Disconnecting In Vitro ESX-1 Secretion from Mycobacterial Virulence

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In this issue of the Journal of Bacteriology, Chen and colleagues (1) report a novel observation regarding the mycobacterial ESX-1 (ESAT-6 system-1/type VII) protein secretion system. Although the mechanisms underlying their observations are not yet understood, their exciting results unlink the in vitro secretion of the two major ESX-1 substrates, ESAT-6 (EspA) and CFP-10 (EspB), from M. tuberculosis virulence for the first time. These findings force us to reconsider how we relate the read-out of in vitro secretion assays to ESX-1 function within an infected host and call into question the roles of ESAT-6 and CFP-10 during infection.

CURRENT UNDERSTANDING OF THE MYCOBACTERIAL ESX-1 SECRETION SYSTEM

The ESX-1 (type VII) secretion system is required for the virulence of several Gram-positive and mycobacterial pathogens, including Mycobacterium tuberculosis, Staphylococcus aureus, and Bacillus anthracis (2–5). ESX-1 is also conserved and functional in non-pathogens, where it plays vital roles in cell conjugation and physiology (6–9). In pathogens, ESX-1 functions early during infection when Mycobacterium is within the phagosome of the macrophage (4, 5). Specifically, ESX-1 is required for lysis of the phagosomal membrane, which allows either mycobacterial products or the bacteria access to the macrophage cytoplasm (10–14). Once lysis occurs, the cytosolic STING/TBK1 pathway is triggered, both promoting a type I interferon response and restricting bacterial growth through autophagy (11, 14, 15). This was recently reviewed in reference 16.

At the molecular level, ESAT-6 and CFP-10 are widely acknowledged as the major substrates of the ESX-1 secretion system. ESAT-6 has been reported to have membrane-lysing activity and may play a direct role in disrupting the phagosomal membrane (17–20). The first papers linking individual genes to the secretion of ESAT-6 and CFP-10 were published about a decade ago (4, 5, 21, 22). The majority of the molecular biology underlying ESX-1 export has been elucidated by turning on the system in vitro, fractionating mycobacterial cells to isolate whole-cell lysates and culture supernatants, and detecting the export of ESX-1 substrates by Western blot analysis or proteomics. Using this in vitro secretion assay, genes located in three distinct chromosomal loci were implicated in the export of ESX-1 substrates during growth in culture. Genes required for ESX-1 export lead to the loss of ESX-1 substrate secretion, most often ESAT-6 and CFP-10, in vitro. Reported loss of ESX-1 function in vitro has corresponded with attenuation of virulence both ex vivo and in vivo (primary examples include references 4, 5, 21, and 22).

UNLINKING IN VITRO ESX-1 SECRETION FROM VIRULENCE: A CHANGE IN PARADIGM

Chen et al. report for the first time a scenario where ESX-1 secretion appears to be disrupted in vitro but does not result in attenuation of M. tuberculosis in various infection models (1). The authors focused on EspA, a known ESX-1 substrate. EspA, ESAT-6, and CFP-10 mutually require each other for export (23, 24). Several of the known ESX-1 substrates contain a WXG motif (25). The authors used site-directed mutagenesis to determine if the WXG motif in EspA was required for the secretion of CFP-10 and ESAT-6 from and virulence of M. tuberculosis. The expected outcome of this experiment was that mutations in EspA that prevented the export of ESAT-6 and CFP-10 would also lead to attenuation of M. tuberculosis in both cellular models of infection and in mice. Indeed, the majority of EspA variants generated in this study that prevented export of ESAT-6 and CFP-10 into the culture supernatant were nontoxic to macrophages, failed to induce proinflammatory cytokines, and were attenuated during acute infection in a mouse model. In short, they exhibited the expected phenotypes associated with ESX-1 deficiency.

Two EspA variants (EspAF5R and EspAK41A) resulted in in vitro protein secretion phenotypes that were identical to that of an espA-null strain (ESX-1 deficient); ESAT-6 and CFP-10 were produced but not exported into the culture supernatant. The EspAF5R and EspAK41A proteins were not detectable by Western blot analysis, indicating that these individual mutations destabilized the EspA proteins. However, although ESX-1 deficient in vitro, the strains expressing EspAF5R and EspAK41 were cytotoxic to macrophages, induced proinflammatory cytokines, and were as virulent as the wild-type strain in a mouse model. Thus, for the first time, the authors report that despite an apparent loss of ESX-1 secretion in vitro, the M. tuberculosis strains were not attenuated for virulence.

ASSAYS FOR MEASURING ESX-1 SECRETION AND FUNCTION

The report by Chen et al. (1) calls into question whether the in vitro secretion assay is a reliable indicator of ESX-1 function within a host. There are two commonly used ways to induce ESX-1 secretion in vitro. First, ESX-1 secretion can be induced by growing the mycobacteria in Sauton’s medium, a general defined broth for bacterial culture. Mycobacterium smegmatis is a nonpathogenic mycobacterial species that serves as a model for ESX-1 expression. In M. tuberculosis, the expected outcome was that mutations in EspA that prevented the export of ESAT-6 and CFP-10 would also lead to attenuation of M. tuberculosis in both cellular models of infection and in mice. Indeed, the majority of EspA variants generated in this study that prevented export of ESAT-6 and CFP-10 into the culture supernatant were nontoxic to macrophages, failed to induce proinflammatory cytokines, and were attenuated during acute infection in a mouse model. Thus, for the first time, the authors report that despite an apparent loss of ESX-1 secretion in vitro, the M. tuberculosis strains were not attenuated for virulence.

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remains unknown. Second, ESX-1 secretion is induced by contact with red blood cells (RBCs). *Mycobacterium marinum* is a pathogenic mycobacterial species that also serves as an established model for ESX-1 secretion. *M. marinum* lyses RBCs in a contact-dependent, ESX-1-dependent manner (27, 28). RBC lysis is used to determine if the membrane lysing activity of the ESX-1 system is functional. The proteins secreted by ESX-1 following each type of induction have not been compared. Therefore, we do not know if both modes of induction are equivalent with respect to the secreted proteome. Additional assays have been proposed to identify secreted substrates of ESX-1, but these have generally failed because tagging ESX-1 substrates with large, enzymatically active proteins generally prevents export (29).

The ESX-1 secretion system causes cytotoxicity in cell-based *ex vivo* models of infection, including macrophages and amoebae (12, 27, 30). Following lysis of the phagosomal membrane, the bacteria induce cytotoxicity, which leads to cell death. Cell death or perforation of the cell membrane is detected by the uptake of membrane-impermeable dyes or live/dead dyes. While the ESX-1 system functions in the phagosome, the proteins actively secreted by ESX-1 within the host cell remain elusive. We as a field have system functions in the phagosome, the proteins actively secreted membrane-impermeable dyes or live/dead dyes. While the ESX-1 proteome generally prevents export (29).

As suggested by Chen et al. (1), the disconnect between *in vitro* secretion and ESX-1 mediated virulence, how do we explain the novel findings of Chen et al.? How can a mycobacterial cell that fails to secrete ESAT-6 and CFP-10 in *in vitro* still be virulent in *in vivo*? Although the studies by Chen et al. do not yet address mechanism, several potential models can be postulated.

Another formal possibility is that expression of EspAF5R or EspAK41A proteins could bypass the need for EspA in ESX-1 secretion (38). The studies by Chen et al. draw attention to the need for continued development of assays for studying type VII secretion. More sensitive assays for detecting secretion are required, as the current assays may not be sensitive enough to detect *in vivo* levels of ESX-1 secretion. Clearly, the research reported here by Chen et al. (1) illustrates the fact that we do not understand all of the players in ESX-1 export, either on the mycobacterial side or on the host side. Therefore, more studies are required to identify bacterial and host genes required for ESX-1 export both *in vitro* and in cell-based models of infection. Finally, this research opens the door to exciting new ways to think about ESX-1 secretion, which will help direct us toward a better understanding of the interplay between mycobacterial pathogens and their hosts.

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