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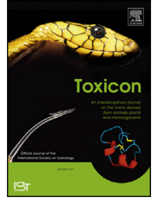

Mechanism of ESAT-6 membrane interaction and its roles in pathogenesis of *Mycobacterium tuberculosis*

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The 6-kDa early secreted antigenic target (ESAT-6; EsxA) of *Mycobacterium tuberculosis* was first identified as a potent T-cell antigen, and it is now recognized as a pore-forming toxin that is essential for virulence of *M. tuberculosis*. ESAT-6 is secreted through the ESX-1 secretion system (Type VII) of *M. tuberculosis* and has been implicated to mediate mycobacterial cytosolic translocation within the host macrophages by rupturing the phagosomal membranes. Recent studies have made significant progresses in understanding of the mechanism of ESAT-6 membrane interaction and its role in *M. tuberculosis* pathogenesis, but important questions still remain to be answered. Here, we summarize the current progress in study of ESAT-6 membrane interaction and its roles in pathogenesis and discuss some of the key remaining questions for future investigation.

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1. Pore-forming toxins

Pore-forming toxin (PFT) is the single largest category of virulence factors and the most common family of bacterial toxins (Gilbert, 2002; Gonzalez et al., 2008; Los et al., 2013). Generally, PFTs contribute to bacterial pathogenicity by forming pores and/or disrupting the host cell membranes, including the plasma membrane and intracellular organelle membranes. Actions of PFTs result in direct lysis of target cells, release of cellular contents, delivery of intracellularly acting bacterial enzymes, bacterial escape from the intracellular compartments (e. g. phagosome and lysosome) to the cytosol, and bacterial cell-to-cell spreading. Due to the critical roles of FPTs in bacterial infection, PFTs become valuable targets for development of novel therapeutics against bacterial pathogens. Studies that lead to better understanding of the mechanism of PFTs action are of great interest for biomedical scientists. Here we discuss 6-kDa early secreted antigenic target (ESAT-6), a newly identified PFT from *Mycobacterium tuberculosis*.

2. ESAT-6 is required for *M. tuberculosis* virulence

ESAT-6 was first identified as a potent T-cell antigen in the short-term culture filtrate of *M. tuberculosis* (Andersen et al., 1995; Sørensen et al., 1995). Since then ESAT-6 has been intensively studied as a potential target for vaccine development against tuberculosis. In parallel to the studies of ESAT-6 as a potential vaccine candidate, comparative and functional genomics of virulent versus attenuated members of mycobacteria complex have opened a novel venue into the roles of ESAT-6 in *M. tuberculosis* pathogenesis. Firstly, subtractive hybridization experiments identified a chromosomal region, named “region of difference 1” (RD1), which was present in virulent *M. tuberculosis*, but not in the attenuated live vaccine *Mycobacterium bovis* bacilli Calmette and Guerin (BCG) (Mahairas et al., 1996). This finding was complemented by the results obtained from screening secreted antigens in different mycobacterial strains, showing that wild type *M. bovis* and *M. tuberculosis* strains have ESAT-6, but BCG does not (Harboe et al., 1996). Later, comparative genomics further revealed that RD1 is part of the *esx-1* locus, which encodes a novel bacterial secretion system (Type VII secretion system) (Behr et al., 1999; Gordon et al., 1999; Pym et al., 2002, 2003). Within the *esx-1* locus, *esat-6* and *cfp-10* (10-kDa culture filtrate protein, or EsxB) are located under the control of a single operon (Berthet et al., 1998). While both ESAT-6 and CFP-10 lack N-terminal Sec or TAT signal sequences, it is believed that they are exported as a heterodimer through the ESX-1 secretion system. This is evidenced by the fact that ESAT-6 and CFP-10 are secreted in a co-dependent manner, and the anti-parallel four-helix bundle of the heterodimer and the C-terminal Y-XXX-

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D/E secretion motif of CFP-10 are the common characteristics shared by the substrates of Type VII secretion system (Atmakuri and Fortune, 2008; Fortune et al., 2005; E. N. G. Houben et al., 2014; Lightbody et al., 2008, 2004; Renshaw et al., 2005, 2002; Veverka and Muskett, 2011).

Studies from several research groups have also demonstrated that deletion of RD1 from *M. tuberculosis* and *M. bovis* resulted in attenuated mycobacterial growth in cultured macrophages and in experimental animals, while introduction of RD1 into BCG increased its virulence (Guinn et al., 2004; Hsu et al., 2003; Lewis et al., 2003; Pym et al., 2003, 2002; Sassetti and Rubin, 2003; Stanley et al., 2003; Wards et al., 2000). More specifically, loss or gain of mycobacterial virulence is closely linked to the ability of mycobacteria to produce and secrete ESAT-6. Mycobacterial strains carrying the mutations that abolish production or secretion of ESAT-6 exhibited attenuated virulence in infection of various animal models, while introduction of ESX-1 or RD1 to restore ESAT-6 secretion resulted in increased virulence of the avirulent vaccine strains BCG and *Mycobacterium microti* (Guinn et al., 2004; Hsu et al., 2003; Lewis et al., 2003; Pym et al., 2002; Wards et al., 2000). The ESX-1 secretion system and release of mycobacterial antigens through ESX-1 have been extensively discussed in several recent excellent reviews (Cambier et al., 2014; E. N. G. Houben et al., 2014; Majlessi et al., 2015; Simeone et al., 2015a; Stanley and Cox, 2013).

3. Roles of ESAT-6 in *M. tuberculosis* pathogenesis

There is a widely accepted perception in the field that after being internalized into the host macrophages, *M. tuberculosis* inhibits phagosome maturation, remains and replicates inside the phagosomes (Kang et al., 2005; Orme, 2004; Pizarro-Cerdá and Cossart, 2006). ESAT-6 has been implicated to inhibit phagosome maturation. *Mycobacterium marinum* is a pathogen that causes tuberculosis-like diseases in fish and contains a highly conserved ESX-1 system. *M. marinum* primarily resides in a poorly acidified, non-lysosomal compartment, but a mutant strain of *M. marinum* defective in secretion of ESAT-6 were found to be mainly in acidified compartments (MacGurn and Cox, 2007; Tan et al., 2006). Recently, however, a series of elegant studies have changed the perception and demonstrated that at the later stage of infection mycobacteria gain access to the cytosol through rupturing the phagosomal membranes. Using sophisticated cryo-electron microscopy van der Wel and colleague showed that *M. tuberculosis* and *Mycobacterium leprae* were able to translocate from the phagolysosomal compartments into the cytosol of myeloid cells, and mycobacterial cytosolic entry was dependent on secretion of ESAT-6 and CFP-10 (van der Wel et al., 2007). Later, the same group showed that ESX-1-mediated mycobacterial cytosolic translocation to the cytosol controlled virulence of mycobacteria (D. Houben et al., 2012). In parallel to those studies, using a β-lactamase-based FRET microscopy Brosch and Enninga groups showed that wild type *M. marinum* induced phagosomal rupture and translocated to the cytosol, while the ESAT-6 secretion-deficient strain did not. Similarly, wild type *M. tuberculosis* translocated from the phagosome to the cytosol, while BCG did not. Introduction of RD1 into BCG conferred it the ability to translocate to the cytosol, while deletion of the C-terminus of ESAT-6, which abolished ESAT-6 secretion, disabled the cytosolic translocation (Simeone et al., 2012). Subsequently, the results obtained in cultured phagocytes were replicated in the infected mouse model, in which mycobacterial cytosolic translocation was detected by a highly sensitive FRET-based flow cytometry (Simeone et al., 2015b). Therefore, it is clear that ESAT-6 plays an essential role in phagosome rupture and cytosolic translocation of mycobacteria.

Gaining cytosolic access has multiple consequences for mycobacterial infection, which include mycobacterial replication in the cytosol and cell-to-cell spreading (Guinn et al., 2004; Hsu et al., 2003) as well as host cell apoptosis (Aguilo et al., 2013), autophagy induction or impairment (Romagnoli et al., 2012; Watson et al., 2012), type-I interferon release (Stanley et al., 2007), and T-cell response (Ryan et al., 2009). It is worth of mentioning that several independent studies have recently made a significant progress in understanding of the ESX-1-mediated host anti-mycobacterial immunity (Collins and Collier, 1984; Wassermann et al., 2015; Watson et al., 2015). Upon cytosolic access mycobacterial DNA is sensed by the nucleotidyl-transferase cGAS, which synthesizes the second messenger cGAMP that in turn activates a series of downstream signaling pathways (reviewed by (Majlessi and Brosch, 2015)). One can imagine that ESAT-6 functions in rupturing the phagosomal membrane to expose mycobacterial DNA to the host cytosolic DNA-sensing mechanisms.

4. Evidence for ESAT-6 membrane-lytic and pore-forming activity

The membrane-lytic activity of ESAT-6 was first reported in a planar lipid bilayer study, in which ESAT-6, either alone or in combination with CFP-10 resulted in major disruptions in conductance, eventually resulting in total destruction of the artificial membranes (Hsu et al., 2003). Later, membrane interaction of ESAT-6 and CFP-10 was tested in a floatation gradient centrifugation experiment using biologically relevant liposomes, in which ESAT-6 exhibited strong association with the liposomes containing 1,2-dimyristoryl-sn-glycero-3-phosphocholine (DMPC) and cholesterol, but CFP-10 interaction with the membranes was weaker and less specific. Moreover, the ESAT-6/CFP-10 heterodimer appeared to interact with the membranes at acidic pH, but not at neutral pH. Finally, electron microscopy revealed that ESAT-6 lysed liposomes, but CFP-10 did not (de Jonge et al., 2007). Using PEG osmoprotection assay, Smith and colleague showed that either *M. marinum* or purified ESAT-6 induced pore formation on the red blood cell membranes with estimated pore size ~4.5 nm in diameter (Smith et al., 2008). Our recent systematic biochemical characterization of the purified recombinant proteins found that ESAT-6, but not CFP-10, induced leakage of liposomes in an acidic pH-dependent manner, which was accompanied by significant conformational changes and increased surface hydrophobicity (De Leon et al., 2012). We have also found that compared to ESAT-6 from *M. tuberculosis* (hereafter termed MtbESAT-6), the orthologous ESAT-6 from non-pathogenic *Mycobacterium smegmatis* (MsESAT-6) did not interact with the membranes, despite that they share over 72% sequence identity. This finding has raised a notion that the ability of ESAT-6 to interact with membranes is the major determinant for virulence phenotype of mycobacterial complex (De Leon et al., 2012) (Fig. 1). Most recently, we labeled ESAT-6 at various positions with NBD (N,N-dimethyl-N-(iodoacetyl)-N-(7-nitrobenz-2-oxa-1,3-diazol) ethylenediamine), which is an environmental sensitive dye that emits strong fluorescence when inserting into lipid membrane. With the NBD-labeled ESAT-6, we mapped the trans-membrane domains of ESAT-6 and presented the first direct evidence that ESAT-6 inserts into membranes and forms a membrane-spanning pore (Ma et al., 2015).

5. Molecular mechanism of ESAT-6 pore formation

ESAT-6 is the prototype of ESAT-6/WXG100 superfamily, which is featured by ~100-residues and a central conserved WXG motif (Pallen, 2002; Poulsen et al., 2014). To date, MtbESAT-6 is the only member that has been reported to exhibit a pH-dependent

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