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 (Mt) 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...
mycobacterial infection and pathogenesis [7]. Thorough knowledge of the mycobacterial protein export systems would help to understand the physiology and pathogenesis of \textit{Mtb} and thereby aid in future drug development [8, 9].

Two protein export pathways conserved in \textit{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-secretory 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 \textit{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]. \textit{Mtb} utilizes the Tat pathway to secrete the virulent phospholipase C [18]. \(\beta\)-lactamase (BlaS) export in \textit{M. smegmatis} is dependent on the Tat pathway as it harbors a signal peptide for the Tat pathway; therefore \textit{tat} mutants are unable to export BlaS [19]. \textit{Mtb} \(\beta\)-lactamase (BlaC), when expressed in the \textit{tat} mutant \textit{M. smegmatis}, failed to get exported indicating as a Tat dependent substrate.

Additionally, the SecA2 pathway is conserved in \textit{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 \textit{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 \textit{Mycobacterium bovis} BCG vaccine, this secA2 mutant \textit{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 \textit{eccA}, \textit{eccC}, and \textit{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
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 TTS 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 phagosomal 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 esxB operon, present in the stated RD1 loci, encodes EsxA and its binding partner EsxB [29], secreted as homodimeric 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 *Mtbc* as well [36]. In *Mtbc*, 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 EspF and EspF proteins [38] while in *Mtbc*, 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_2984 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

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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. *Mtbc* 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, *Mtbc* can come out by making the damage of macrophage

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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 PE-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 IFNγ 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 oloxacin mono-resistant \( \text{Mtb} \) displays mutations in ESX-5 loci. In clinical strains, the V762G mutation in PE/PPE protein [56].

Extracellular locations

In \textit{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 secreted effectors and 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].

**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. \textit{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 \( \text{Mtb} \) with its substrates being EspA and EspD obtained from the same operon [70]. Since EspC filaments are visualized on the \( \text{Mtb} \) 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, EssA 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, anti-virulence 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.
ESX-5 substrates are transported and localized on to this capsule layer [74]. Part of these substrates belong to the class of PE/PEP/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/PEP 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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