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Immune regulatory activities of early antigenic target of 6-kD protein of *Mycobacterium tuberculosis* and implications for tuberculosis vaccine design

Buka Samten[a,b,*], **Xisheng Wang**[a,b], and **Peter F. Barnes**[a,b,c]

[a]Center for Pulmonary and Infectious Disease Control, Tyler, TX 75708

[b]Departments of Microbiology and Immunology, and Medicine, Tyler, TX 75708

[c]University of Texas Health Science Center, Tyler, TX 75708

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Introduction

Tuberculosis remains an urgent public health threat world-wide despite the tremendous progress has been made in understanding the biology of tuberculosis since its causative agent, *Mycobacterium tuberculosis (Mtb)*, was identified over a century ago. The world-wide spread of human immune deficiency virus infection and lack of effective vaccine against tuberculosis have limited tuberculosis control efforts. Recent development of multi-drug-resistant and extensively drug-resistant strains of *Mtb*[1] have worsened the situation in countries where tuberculosis is endemic. Therefore, design of more effective antituberculosis vaccines and identification of novel drug targets are imperative to control tuberculosis. In this review, we discuss the identification of early secreted antigenic target of 6 kD (ESAT-6) from the culture supernatants of *Mtb*, its immune regulatory effects on macrophages and T cells, and the implications for tuberculosis vaccine design.

1. Discovery of ESAT-6 and the ESX-1 secretion system

Since T cells provide protection against *Mtb* infection, induction of enhanced T cell immunity against *Mtb* antigens has been central to develop a protective vaccine. *Mtb* is transmitted through aerosols generated by tuberculosis patients, and establishes infection in the lungs when the bacilli are ingested by alveolar macrophages. Here, the organisms reside in membrane-coated phagosomes, grow and cause disease in the lungs. Thus, accessibility of *Mtb* antigenic proteins to immune cells during infection is crucial for inducing T cell responses. In animal models, inoculation of live bacilli provides greater levels of protection against subsequent challenge with *Mtb* than administration of dead organisms,[2] suggesting

*Address correspondence to: Buka Samten, CPIDC, UT Health Science Center, 11937 US Hwy 271, Tyler, TX 75708-3154, phone (903) 877-7665, fax (903) 877-7989, buka.samten@uthct.edu.

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that proteins secreted by live bacilli are critical for induction of protective T cell immunity. Because short-term (10–14 day) culture filtrates of *Mtb* are enriched with secreted proteins[3] and with minimal contamination of structural proteins from the bacilli due to degradation, the latter are believed to have high vaccine potential. Based on this notion, Peter Andersen's group from the Statens Serum Institut identified and purified a 95-amino acid protein from the short-term culture filtrate of *Mtb* that induced high concentrations of IFN-γ production by T cells from *Mtb*-infected mice,[4] a cytokine that is essential for resistance to mycobacterial infection[5]. This 95-amino acid protein, named the 6-kDa early secreted antigenic target (ESAT-6), is present in both cytosolic and cell wall fractions of *Mtb*, when analyzed its distribution in cellular compartments by Western blotting with HYB 76-8, anti-ESAT-6 monoclonal antibody.[4, 6]

ESAT-6 is produced by clinical isolates of *Mtb* and by virulent *M. bovis*, but not by all substrains of attenuated bacillus Calmette Guerin (BCG) vaccine strain of *M. bovis*, due to genomic deletion of a region that contains the gene encoding ESAT-6.[7] Genomic analysis of mycobacterial strains determined that the region of difference (RD)1 is deleted in all BCG strains and in the naturally-occurring less virulent *M. microti*, but present in all virulent strains of *M. bovis*, all clinical isolates of *Mtb*, and the virulent laboratory strain, H37Rv.[8] This was further confirmed by microarray and bacterial artificial chromosomal analysis.[9–11] H37Ra, the most widely used attenuated laboratory strain *of Mtb*, also has a defect in secretion of ESAT-6, due to a point mutation in the DNA-binding domain of the two-component regulator protein, PhoP. Reintroduction of the wildtype *PhoP* gene into H37Ra resulted in recovery of virulence, with secretion of ESAT-6, both *ex vivo* and *in vivo*,[12] further demonstrating the critical role of ESAT-6 secretion in virulence of *Mtb*.

The studies with ESAT-6 gene regulation in *Mtb* identified its molecular partner,[13] culture filtrate protein of 10 kDa (CFP10), and its encoding gene, *esxB* (Rv3874), located immediately upstream of *esat-6 or esx*A (Rv3875).[14] Structural studies and analysis of the genes revealed that ESAT-6 and CFP10 do not possess classical signal peptides required for secretion, despite their presence in short-term culture filtrates of *Mtb*. The studies to understand this phenomenon led to the identification of a special ESAT-6 secretion system, designated as ESX-1, composed of several genes located within the RD1 region.[15, 16] Gene deletion and reintroduction studies with the RD1 region of *Mtb* demonstrated that the ESX-1 secretion system and its substrates, ESAT-6 and CFP10, are required for virulence and pathogenicity in murine infection.[11, 17, 18] ESAT-6 is also secreted by *Mtb in vivo*, as it is present in the lungs of mice infected with RD1-complemented BCG, from 3 weeks to 6 weeks post-infection, indicating that ESAT-6 is expressed during the critical phases of establishment of pulmonary infection and has the potential to interact with lung cells, including immune cells.[18] Upon interaction with cells, ESAT-6 mediates lysis and apoptosis of lung epithelial cells[19] and macrophages,[20] permits cell-to-cell spread of *Mtb* without lysing infected cells through eliciting formation of cellular ejectosomes,[21] and stimulates granuloma formation.[22] Other pathogens, such as *Staphylococcus aureus*[23] and *Bacillus anthracis*[24], also produce ESAT-6 like or homologues that are secreted through an ESX-1-like secretion system and are required for pathogenicity. These suggest that the ESX-1 secretion system and its substrates are evolutionarily conserved among many human pathogens, and constitute a general mechanism for virulence.

Because the genes of ESAT-6 and CFP10 are present in clinical isolates of *Mtb* but not in BCG or most nontuberculous mycobacteria,[24] and these proteins known to induce T cell immune responses, therefore positive ESAT-6 and CFP10 specific T cell responses in the patient blood samples could indicate infection of *Mtb*. Based on this, ESAT-6- and CFP10-based diagnostic tests have been developed to identify persons with latent tuberculosis infection and active tuberculosis disease, based on the positive reactivity of T cells with

IFN-γ production when stimulated with peptides of ESAT-6 and CFP10 *in vitro* and detected by ELISA or ELISPOT.[25]

Because ESAT-6 elicits potent T-cell responses in mice, it has long been considered a potential vaccine candidate, and ESAT-6-based subunit vaccines conferred protection against challenge with *Mtb* in mice.[26] A vaccine construct that includes ESAT-6 and Antigen 85 reduced the bacillary burden after challenge with *Mtb* in guinea pigs and nonhuman primates.[27,28] In summary, identification of ESAT-6 has improved our knowledge about *Mtb* physiology, virulence and pathogenesis, and could lead to development of ESAT-6-based tuberculosis vaccines. However, recent studies demonstrate that ESAT-6 also has immune regulatory activities.

2. Effects of ESAT-6 on macrophage activation and cytokine production

2.1 Inhibition of macrophage activation

Macrophages, especially alveolar macrophages, are believed to play a critical role in limiting growth of *Mtb*, initially through phagocytosis, followed by fusion of bacilli-containing phagosomes with lysosomes that contain enzymes to degrade bacilli, and by producing bactericidal molecules, such as, reactive oxygen species and reactive nitrogen species.[26] Macrophages also initiate adaptive immunity by producing chemokines that attract T cells to the site of infection and by antigen presentation and costimulation to activate recruited T cells to elicit protective immune responses in the lungs. Activated T cells in turn produce IFN-γ, which further potentiates the bactericidal effects of macrophages to eliminate infection[27]. However, *Mtb* has evolved mechanisms to manipulate the functions of macrophages and to evade host immunity. It is therefore of interest to determine if ESAT-6 contributes to *Mtb*-mediated immune evasion and pathogenicity through specific effects on macrophage function.

Studies led by Dr. Sharma showed that ESAT-6 inhibits LPS-induced macrophage gene expression by regulating mitogen-activated protein kinase (MAPK) extracellular signal regulated kinase 1/2,[28] probably through reduction of reactive oxygen species.[29] The same group has demonstrated that ESAT-6 decreases IFN-γ induced expression of MHC class II expression by inhibiting MHC class II transactivator expression through regulating chromatin remodeling. [30] These changes reduce the efficiency of antigen presentation by macrophages, therefore may affect effective induction of T cell immune responses against tuberculosis infection. Studies led by Dr. Basu demonstrated that ESAT-6 decreases LPS-stimulated IL-12 p40 production by mouse macrophages through inhibiting TLR4-mediated MAPK activation and NF-κB activation. This inhibition is mediated by the C-terminal 20 amino acid residues (76–95) of ESAT-6 through binding to macrophage TLR2.[31] Since production of IL-12 is crucial for T cell production of IFN-γ, this finding suggests that ESAT-6 may also inhibit Th1 immune responses through affecting cytokine production of macrophages.

Collectively, these studies suggest that ESAT-6 modulates macrophage functions through perturbation of intracellular signaling pathways to reduce antigen presentation and cytokine production to indirectly inhibit T cell mediated protective immunity against tuberculosis infection. Thus provide a potential role for ESAT-6 in immune evasion of *Mtb* during infection.

2.2 Activation of the inflammasome

Recent studies of innate immunity to infectious pathogens have uncovered the nucleotide-binding oligomerization domain-like receptors or NLRs. NLRs are expressed predominantly in the cytoplasm, whereas Toll-like receptors (TLRs) are present on the cell membrane.

Besides recognizing pathogen-associated molecular patterns released by intracellular organisms into the cytoplasm of infected cells, NLRs also detect host molecules released into the cytoplasm in response to molecular stress or cell damage, so-called “danger signals” or danger-associated molecular patterns, such as ATP.[32] Activation of NLRs initiates a cascade of intracellular events that result in activation of NF-κB, which in turn leads to expression of the proinflammatory cytokine precursors, pro-IL-1β and pro-IL-18, and assembly of the inflammasome, a tripartite protein complex that includes a sensor protein (NLR), an adaptor protein (apoptosis associated speck-like protein containing a caspase-recruitment domain), and an effector molecule (procaspase-1). Assembly of inflammasome is required for conversion of procaspase-1 into active caspase-1, which processes pro-IL-1β and pro-IL-18 into mature, active and secreted forms of IL-1β and IL-18. Therefore, activation of inflammasome by NLR controls secretion of IL-1β and IL-18, and is important in regulation of macrophage activation and secretion of proinflammatory cytokines.[33] So far, four different inflammasomes have been identified, and Nlrp3 is the most documented one. Aberrant activation of the inflammasome, due to genetic mutations in Nlrp3, causes enhanced activation of caspase-1 and elevated IL-1β levels in humans, resulting in an autoinflammatory disorder known as cryopyrin-associated periodic syndrome.[34]

Activation of the inflammasome, followed by production of IL-1β and IL-18, is also critical for innate immunity against many human pathogens.[33] In response to *Mtb* infection, macrophages produce IL-1β and IL-18,[35, 36] which may contribute to chronic inflammation. Therefore, it is important to understand the mechanisms through which *Mtb* induces production of IL-1β and IL-18. Infection of macrophages with wild type *Mtb* and *M. marinum* induced potent IL-1β secretion, compared to their respective ESX-1 gene deletion strains,[36–38] suggesting that macrophage production of IL-1β requires secretion of one of the ESX-1β substrate proteins. Indeed, recombinant ESAT-6 directly activates Nlrp3 inflammasome and elicits secretion of IL-1β by macrophages.[39] ESAT-6 activates Nlrp3 inflammasome probably through permeabilization of phagosomes and activation of Syk tyrosine kinase in *Mtb* infected macrophages, and activation of Nlrp3 may be associated with necrotic death of *Mtb* infected macrophages[40]. These findings further suggest that ESAT-6 contributes to initiation and regulation of inflammatory responses in tuberculosis infection through activation of inflammasome in macrophages. In conclusion, ESAT-6 may play a critical role in lung pathology and chronic inflammation by activation of inflammasome in alveolar macrophages during tuberculosis infection.[41, 42]

3. Inhibition of T cell immune responses

Because T cells are critical for protection against tuberculosis infection, we investigated the direct effect of ESAT-6 on human peripheral blood $CD3^+$ T cell functions in response to TCR stimulation with plate-bound α-CD3 and α-CD28. ESAT-6 inhibits T cell IFN-γ production in a dose-dependent manner.[43] ESAT-6 also reduces α-CD3 and α-CD28 stimulated T cell production of TNF-α, IL-10 and IL-17, but does not affect IL-2 production, indicating that ESAT-6 inhibits production of some cytokines but not others, and this inhibition is unlikely to be due to a general cytotoxic effect. ESAT-6 also decreases T cell proliferation. We found that ESAT-6 inhibits IFN-γ production through activating p38 MAPK, as blocking p38 MAPK activity with a chemical inhibitor or reducing its expression in T cells with siRNA reversed the inhibitory effect of ESAT-6 on IFN-γ production, but not on production of IL-10 and IL-17, or the effects on T cell proliferation.[44] Incubation of human T cells directly with ESAT-6 induced phosphorylation and functional kinase activity of p38 MAPK, and activation of p38 MAPK was not due to increased Ca^{2+} levels in T cells, as is the case for most pore-forming toxins of pathogenic bacteria.[45, 46] Thus our results suggest the central role of p38 MAPK in ESAT-6 inhibition of T cell IFN-γ production, and activation of p38 MAPK by *Mtb* may play role in pathogenesis of tuberculosis. Indeed,

activation of p38 MAPK in *Mtb*-infected mononuclear phagocytes blocks phagosomal maturation and leads to reduced expression of CD1 and antigen-presenting capacity of dendritic cells,[47, 48] and activation of p38 MAPK by *Mtb* through TLR2 pathway also causes apoptosis of neutrophils.[49] These studies together with ours suggest that *Mtb* activates p38 MAPK to subvert host immune response through multiple effects. p38 MAPK is also the intracellular target of toxins from other pathogens, such as the Shiga toxin of *Shigella dysenteriae*[50] and the lethal toxin of *B. anthracis*,[51] further suggesting that p38 MAPK lies on an intracellular signaling pathway that is targeted by many bacterial pathogens, including *Mtb* by secretion of ESAT-6.

4. Implications for development of antituberculosis vaccines

Because of its active secretion by *Mtb* and strong induction of recall T-cell IFN-γ responses in infected mice[6], ESAT-6 has been the focus of vaccine studies against tuberculosis infection in multiple animal models.[25–28] On the other hand, the data summarized above demonstrate that ESAT-6 can contribute to virulence, inhibit macrophage activation, reduce production of IL-12 and directly suppress T-cell production of IFN-γ. Although ESAT-6 contains only 95 amino acids, different portions of the molecule have been found to have distinct effects on specific cell types. The amino terminal section is highly immunogenic for T cells,[53.54] whereas the C-terminus inhibits IL-12 production in macrophages[33] and contributes to virulence in animals.[55] To improve ESAT-6-based vaccine, it would be desirable to identify the mechanisms through which ESAT-6 regulates the function of T cells, macrophages and dendritic cells, and to identify the amino acid residues of ESAT-6 that mediate these effects. Indeed, *Mtb* strains that differ only in point mutations of ESAT-6 have shown differential virulence and pathogenicity in a mouse model of infection,[52] and lost activation of Nlrp3 in *Mtb* infected macrophages.[40] Optimized ESAT-6-based vaccines would include those portions of ESAT-6 that stimulate strong T-cell responses but exclude sections that inhibit macrophage antigen presentation and T cell IFN-γ production.

Summary

Although ESAT-6 was originally identified as a strong T cell immunogen in short-term culture filtrate of *Mtb*, and has therefore been a candidate vaccine antigen for many years, recent work has demonstrated that ESAT-6 is also a virulence factor that mediates pathogenicity of *Mtb*. The studies described in this review suggest that ESAT-6 secreted by *Mtb* subverts host immunity by manipulating intracellular signaling pathways in macrophages and T cells, which are critical in protection against *Mtb*. Furthermore, ESAT-6 elicits pro-inflammatory responses that can be detrimental to the host. Understanding the molecular mechanisms through which ESAT-6 inhibits immunity will permit design of ESAT-6-based vaccine constructs that elicit protective immune responses with minimal negative effects.

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