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REVIEW PAPER

ESX secretion system: The gatekeepers of mycobacterial survivability and pathogenesis

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ABSTRACT

Mycobacterium tuberculosis, the causative agent of Tuberculosis has plagued humankind for ages and has surfaced stronger than ever with the advent of drug resistance. Mycobacteria are adept at evading the host immune system and establishing infection by engaging host factors and secreting several virulence factors. Hence these secretion systems play a key role in mycobacterial pathogenesis. The type VII secretion system or ESX (early secretory antigenic target (ESAT6) secretion) system is one such crucial system that comprises five different pathways having distinct roles in mycobacterial proliferation, pathogenesis, cytosolic escape within macrophages, regulation of macrophage apoptosis, metal ion homeostasis, etc. ESX 1–5 systems are implicated in the secretion of a plethora of proteins, of which only a few are functionally characterized. Here we summarize the current knowledge of ESX secretion systems of mycobacteria with a special focus on ESX-1 and ESX-5 systems that subvert macrophage defenses and help mycobacteria to establish their niche within the macrophage.

KEYWORDS

mycobacterium, ESX secretion system, phagosomal rupture, cell death

INTRODUCTION

Tuberculosis, the leading cause of death amongst the infectious diseases worldwide, has risen to alarming proportions owing to the emergence of extreme and total drug-resistant mycobacterial species (1). Healthy individuals pick up mycobacteria containing aerosols released during coughing or sneezing of an infected individual, whereupon the mycobacteria reach the lung and are therefore engulfed by alveolar macrophages. Once inside the macrophage phagosome, mycobacteria hijack several host factors directly or indirectly through the secretion of several virulence factors to prevent phagosome maturation or escape into the cytosol and thereby prevent their elimination and establish their niche within the macrophage [1]. Due to harsh intramacrophage conditions of immunocompetent individuals, mycobacteria activate their Dormancy Survival Regulator (DosR) regulon to trigger dormancy and stay latent for an extended period until the individual becomes immunocompromised [2]. *Mycobacterium tuberculosis* (*Mtb*) is known to adopt some exclusive strategies to replicate and maintain its intracellular persistence.

Protein export systems are utilized by pathogenic bacteria to unleash their virulence repertoire [3]. Since a hydrophobic mycolic acid layer on the mycobacterial outer layer acts as a shield against the harsh macrophage conditions, regulation of the protein secretion system through this impermeable cell envelope remains an enigma [4]. Though several outer membrane proteins (OMPs), cell envelope, or surface-exposed proteins are involved in host immune modulation and exhibit virulence [5, 6], the role of secreted proteins depends on its export to bacterial outer spaces for which they harbor specialized transportation system. Hence such export systems play crucial roles in protein export through the mycobacterial membrane, thus leading to appropriate mycobacteria-host interaction and thereby enabling

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mycobacterial infection and pathogenesis [7]. Thorough knowledge of the mycobacterial protein export systems would help to understand the physiology and pathogenesis of *Mtb* and thereby aid in future drug development [8, 9].

Two protein export pathways conserved in *Mtb* are the general secretion (Sec) and the twin-arginine translocation (Tat) export pathways [10]. Additionally, two specialized export systems include the 6 kDa early secretory antigenic target (ESAT6) secretion (ESX) systems and the accessory Sec (SecA2) export pathway [11]. The Sec pathway, which translocates proteins in their unfolded state, has three components, one that targets the substrate, a motor protein, and a SecYEG translocase, which is a conducting channel integrated within the membrane [12]. The periplasm destined proteins possess a signal sequence recognized by SecB, which acts as a chaperone by binding to the pre-secretary proteins and thereby prevents the folding of those proteins [13]. The substrates are then delivered to the multifunctional SecA protein, which then guides the proteins to the SecYEG channel and acts as an ATPase to provide energy for protein translocation [14]. The attribution of SecA in mycobacterial protein export has been shown in *Mycobacterium smegmatis* by performing conditional silencing of *secA1* [15]. Depletion of SecA1 using a tetracycline repressor resulted in growth inhibition and reduced export of the cell wall porin MspA [16].

On the other hand, the Tat pathway comprises of TatA, TatB, and TatC. For folded proteins, the Tat signal sequence pairs with a "twin" arginine at the N-terminal S-R-R motif [17]. *Mtb* utilizes the Tat pathway to secrete the virulent phospholipase C [18]. β-lactamase (BlaS) export in *M. smegmatis* is dependent on the Tat pathway as it harbors a signal peptide for the Tat pathway; therefore *tat* mutants are unable to export BlaS [19]. *Mtb* β-lactamase (BlaC), when expressed in the *tat* mutant *M. smegmatis*, failed to get exported indicating as a Tat dependent substrate.

Additionally, the SecA2 pathway is conserved in *Mycobacterium*, along with the SecY2 protein, that constitutes the SecA2/Y2 system, capable of exporting large glycosylated proteins [20]. Site-directed mutagenesis of K129R in the ATP-binding site of the SecA2 export system renders it defective in protein export. This leads to the accumulation of the SecA2 substrates at the membrane instead of being exported to the macrophage cytosol [21]. So it would be intriguing to explore the presence of signal peptides in the substrates exported by SecA2. Since *Mtb* SecA2 can inhibit the host innate immune response, *secA2*-mutant mycobacteria-infected macrophages produce more pro-inflammatory cytokines resulting in the apoptotic death of these infected macrophages as compared to wild-type mycobacteria infected macrophages [22]. Interestingly compared to the *Mycobacterium bovis* BCG vaccine, this *secA2* mutant *Mtb* provides better protective immunity against TB [23]. Among the secretion systems, an essential secretory pathway is ESX, a type seven secretion system (T7SS), possessing five pathways, namely ESX1-5, wherein ESX-3 and ESX-5 are mainly used in protein transportation while being associated with ESX-1. Proteins that harbor WXG motifs are marked as substrates for transportation through the ESX system (Fig. 1A). The ESX systems secrete small proteins having homology to ESAT-6, and this is the hallmark of exportation through this system as these ESAT-6-like proteins (Esx) do not possess Sec or Tat signal peptides and rely on the ESX system for their secretion [24]. Here we have comprehensively discussed the contribution of the ESX pathway in the mycobacterial pathogenesis.

The components and substrates of the T7S system are present in various orders and families. BLAST search identifies T7S system-specific homologous genes *eccA*, *eccC*, and *mycP* in similar T7S specific loci in the Actinobacterium genome. These ESX proteins and EccC components of Actinobacteria are conserved with mycobacterial T7S system (Fig. 1B). Besides, the Pfam family proteins are also associated with the T7SS. Although the T7S system is also found in Firmicutes, there are some distinctive differences in the mode of secretion by Firmicutes and Actinobacteria [25]. The functional ability of the T7S system is exhibited in different species through the secretion of ESX proteins (9).

The chromosomal ESX loci of mycobacteria show the existence of five classical types of ESX systems. Based on phylogenetic and gene order analysis, ESX systems can be

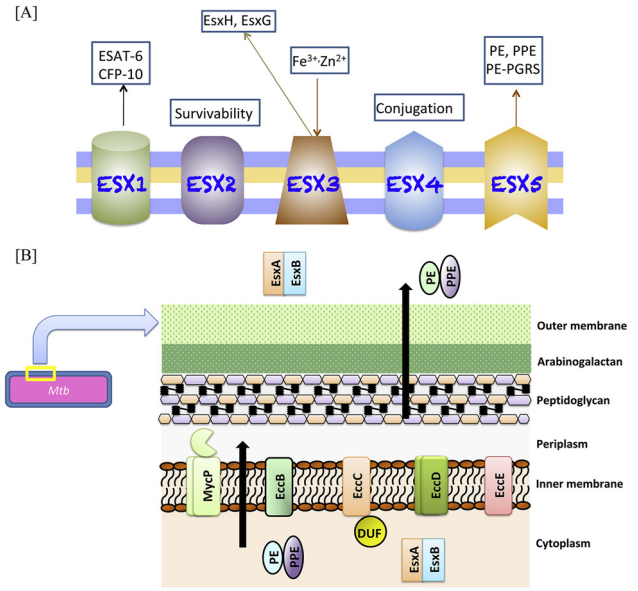

Fig. 1. **[A]** The ESX system comprises of five pathways-ESX-1, which secretes antigens that lead to the phagosomal membrane rupture, whereas ESX-2 is responsible for survivability, and ESX-3 is involved in iron and zinc uptake and hindering of phagosome maturation. Additionally, ESX-4 is required in conjugation, and ESX-5 releases PE/PPE and PE-PGRS proteins, which play a role in immunomodulation. **[B]** Schematic representation of the conserved components of the ESX secretion system showing the presence of conserved, transmembrane domain-containing proteins EccB, EccC, EccD, and EccE. Upon binding to EccC, cytoplasmic substrates undergo a conformational change through ATP hydrolysis by the DUF domain and, thereafter get transported. The EsxA and EsxB components are shown to cross all the layers and finally rupture the phagosome membrane. The PE and PPE domain-containing proteins end up in the capsule layer from where they can control TLR binding and, thereafter, immune subversion

categorized into two groups: (i) the ancestral type, which includes the ESX-4 and systems resembling ESX-4 from both mycobacterial and non-mycobacterial source, and (ii) the mycobacteria-specific ESX system, which includes ESX-1-3, ESX-5 and the plasmid-encoded (ESX-P) [26]. ESX-P systems are derived from a common ancestor and diversified through extensive gene rearrangements to diversify the mycobacterial T7S systems and help in mycobacterial adaptation to new environments, including the host [26].

ESX-1: RESPONSIBLE FOR MEMBRANE LYSIS

In mycobacterial pathogenesis, the ESX-1 system induces phagosome membrane lysis and interaction of bacterial components with macrophage cytoplasm, thus imparting complete virulence to *Mtb*. ESX-1 secretes ESAT-6/EsxA, a 6 kDa early secreted antigenic target, and CFP-10/EsxB, a 10 kDa culture filtrate protein, which exhibits highly immunogenic properties [27]. The tuberculosis vaccination strain *M. bovis* BCG, which lacks the ESX-1 system, was generated by deleting the genomic loci corresponding to the region of difference (RD1) that harbors the ESX-1 specific genes [28]. The esxBA operon, present in the stated RD1 loci, encodes EsxA and its binding partner EsxB [29], secreted as heterodimeric proteins, and is also known to act as pore-forming toxins (PFTs). PFTs generally rupture or form pores in the phagosome membrane, plasma membrane, and cell organelle membranes that help in mycobacterial pathogenesis. It has been observed that part of the infected phagosome-resident mycobacteria escapes into the cytosol using the ESX-1 system [30]. Besides, upon proliferation inside the phagosomes, the dissolution of the phagosomes and plasma membrane is required for bacterial dissemination and cell-to-cell spreading [30]. The ESX-1 secreted ESAT-6 and CFP-10 are also involved in the cytosolic escape of *Mtb* and *M. Leprae* inside myeloid cells [31]. Apart from that, *M. bovis* BCG strain lacking the RD1 loci cannot secrete the above effectors, and mycobacteria are restricted within the phagosomes without cytosolic escape, but the integration of the RD1 loci into the BCG genome enables them to escape into the cytosol [32].

After the escape of mycobacteria into the cytosol, when mycobacterial DNA gets access to the cytosol, cGAS (cyclic GMP–AMP synthase) a nucleotidyl transferase can recognize it and trigger a downstream signaling cascade through the second messenger cyclic GMP-AMP/cGAMP, which then triggers ESX-1 mediated secretion of some virulence factors. cGAMP also activates the STING-TBK1-IRF3 cascade, which then induces the expression of IFN-β [33]. These indicate that *Mtb* restrains the host from mounting an immune response by preventing the secretion of pro-inflammatory cytokines and inhibiting the antigen presentation. AIM-2, an NLRP3 inflammasome complex sensor, is responsible for the differentiation of cytokines protective against the pathogen, such as IL-1β, thus leading to autophagy. Parallelly it is also known to detect the secreted mycobacterial DNA [33] (Fig. 2A).

Studying the pathogenesis of *Mycobacterium marinum* has played a significant role in understanding the mycobacterial pathogenesis. The secreted EspF [34] and EspE [35] proteins were first identified as a substrate of ESX-1 in *M. marinum* and secreted by *Mtb* as well [36]. In *Mtb*, the 103 amino acids long EspF protein is homologous to EspC protein, whereas the 402 amino acids long EspE protein is similar to the EspA protein [37]. *M. marinum* requires EspH to secrete EspE and EspF proteins [38] while in *Mtb*, the EspL protein maintains the stability of EspE and EspF substrates by acting as a chaperone for the ESX-1 system. Proteomic screen identified MMAR_2984 as a substrate of the ESX-1 system, which was later validated through experiments [39]. The deletion of MMAR_2894 reduced the optimal secretion of ESX-1 substrates relative to the wild-type mycobacteria, but it was sufficient to promote phagosomal escape and apoptosis. In the context of mycobacterial pathogenesis, granuloma formation is an immunological standpoint where the host tries to contain the disease while mycobacteria gets an optimal condition to trigger necrosis and escape to other loci. Experiments in the zebrafish: *M. marinum* model shows that the ESX-1 system has a defined role in granuloma formation [40].

Upon stress, the virulent secretory substrates of ESX-1 are upregulated by the iron-sulfur cluster transcription factor WhiB6 [41]. Further studies demonstrated that in the ESX-1

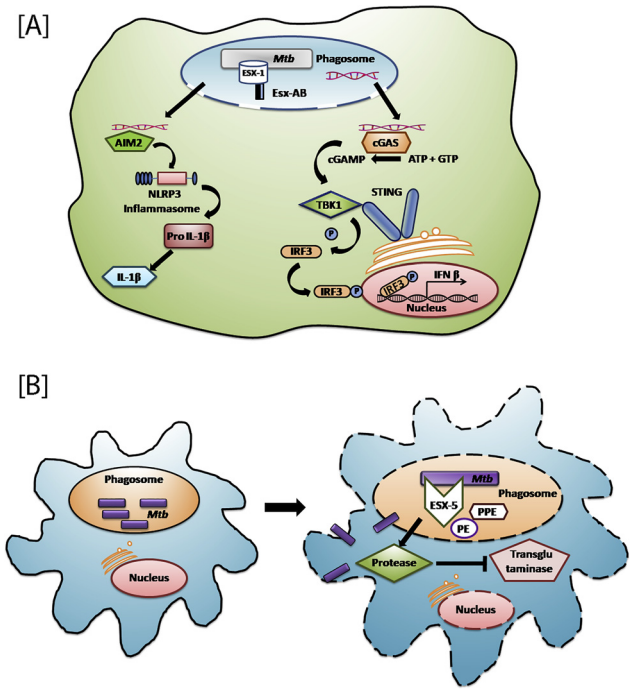

Fig. 2. **[A]** ESAT-6 and CFP-10, which are substrates of ESX-1, are responsible for phagosomal rupture that leads to the cytosolic escape of mycobacteria and subsequent release of its DNA into the macrophage cytosol and its sensing by cGAS leading to activation of the second messenger cGAMP. Thereafter cGAMP activated STING phosphorylates IRF3 and induces the production of IFNβ. *Mtb* DNA is also sensed by AIM2, which helps in the maturation of IL-1β. **[B]** PE and PPE substrates of ESX-5 act as an immunomodulator, and by inhibiting PAI2 production and cleaving the amino-terminal of annexin-1, a porous apoptotic body is formed. As a result, *Mtb* can come out by making the damage of macrophage

mutant *M. marinum*, the expression of the *whiB6* gene was repressed due to the binding of the regulatory transcription factor EspM at the intergenic region of *espM-whiB6* genes. Hence *M. marinum* strains lacking the *espM* gene exhibited elevated levels of WhiB6, which was restored to normal levels or even repressed when EspM from *M. marinum*, *Mtb*, or *M. smegmatis* were exogenously expressed. This cross-species complementation of the *espM* gene confirms the functional conservation of the EspM protein across all three species [42].

LESS EXPLORED ESX PATHWAYS

ESX-2

The ESX-2 system has not yet been linked to protein secretion and has been associated with slow-growing mycobacterial species. The genes encoding the ESX system, which leads to transcriptional regulation, also includes the ESX-2 genes for the co-regulation of transcription and enables the survival of mycobacteria within dendritic cells [43]. EspR genes are known to regulate the genes at the ESX-2 loci. The ESX-2 system lacks the counterparts of the crucial EspACD proteins of the ESX-1 system, and it is one of the later emerged ESX systems that may have evolved from the plasmid dependent genome duplication and alterations in the genomic loci of earlier evolved ESX-1 and ESX-3 systems [43]. EccB2 has been identified as an ESX-2 system-specific ATPase [44], while the protein EccE2 forms an integral transmembrane component of the ESX-2 secretion system [44]. Analysis of the genetic loci of the ESX-2 system shows the presence of *pe* and *ppe68* fragments that code for PE and PPE68 proteins, respectively. Recent observations suggest that the ESX-2 secretion system is non-functional for *Mycobacterium leprae*. This indicates that the ESX-2 system does not have any role in pathogenesis.

ESX-3 HINDERS PHAGOSOME MATURATION

ESX-3 regulates iron homeostasis and zinc acquisition in *Mtb* through the secretion of PE-PPE family protein PE5-PPE4 [45]. It enhances mycobacterial virulence by restricting the host defense mechanism of iron acquisition. ESX-3 is an essential system regulating metal ion homeostasis; hence its mutants were difficult to generate in *Mtb* until recently when supplementation with hemin complexes allowed the ESX-3 mutants to survive. The four conserved components of its core include—EccB3, EccC3, EccE3 (single copy), and EccD3 (dimer) [46]. EccC3 consists of a flexible arrangement of four ATPase domains in the inner membrane, which are connected to the membrane through the stalk domain. In the inner membrane, the EccD3 dimer is embedded along with EccE3's transmembrane helix to form a stable, rigid core. EccB3 located at the inner membrane traverses through the membrane to make contact with the stalk domain of EccC3. After binding of a substrate to EccC3, EccB3, and stalk domain, conformational changes occur for ATP hydrolysis in DUF (the domain of unknown function), which then helps in protein transport from cytosol to periplasmic space [47].

The EsxG–EsxH heterodimer regulates iron acquisition through the secretion of PE15-PPE20 protein via the ESX-3 system and contributes to the virulence mechanisms of mycobacteria. Additionally, EsxH can trigger the secretion of IFNg from T-cells and inhibit the endosomal sorting complex required for transport (ESCRT). The ESCRT factor-Hrs, is targeted by EsxG and EsxH where a complex of four proteins, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, is engaged to the endosomal membrane step by step. This machinery directs the predetermined cargo in the multi-vesicular bodies (MVBs) to fuse with lysosomes for subsequent degradation [48]. EsxG–EsxH hinders ESCRT-mediated endomembrane repair, where ESCRT is recruited at the phagosome damage sites, and it indicates a host-pathogen conflict over endomembrane integrity [49]. Interestingly, *M. smegmatis*, which lacks ESX-3 when made to express it through genome integration, provides effective protection against active TB and therefore exhibits possibility as a vaccine candidate [50]. The ESX-3 system is equally important for environmental as well as pathogenic mycobacterial species.

ESX-4

The ESX-4 system is involved in the secretion of effectors that are required in the conjugation process, especially in the mycobacterial receiver strains. From a donor: recipient co-culture study, an ESX-1 mediated expression of ESX-4 genes was observed in recipient cells [51]. Thus the ESX-1 and ESX-4 systems could enable mutual communication. Supposedly, *Mycobacterium canetti* acquired the ESX-4 system through horizontal gene transfer from *Mtb* [52], and in general, the ESX-4 systems are dispensable both for *in vitro* growth and virulence in most mycobacteria. *M. leprae* genome does not harbor any ESX-4 system. A recent study using *Mycobacterium abscessus* genome-scale Himar mariner transposon library has shown that in these fast-growing mycobacteria, intact ESX-4 systems are required for virulence-associated phagosomal rupture and cytosolic escape of mycobacteria, both in amoeba and human macrophages [53]. This indicates that in *M. abscessus*, the lack of an ESX-1 system makes the ESX-4 system its surrogate. Conjugal contact of two mycobacterial cells activates the extracytoplasmic transcription factor SigM, which then triggers the activation of ESX-4 in *M. smegmatis*. Altogether, it can be stated that the ESX-4 system is ancient, and the remaining systems have arisen through gene duplication and gene diversification. Once this diversification occurred, the ESX-4 system was either genomically lost in these organisms or remained as a non-functional unit [54].

ESX-5: THE IMMUNOMODULATORY ESX SECRETION SYSTEM

ESX-5 is the next most studied system after the ESX-1 and purportedly originated through conjugation mediated transfer

of its precursor components from the plasmid-encoded ESX-P1 fragments into the genomes of the slow-growing mycobacteria. The high sequence similarity between the Ecc and MycP proteins of ESX-P1 and ESX-5 confirms this plausibility. ESX-5 plays a pivotal role in the secretion of proteins containing Pro-Glu (PE), polymorphic GC-rich sequences (PGRSs), Pro-Pro-Glu (PPE) motifs, and mycobacterial mutants lacking a functional ESX-5 fail to secrete these PE and PPE protein repertoire, thus making them avirulent and exhibiting a small colony phenotype [55]. ESX-5 secretes LipY, which bears a C-terminal lipase domain that functions as a PE/PPE protein [56]. ESX-5 has also been linked to drug resistance since ofloxacin mono-resistant *Mtb* displays mutations in ESX-5 loci. In clinical strains, the V762G mutation in ESX-5 specific *eccC5* loci is efficient in displaying ofloxacin resistance [57]. Disruption of *espG5* and *ppe10* has been shown to alter the mycobacterial capsule composition, thus affecting its integrity and thereby modulating innate immune responses by affecting IL-1β production and apoptosis induction [58]. This indicates a role of ESX-5 in capsule maintenance. For cells undergoing apoptosis, plasminogen activator inhibitor type 2 (PAI2) protects annexin-1 from protease attack at the cell surface. Although mycobacterial lipopolysaccharides induce PAI2 production, virulent *Mtb* downregulates PAI2 production [59]. *Mtb* infected cells secrete a protease, possibly through the ESX-5 system, that cleaves the amino-terminal fragment of annexin-1 and reduces its ability as tissue transglutaminase [60]. As a result, the cross-linking of molecules is hindered, and apoptotic bodies cannot form. This proteolytic activity does not hamper the capacity of annexin-1 to bind to the cell surface and associate with phosphatidylserine. Therefore, instead of a rigid, impermeable apoptotic body, a fragile, porous necrotic body is formed (Fig. 2B). Hence, *Mtb* easily avoids the macrophage's programmed cell death and thereby evades the immune system [61].

Interestingly the same ESX-5 system has been implicated in the activation of caspase-independent cell-death of *Mtb* infected macrophages upon proliferation to spread the infection. To escape from the activated macrophage through necrosis, or to infect new macrophages, *Mtb* utilizes ESX-5 system secreted effectors and find a new niche for its survivability once it has proliferated inside an infected macrophage. *Mtb*, through MyD88/IL-1 receptor-dependent manner, joyrides the naive alveolar macrophages into the lung interstitium to spread its population, and ESX-5 induced secretion of IL-1b promotes this process [62].

LINKING THE ESX SYSTEM AND THE CELL ENVELOPE

The presence of several lipids such as trehalose, phenolic glycolipids (PGLs), contained-lipids [polyacyltrehalose (PAT), sulfolipids, diacyltrehalose (DAT), lipooligosaccharides (LOS)], and phthiocerol dimycocerosates (PDIMs) on the mycobacterial cell envelope along with the capsule layer, makes mycobacterial envelope less permeable to hydrophilic solutes [63]. The periplasmic space is formed by both outer and inner membranes, where at the innermost region, a thin peptidoglycan layer persists, which is covalently linked with lipoarabinomannan and arabinogalactan. Taken together ESX system, secreted proteins, has to cross several layers of the mycobacterial envelope before reaching the macrophage cytosol. Upon mycobacterial infection, macrophage TLR2 is known to induce an innate immune response by activating NF-kB. To dampen this response, mycobacteria harbor sulfoglycolipids on its cell envelope [64]. These sulfoglycolipids being competitive antagonists for TLR2, prevent the TLR2/TLR6 or TLR2/TLR1 heterodimer formation and thereby repress NF-kB expression, which dampens the pro-inflammatory cytokine driven innate immune response [65].

Cellular membrane (CM)

There are four ESX-conserved proteins EccB-E complex at the membrane that putatively functions in protein translocation [66]. Cleavage of EspB, an ESX-1 secreted protein, by mycosin (MycP1), enables it to bind phosphatidylserine and phosphatidic acid. This indicates that EspB interacts with the phospholipids on the macrophage cell membrane or phagosome membrane and triggers intracellular signaling pathways that help spread the pathogenicity of *Mtb* [67].

Cell wall

Interaction of the ESX-1 component EccB1 with peptidoglycan or arabinogalactan occurs via extension across the periplasm [68], but the binding characteristics of EccB1 with peptidoglycan have not yet been studied. *In silico* studies show that Mce1 family proteins, which play a role in lipid homeostasis and transport, may be involved in ESX-1 mediated transport of factors across the mycolate outer membrane (MOM), thus implies that Mce1 proteins are MOM pore-forming proteins [69]. The espACD operon encodes espC, a substrate of ESX-1, in *Mtb* with its substrates being EspA and EspD obtained from the same operon [70]. Since EspC filaments are visualized on the *Mtb* capsule, it is thought to be a MOM channel component and the "needle" of ESX-1 [71]. A recent study showed that the ESX-1 secreted substrates, EsxA and EspE, and the ESX-5 secreted PE-PGRS, PPE-MPTR motif-containing proteins are localized on the mycobacterial surface [72]. The PE/PPE proteins play crucial roles in immunomodulation, prevention of host cell death, maintenance of mycobacterial cell wall and capsule integrity, antivirulence to dampen hypervirulent response and nutrient transport across the mycobacterial outer membrane [73]. How these ESX substrates are destined, and their exact relationship of the different ESX system with their secreted substrates remains an enigma.

Extracellular locations

In *in vitro* cultures, the mycobacterial capsule is not retained and released in the growth media owing to the presence of Tween-80 or Tyloxapol, which is used to prevent bacterial clumping. In the absence of detergents, several ESX-1 and

ESX-5 substrates are transported and localized on to this capsule layer [74]. Part of these substrates belong to the class of PE/PPE/PE-PGRS or PPE-MPTR motif-containing proteins and are secreted primarily by the ESX-5 and minimally by the ESX-1 secretory systems.

CONCLUSION

Even though substantial information is available on the ESX protein export system, it is just the tip of an iceberg, and only meticulous studies will help us explore this abyss. How the substrates of ESX-1 cause mycobacterial DNA to pass through its pore and access the host cytosol still needs to be addressed. How mycobacterial virulence factors from the mycobacterial lumen gradually cross the mycobacterial cell membrane, mycobacterial cell wall, mycobacterial capsular layer, macrophage phagosome membrane to arrive at the macrophage cytosol finally remains an enigma. The precise and sequential role of one or more mycobacterial secretory pathways, including the ESX system, as gatekeepers to this secretion, is inevitable but yet an enigma. The gross lacuna of knowledge of the components and intricate functionalities of ESX-2, ESX-3, and ESX-4 must be explored to better understand their role in mycobacterial pathogenesis. The PE/PPE or PE-PGRS domain-containing proteins are substrates for the ESX-5 secretory system, but the mechanism through which these domains serve as a signal for secretion remains to be unraveled, and thereafter the precise mechanism of secretion needs to be resolved. Continued study of the mycobacterial protein export systems is crucial to have a holistic knowledge of their export mechanisms and the role of each system in virulence or growth in general. Since these secretory machineries are associated with mycobacterial viability and pathogenicity, they are attractive therapeutic targets. Chemotherapeutic interventions targeting the protein export pathways, especially those involved in secreting essential virulence factors can help us tackle the tuberculosis menace.

Conflict of interest: The authors declare that there is no conflict of interest in context of the information provided in the manuscript.

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REFERENCES

1. Korch SB, Contreras H, Clark-Curtiss JE. Three *Mycobacterium tuberculosis* Rel toxin-antitoxin modules inhibit mycobacterial growth and are expressed in infected human macrophages. J Bacteriol 2009 Mar;191(5):1618–30. Epub 2008 December 29.
2. BoseDasgupta S, Pieters J. Macrophage-microbe interaction: Lessons learned from the pathogen *Mycobacterium tuberculosis*. Semin Immunopathol 2018 Nov;40(6):577–1. Epub 2018 October 10.
3. Unnikrishnan M, Constantinidou C, Palmer T, Pallen MJ. The enigmatic Esx proteins: Looking beyond mycobacteria. Trends Microbiol 2017 Mar;25(3):192–204. Epub 2016 Novemebr 25.
4. Hett EC, Rubin EJ. Bacterial growth and cell division: Amycobacterial perspective. Microbiol Mol Biol Rev 2008;72:126–56.
5. Ly A, Liu J. Mycobacterial virulence factors: Surface-exposed lipids and secreted proteins. Int J Mol Sci 2020;21(11):3985.
6. Forrellad MA, Klepp LI, Gioffré A, Sabio y García J, Morbidoni HR, de la Paz Santangelo M, et al. Virulence factors of the *Mycobacterium tuberculosis* complex. Virulence 2013;4(1):3–66.
7. Raghavan S, Manzanillo P, Chan K, Dovey C, Cox JS. Secreted transcription factor controls *Mycobacterium tuberculosis* virulence. Nature 2008;454:717–21.
8. Yang H, Tang H, Chen XX, Zhang CJ, Zhu PP, Ding H, et al. Identification of secretory proteins in *Mycobacterium tuberculosis* using pseudo amino acid composition. Biomed Res Int 2016;2016: 5413903.
9. Houben EN, Korotkov KV, Bitter W. Take five – Type VII secretion systems of Mycobacteria. Biochim Biophys Acta 2014 Aug;1843(8): 1707–16. Epub 2013 November 18.
10. Natale P, Brüser T, Driessen AJ. Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane–distinct translocases and mechanisms. Biochim Biophys Acta 2008 Sep; 1778(9):1735–56. Epub 2007 August 9.
11. Green ER, Mecsas J. Bacterial secretion systems: An overview. Microbiol Spectr 2016;4. https://doi.org/10.1128/microbiolspec.VMBF-0012-2015.
12. Papanikou E, Karamanou S, Economou A. Bacterial protein secretion through the translocase nanomachine. Nat Rev Microbiol 2007;5:839–51.
13. Randall LL, Hardy SJ. SecB, one small chaperone in the complex milieu of the cell. Cell Mol Life Sci 2002;59:1617–23.
14. Brundage L, Hendrick JP, Schiebel E, Driessen AJ, Wickner W. The purified *E. coli* integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependant precursor protein translocation. Cell 1990;62:649–57.
15. Braunstein M, Brown AM, Kurtz S, Jacobs WR Jr. Two nonredundant SecA homologs function in mycobacteria. J Bacteriol 2001; 183:6979–90.
16. Sassetti CM, Boyd DH, Rubin EJ. Comprehensive identification of conditionally essential genes in mycobacteria. Proc Natl Acad Sci USA 2001 Oct 23;98(22):12712–7. Epub 2001 October 16.
17. Müller M. Twin-arginine-specific protein export in Escherichia coli. Res Microbiol 2005 Mar;156(2):131–6. Epub 2005 January 28.
18. Raynaud C, Guilhot C, Rauzier J, Bordat Y, Pelicic V, Manganelli R, et al. Phospholipases C are involved in the virulence of *Mycobacterium tuberculosis*. Mol Microbiol 2002;45:203–17.
19. McDonough JA, Hacker KE, Flores AR, Pavelka MS Jr, Braunstein M. The twin-arginine translocation pathway of Mycobacterium smegmatis is functional and required for the export of mycobacterial beta-lactamases. J Bacteriol 2005;187:7667–79.

20. Chen Q, Wu H, Fives-Taylor PM. Investigating the role of secA2 in secretion and glycosylation of a fimbrial adhesin in Streptococcus parasanguis FW213. Mol Microbiol 2004;53:843–56.
21. Rigel NW, Gibbons HS, McCann JR, McDonough JA, Kurtz S, Braunstein M. The accessory SecA2 system of mycobacteria requires ATP binding and the canonical SecA1. J Biol Chem 2009 Apr 10;284(15):9927–36. Epub 2009 February 23.
22. Kurtz S, McKinnon KP, Runge MS, Ting JP, Braunstein M. The SecA2 secretion factor of *Mycobacterium tuberculosis* promotes growth in macrophages and inhibits the host immune response. Infect Immun 2006 Dec;74(12):6855–64. Epub 2006 October 9.
23. Hou JM, D'Lima NG, Rigel NW, Gibbons HS, McCann JR, Braunstein M, et al. ATPase activity of *Mycobacterium tuberculosis* SecA1 and SecA2 proteins and its importance for SecA2 function in macrophages. J Bacteriol 2008 Jul;190(14):4880–7. Epub 2008 May 16. Erratum in: J Bacteriol. 2009 Jun;191(12):4051.
24. Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CM, et al. Type VII secretion – mycobacteria show the way. Nat Rev Microbiol 2007;5:883–91.
25. Burts ML, Williams WA, DeBord K, Missiakas DM. EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of Staphylococcus aureus infections. Proc Natl Acad Sci USA 2005 Jan 25;102(4):1169–74. Epub 2005 January 18.
26. Dumas E, Christina Boritsch E, Vandenbogaert M, Rodríguez de la Vega RC, Thiberge JM, Caro V, et al. Mycobacterial pan-genome analysis suggests important role of plasmids in the radiation of Type VII secretion systems. Genome Biol Evol 2016;8:387–402.
27. McLaughlin B, Chon JS, MacGurn JA, Carlsson F, Cheng TL, Cox JS, et al. A mycobacterium ESX-1-secreted virulence factor with unique requirements for export. PLoS Pathog 2007;3:e105.
28. Pym AS, Brodin P, Brosch R, Huerre M, Cole ST. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines Mycobacterium bovis BCG and Mycobacterium microti. Mol Microbiol 2002;46:709–17.
29. Berthet FX, Rasmussen PB, Rosenkrands I, Andersen P, Gicquel B. A *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiology 1998;144:3195–203.
30. Los FC, Randis TM, Aroian RV, Ratner AJ. Role of pore-forming toxins in bacterial infectious diseases. Microbiol Mol Biol Rev 2013; 77:173–207.
31. Flint JL, Kowalski JC, Karnati PK, Derbyshire KM. The RD1 virulence locus of *Mycobacterium tuberculosis* regulates DNA transfer in Mycobacterium smegmatis. Proc Natl Acad Sci USA 2004 Aug 24;101(34):12598–603. Epub 2004 August 16.
32. van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, Pierson J, et al. *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. Cell 2007;129: 1287–98.
33. Wassermann R, Gulen MF, Sala C, Perin SG, Lou Y, Rybniker J, et al. *Mycobacterium tuberculosis* differentially activates cGAS- and inflammasome-dependant intracellular immune responses through ESX-1. Cell Host Microbe 2015 Jun 10;17(6):799–810. Epub 2015 June 2.
34. Champion PA, Champion MM, Manzanillo P, Cox JS. ESX-1 secreted virulence factors are recognized by multiple cytosolic AAA ATPases in pathogenic mycobacteria. Mol Microbiol 2009 Sep; 73(5):950–62. Epub 2009 August 4.
35. Carlsson F, Joshi SA, Rangell L, Brown EJ. Polar localization of virulence-related Esx-1 secretion in mycobacteria. PLoS Pathog 2009 Jan;5(1):e1000285. Epub 2009 January 30.
36. Sala C, Odermatt NT, Soler-Arnedo P, Gülen MF, von Schultz S, Benjak A, et al. EspL is essential for virulence and stabilizes EspE, EspF and EspH levels in *Mycobacterium tuberculosis*. PLoS Pathog 2018;14:.
37. Kapopoulou A, Lew JM, Cole ST. The MycoBrowser portal: a comprehensive and manually annotated resource for mycobacterial genomes. Tuberculosis (Edinb) 2011 Jan;91(1):8–13. Epub 2010 Octber 25.
38. Phan TH, van Leeuwen LM, Kuijl C, Ummels R, van Stempvoort G, Rubio-Canalejas A, et al. EspH is a hypervirulence factor for Mycobacterium marinum and essential for the secretion of the ESX-1 substrates EspE and EspF. PLoS Pathog 2018;14:.
39. Bosserman RE, Nicholson KR, Champion MM, Champion PA. A new ESX-1 substrate in mycobacterium marinum that is required for hemolysis but not host cell lysis. J Bacteriol 2019;201: e00760–18.
40. Pagán AJ, Ramakrishnan L. The formation and function of granulomas. Annu Rev Immunol 2018 Apr 26;36:639–65. Epub 2018 February 5.
41. Bosserman RE, Nguyen TT, Sanchez KG, Chirakos AE, Ferrell MJ, Thompson CR, et al. WhiB6 regulation of ESX-1 gene expression is controlled by a negative feedback loop in *Mycobacterium marinum*. Proc Natl Acad Sci USA 2017 Dec 12;114(50): E10772–81. Epub 2017 November 27.
42. Sanchez KG, Ferrell MJ, Chirakos AE, Nicholson KR, Abramovitch RB, Champion MM, et al. EspM is a conserved transcription factor that regulates gene expression in response to the ESX-1 system. mBio 2020;11:e02807–19.
43. Newton-Foot M, Warren RM, Sampson SL, van Helden PD, Gey van Pittius NC. The plasmid-mediated evolution of the mycobacterial ESX (Type VII) secretion systems. BMC Evol Biol 2016;16:62.
44. Serafini A, Pisu D, Palù G, Rodriguez GM, Manganelli R. The ESX-3 secretion system is necessary for iron and zinc homeostasis in *Mycobacterium tuberculosis*. PLoS One. 2013;8:.
45. Bitter W, Houben EN, Bottai D, Brodin P, Brown EJ, Cox JS, et al. Systematic genetic nomenclature for type VII secretion systems. PLoS Pathog 2009 Oct;5(10):. Epub 2009 October 30.
46. Poweleit N, Czudnochowski N, Nakagawa R, Trinidad DD, Murphy KC, Sassetti CM, et al. The structure of the endogenous ESX-3 secretion system. Elife 2019;8:.
47. Famelis N, Rivera-Calzada A, Degliesposti G, Wingender M, Mietrach N, Skehel JM, et al. Architecture of the mycobacterial type VII secretion system. Nature 2019;576(7786):321–5. Epub 2019 October 9.
48. Raiborg C, Stenmark H. The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature 2009;458:445–52.
49. Mittal E, Skowyra ML, Uwase G, Tinaztepe E, Mehra A, Köster S, et al. *Mycobacterium tuberculosis* Type VII secretion system effectors differentially impact the ESCRT endomembrane damage response. mBio 2018;9:e01765–18.
50. Portal-Celhay C, Tufariello JM, Srivastava S, Zahra A, Klevorn T, Grace PS, et al. *Mycobacterium tuberculosis* EsxH inhibits ESCRT-dependant $CD4^+$ T-cell activation. Nat Microbiol 2016;2:16232.
51. Gray TA, Clark RR, Boucher N, Lapierre P, Smith C, Derbyshire KM. Intercellular communication and conjugation are mediated by ESX secretion systems in mycobacteria. Science 2016;354:347–50.

52. Gray TA, Krywy JA, Harold J, Palumbo MJ, Derbyshire KM. Distributive conjugal transfer in mycobacteria generates progeny with meiotic-like genome-wide mosaicism, allowing mapping of a mating identity locus. PLoS Biol 2013 Jul;11(7):. Epub 2013 July 9.
53. Laencina L, Dubois V, Le Moigne V, Viljoen A, Majlessi L, Pritchard J, et al. Identification of genes required for *Mycobacterium abscessus* growth in vivo with a prominent role of the ESX-4 locus. Proc Natl Acad Sci USA 2018 Jan 30;115(5):E1002–11. Epub 2018 January 17.
54. Clark RR, Judd J, Lasek-Nesselquist E, Montgomery SA, Hoffmann JG, Derbyshire KM, et al. Direct cell-cell contact activates SigM to express the ESX-4 secretion system in *Mycobacterium smegmatis*. Proc Natl Acad Sci USA 2018 Jul 10;115(28):E6595–603. Epub 2018 June 25.
55. Bottai D, Brosch R. Mycobacterial PE, PPE, and ESX clusters: novel insights into the secretion of these most unusual protein families. Mol Microbiol 2009 Aug;73(3):325–8. Epub 2009 July 7.
56. Daleke MH, Cascioferro A, de Punder K, Ummels R, Abdallah AM, van der Wel N, et al. Conserved Pro-Glu (PE) and Pro-Pro-Glu (PPE) protein domains target LipY lipases of pathogenic mycobacteria to the cell surface via the ESX-5 pathway. J Biol Chem 2011 May 27;286(21):19024–34. Epub 2011 April 6.
57. Eilertson B, Maruri F, Blackman A, Guo Y, Herrera M, van der Heijden Y, et al. A novel resistance mutation in eccC5 of the ESX-5 secretion system confers ofloxacin resistance in *Mycobacterium tuberculosis*. J Antimicrob Chemother 2016 Sep;71(9):2419–27. Epub 2016 June 3.
58. Ates LS, van der Woude AD, Bestebroer J, van Stempvoort G, Musters RJ, Garcia-Vallejo JJ, et al. The ESX-5 system of pathogenic mycobacteria is involved in capsule integrity and virulence through its substrate PPE10. PLoS Pathog 2016;12:.
59. Velmurugan K, Chen B, Miller JL, Azogue S, Gurses S, Hsu T, et al. *Mycobacterium tuberculosis* nuoG is a virulence gene that inhibits apoptosis of infected host cells. PLoS Pathog 2007;3:e110.
60. Gan H, Lee J, Ren F, Chen M, Kornfeld H, Remold HG. *Mycobacterium tuberculosis* blocks the crosslinking of annexin-1 and apoptotic envelope formation on infected macrophages to maintain virulence. Nat Immunol 2008 Oct;9(10):1189–97. Epub 2008 September 14.
61. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 2003;67:593–656.
62. Cohen SB, Gern BH, Delahaye JL, Adams KN, Plumlee CR, Winkler JK, et al. Alveolar macrophages provide an early *Mycobacterium tuberculosis* niche and initiate dissemination. Cell Host Microbe 2018 Sep 12;24(3):439–46. . Epub 2018 August 23.
63. Abdallah AM, Verboom T, Hannes F, Safi M, Strong M, Eisenberg D, et al. A specific secretion system mediates PPE41 transport in pathogenic mycobacteria. Mol Microbiol 2006;62:667–79.
64. Mishra M, Adhyapak P, Dadhich R, Kapoor S. Dynamic remodeling of the host cell membrane by virulent mycobacterial sulfoglycolipid-1. Sci Rep 2019;9:12844.
65. Blanc L, Gilleron M, Prandi J, Song OR, Jang MS, Gicquel B, et al. *Mycobacterium tuberculosis* inhibits human innate immune responses via the production of TLR2 antagonist glycolipids. Proc Natl Acad Sci USA 2017 Oct 17;114(42):11205–10.
66. Ohol YM, Goetz DH, Chan K, Shiloh MU, Craik CS, Cox JS. *Mycobacterium tuberculosis* MycP1 protease plays a dual role in the regulation of ESX-1 secretion and virulence. Cell Host Microbe 2010;7:210–20.
67. Chen JM, Zhang M, Rybniker J, Boy-Röttger S, Dhar N, Pojer F, et al. *Mycobacterium tuberculosis* EspB binds phospholipids and mediates EsxA-independent virulence. Mol Microbiol 2013;89:1154–66.
68. Sani M, Houben EN, Geurtsen J, Pierson J, de Punder K, van Zon M, et al. Direct visualization by cryo-EM of the mycobacterial capsular layer: A labile structure containing ESX-1-secreted proteins. PLoS Pathog 2010;6:.
69. Forrellad MA, McNeil M, Santangelo Mde L, Blanco FC, García E, Klepp LI, et al. Role of the Mce1 transporter in the lipid homeostasis of *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 2014 Mar;94(2):170–7. Epub 2014 January 2.
70. Wagner JM, Chan S, Evans TJ, Kahng S, Kim J, Arbing MA, et al. Structures of EccB1 and EccD1 from the core complex of the mycobacterial ESX-1 type VII secretion system. BMC Struct Biol 2016;16:5.
71. Ates LS, Brosch R. Discovery of the type VII ESX-1 secretion needle? Mol Microbiol 2017 Jan;103(1):7–12. Epub 2016 November 25.
72. Abdallah AM, Verboom T, Weerdenburg EM, Gey van Pittius NC, Mahasha PW, Jiménez C, et al. PPE and PE_PGRS proteins of Mycobacterium marinum are transported via the type VII secretion system ESX-5. Mol Microbiol 2009 Aug;73(3):329–40. Epub 2009 July 7.
73. Ates LS, Dippenaar A, Ummels R, Piersma SR, van der Woude AD, van der Kuij K, et al. Mutations in ppe38 block PE_PGRS secretion and increase virulence of *Mycobacterium tuberculosis*. Nat Microbiol 2018 Feb;3(2):181–8. Epub 2018 January 15.
74. Kinhikar AG, Verma I, Chandra D, Singh KK, Weldingh K, Andersen P, et al. Potential role for ESAT6 in the dissemination of *M. tuberculosis* via human lung epithelial cells. Mol Microbiol 2010 Jan;75(1):92–106. Epub 2009 November 10.