstream pathways involved in lipid biosynthesis and protects against stress. Rip1 is required for M. tuberculosis virulence since Rip1 deletion impairs *M. tuberculosis* growth in mouse lungs and persistence of chronic infection (Schneider et al., 2014).

The aminopeptidases belonging to the M17 metalloprotease family has been shown to be a potential therapeutic target (Matsui et al., 2006). Through this information, Correa and colleagues observed that MtLAP (Rv2213) from *M. tuberculosis* is a metal dependent protease available in cytosol which exhibited strong sensitivity to bestatin, a strong aminopeptidase inhibitor. Testing its effect on *M. tuberculosis* infected macrophages, bestatin treatment strongly inhibited the bacterial growth, and, in the murine model of tuberculosis, this drug caused a reduction in bacterial load and lung injury. These effects on the growth and infection by M. tuberculosis confirm aminopetidases as a strong therapeutic potential target for the control of the disease (Correa et al., 2017).

Proteases involved in mycobacterial stress

M. tuberculosis has created mechanisms to assist its resistance to stress conditions, such as; acid pH, low oxygen content, low nutrient availability, reactive oxygen and nitrogen intermediates as well as other products generated by the host. These stress conditions lead to changes in protein conformation, such as; poor folding and aggregation (Rohde et al., 2007) and, consequently, the removal of damaged proteins is necessary for the establishment of infection. An *M. tuberculosis* gene called PepD (Rv2745c) has demonstrated the possibility of performing this function. This gene encodes a serine protease type, HtrA, responsible for the processing of altered *M. tuberculosis* proteins due to exposure to stress. PepD is directly regulated by the stress-responsive two-component signal transduction system, MprAB and indirectly by the sigma factor of the extracytoplasmic function (ECF) SigE. In a genomic analysis, McGillivray and colleagues (2014) observed mechanisms in which the Rv2745c gene product helps *M. tuberculosis* to respond to redox stress. The protein encoded by Rv2745c facilitates signaling downstream in response to stress, and its suppression leads to disruption of important regulators. Understanding the response to this stress may help to better understand the ability of *M. tuberculosis* to survive in macrophages (McGillivray et al., 2014).

Proteases involved in protein secretion and signal transduction

An important mechanism of virulence in *M. tuberculosis* is the ESX-1 type VII secretion system. This system transports virulence factors important for the pathogen replication in macrophages causing effects on host cells (Stanley et al., 2003). This system has as substrates ESAT-6 and CFP-10, two proteins secreted and identified as immunodominant antigens of *M. tuberculosis*. The deletion of these proteins in virulent strains resulted in a decrease in their virulence, suggesting that these proteins are important for *M. tuberculosis* virulence. The ESAT-6 and CFP-10 proteins are encoded by the esxA (Rv3870) and esxB (3871) genes, respectively, with various activities including inhibition of phagosome maturation and cytokine signaling by infected macrophages. However, some proteins are required for their secretion such as MycP1 (Rv3883), a serine protease similar to subtilisin, which is secreted and degrades non-specific proteins. Ohol et al. demonstrated the importance of MycP1 for ESX-1 secretion in *M. tuberculosis* and suggestted that MycP1 has defined substrate specificity and cleaves substrates containing proline residues. MycP1 protein could perhaps be an interesting drug target for latent infection, as it is predicted to be outside the cell membrane and therefore accessible to small molecules (Ohol et al., 2010., van Winden et al., 2016).

Originally Hip1 was thought to be a carboxylesterase because it was unable to cleave commonly used trypsin-like protease substrates in vitro. However, a transposon mutant Hip1-KO strain was found to be deficient in proteolytic processing of the mycobacterial heat-shock protein GroEL2. A later study revealed that Hip1 protease (Rv2224c) is indeed a serine protease that can cleave GroEL2 in vitro and in vivo, resulting in the extracellular release of processed monomeric GroEL2 as a mediator of Hip1-dependent immunomodulatory activities. This serine protease associated with the cellular envelope is required for the immunomodulation of host inflammatory responses. Because of its role in mycobacterial virulence, it becomes a potential drug target. Lentz et al. demonstrated a strategy to develop chemical probes (irreversible inhibitors, ABPs and selective substrate probes) for Hip1 activity in *M. tuberculosis* by combining compound library selection with focus on multiple types of substrate selectivity profiles. Important determinants of the substrate were identified that are important for the recognition of Hip1, as well as a chloroisocoumarin compound that irreversibly inhibits Hip1 (Lentz et al., 2016).

It is known that the *M. tuberculosis* genome encodes approximately 70 lipoproteins, many of which are important for virulence, mediating pathogen-host interactions. They are involved in various functions such as; cell wall synthesis, nutrient uptake, transmembrane signaling and adhesion, and their secretion depends on the action of signal peptidases which can be found in types I and II (Sutcliffe & Russell, 1995). Type II signal peptidases were excluded from this analysis because they were not studied in the period comprised in this review. Type I signal peptides (Spase type I) are membrane bound endopeptidases responsible for cleaving signal peptide of secreted proteins during membrane translocation through the general

secretion pathway (Sec). They are extremely important, since the cleavage of the N-terminal signal peptide allows the release of the mature protein from the cytoplasmic membrane to the external environment. An example of *M. tuberculosis* Spase type I is the LepB protease (Rv2903c) which plays a key role in bacterial growth. Its inhibition leads to death of both replicating and non-replicating bacteria. It has a transmembrane domain that allows its anchorage in the cytoplasmic membrane. Some potential compounds with inhibitory activity to this protease have been discovered and are promising therapeutic drugs (Ollinger et al., 2012; Bonnett et al., 2016).

CONCLUDING REMARKS

This review has consolidated knowledge about the various proteases involved in *M. tuberculosis* pathogenesis which were investigated from 2014 to 2018.

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