Microbiology Spectrum
American Society for Microbiology Press

The Role of ESX-1 in *Mycobacterium tuberculosis* Pathogenesis

KA-WING WONG

Shanghai Public Health Clinical Center, Key Laboratory of Medical Molecular Virology, School of Basic Medical Sciences, Shanghai Medical College of Fudan University, Shanghai 200032, People's Republic of China

ABSTRACT In this article, we have described several cellular pathological effects caused by the *Mycobacterium tuberculosis* ESX-1. The effects include induction of necrosis, NOD2 signaling, type I interferon production, and autophagy. We then attempted to suggest that these pathological effects are mediated by the cytosolic access of *M. tuberculosis*-derived materials as a result of the phagosome-disrupting activity of the major ESX-1 substrate ESAT-6. Such activity of ESAT-6 is most likely due to its pore-forming activity at the membrane. The amyloidogenic characteristic of ESAT-6 is reviewed here as a potential mechanism of membrane pore formation. In addition to ESAT-6, the ESX-1 substrate EspB interferes with membrane-mediated innate immune mechanisms such as efferocytosis and autophagy, most likely through its ability to bind phospholipids. Overall, the *M. tuberculosis* ESX-1 secretion system appears to be a specialized system for the deployment of host membrane-targeting proteins, whose primary function is to interrupt key steps in innate immune mechanisms against pathogens. Inhibitors that block the ESX-1 system or block host factors critical for ESX-1 toxicity have been identified and should represent attractive potential new antituberculosis drugs.

BACKGROUND

The identification of ESAT-6 secretion system-1 (ESX-1) as a virulence determinant of *Mycobacterium tuberculosis* is a major discovery in the history of tuberculosis research. ESX-1 is encoded by a genetic locus known as RD1, which stands for "region of difference" and is one of the deleted regions in the vaccine strain *Mycobacterium bovis* bacille Calmette-Guérin (BCG) for humans (1). The first evidence emerges from the finding that the absence of RD1 is responsible for the attenuation of BCG's virulence (2–4). Introduction of RD1 into BCG is sufficient to induce BCG growth in lung and spleen, granuloma formation in lung, splenomegaly, and inflammation and abscesses in liver and kidney in mice (4). Conversely, deletion of RD1 in the virulent H37Rv *M. tuberculosis* strain inactivates the ability of H37Rv to enable rapid bacterial replication in lung and spleen, to cause lung histopathology and death in mice (2, 3). Lung sections from infected mice show evidence of macrophage lysis, which is a RD1-dependent process (2). Consistent with this observation, Lewis et al. describe the requirement of RD1 for H37Rv to grow within and kill human macrophages (3).

Macrophage is the primary cell harboring *M. tuberculosis* during tuberculosis infection in humans (5). Accordingly, the discovery that RD1 mediates the induction of macrophage death by *M. tuberculosis* suggests that the outcome of the interaction between RD1 and macrophage host cell will be a key determinant of the virulence of *M. tuberculosis* pathogenesis. Significant progress toward understanding how ESX-1/RD1

Received: 1 October 2015, **Accepted:** 31 March 2017, **Published:** 19 May 2017

Editors: William R. Jacobs Jr., Howard Hughes Medical Institute, Albert Einstein School of Medicine, Bronx, NY 10461; Helen McShane, University of Oxford, Oxford OX3 7DQ, United Kingdom; Valerie Mizrahi, University of Cape Town, Rondebosch 7701, South Africa; Ian M. Orme, Colorado State University, Fort Collins, CO 80523.

Citation: Wong K-W. 2017. The Role of ESX-1 in *Mycobacterium tuberculosis* Pathogenesis, *Microbiol Spectrum* 5(3):TBTB2-0001-2015. doi:10.1128/microbiolspec.TBTB2-0001-2015.

Correspondence: Ka-Wing Wong, kwwong@gmail.com

© 2017 American Society for Microbiology. All rights reserved.

facilitates tuberculosis pathogenesis has been made recently. In this article, we aim to provide an updated overview about the progress and put forward emerging concepts for improving our understanding of the nature of ESX-1-induced pathogenesis. In doing so, we also highlight some recent developments in designing intervention strategies based on targeting the RD1-encoded proteins.

ESX-1 SECRETION SYSTEM

The RD1 locus encodes a type VII secretion system known as ESAT-6 secretion system-1 (ESX-1) in *Mycobacterium* species (6). RD1 comprises nine genes, two of which are the secreted protein ESAT-6 (early secreted antigenic target of 6 kDa) and CFP-10 (culture filtrate protein of 10 kDa). Both of these proteins are immunodominant antigens and major virulence factors of *M. tuberculosis*. ESAT-6 and CFP-10 are cosecreted as a heterodimeric complex and are also essential components of the ESX-1 secretion system. Other RD1 genes encode the ATPase Rv3871, the transmembrane protein Rv3870, and the channel protein Rv3877. The ESAT-6-CFP-10 complex is recognized by the ATPase Rv3871, which is localized to the inner membrane through its interaction with Rv3870. The complex is then translocated across the inner membrane, most likely through the channel protein Rv3877. Other RD-1 genes might facilitate the biogenesis of the secretion system. Non-RD-1 genes are also required for proper functioning of ESX-1. EspR is a transcriptional regulator of ESX-1. EspA is an ESX-1-secreted substrate, and its secretion is required for the secretion of ESAT-6 and CFP-10. Additional detail about how the ESX-1 secretion system works is beyond the scope of this article. Interested readers should consult the comprehensive review by Abdallah et al. (6).

KEY OBSERVATIONS LEADING TO RECENT EVIDENCE OF PHAGOSOME DISRUPTION BY ESX-1

M. tuberculosis is phagocytized by alveolar macrophage after it is inhaled into the lungs of an individual. The internalized bacteria then reside within a membrane-bound compartment called phagosome. Armstrong and Hart used electron microscopy to examine these compartments in detail (7). Phagosomes containing nonpathogenic BCG normally undergo maturation, which is then followed by fusion with multiple lysosomes. The fusion events bring lysosomal digestive enzymes into the lumen of the phagosome for degradation. Indeed, damaged BCG is frequently observed. Using a sophisticated electron microscopy technique, Peters' group revisited the pioneer work of Armstrong and Hart (8). To their surprise, they found evidence of phagosome maturation and lysosome fusion in phagosomes containing H37Rv that contain ESX-1 (8). These are transient events and are only observed during early infections. In contrast, phagosome maturation and lysosome fusion are uninterrupted and occur continuously in macrophages infected with BCG. The authors show that, after the early engagement with lysosome, *M. tuberculosis* translocates from phagosome into cytosol and this process requires ESX-1 (8). This translocation has been proposed as a virulence mechanism (9). Similar observations were made earlier by McDonough et al. (10).

How does *M. tuberculosis* impair phagosome-lysosome fusion? The fusion requires membrane localizations of lysosome-associated proteins (LAMP)-1 and LAMP-2 on phagosomes (11). Membrane disruption should therefore affect phagosome-lysosome fusion. Importantly, membrane disruption by *M. tuberculosis* was first observed in 1984 by Leake et al. and Myrvik et al. using electron microscopy (12, 13). The disruption is observed only in macrophages infected with virulent *M. tuberculosis* H37Rv but not with attenuated BCG or H37Ra, consistent with the results of Armstrong and Hart. This finding has recently been confirmed by other methods. Damaged phagosomes can be marked by galectin-3 and are indeed observed in *M. tuberculosis*-infected macrophages in an-ESX-1-dependent fashion (14, 15). Simeone et al. have developed a single-cell fluorescence resonance energy transfer assay to monitor phagosome disruption (16). Using this assay, the authors demonstrate phagosome disruption in mouse spleen and lungs as revealed by the assay based on fluorescence resonance energy transfer (17). *In vitro*, the assay reveals evidence of phagosome disruption within 24 h of macrophage infections (17). Interestingly, most of the cytosolic translocation event for *M. tuberculosis* requires 2 or 3 days to occur (9).

It is possible that the appearance of cytosolic localization of *M. tuberculosis* arises from collapse of phagosomal membrane as a result of continuous disruption of the phagosomal membrane. According to this scenario, cytosolic translocation of *M. tuberculosis* and impairment of phagosome-lysosome fusion can be seen as downstream effects of phagosome disruption. This has an important implication in *M. tuberculosis* pathogenesis. Phagosome disruption is a proposed mechanism of "patterns of pathogenesis." This concept was

proposed by Vance, Isberg, and Portnoy to help in understanding pathogenic host responses to virulent microorganisms (18). In the next section, we will present new evidence that fits the idea that phagosome disruption is responsible for all major pathogenic responses as a result of tuberculosis infections. Therapeutic implication of this idea will then be discussed.

DAMAGE OF THE *M. TUBERCULOSIS*-CONTAINING PHAGOSOME: CONSEQUENCES OF CYTOSOLIC ACCESS BY *M. TUBERCULOSIS*

Phagosome disruption compromises the integrity of the phagosomal membrane and can lead to cytosolic access of the lumen content within the phagosome compartments. There are at least four major cellular responses as a result of such cytosolic access of *M. tuberculosis*-derived materials. And for each of the responses, the element of pathogenesis can be readily identified. First, phagosome disruption can lead to activation of the cytosolic inflammasome receptor NLRP3 (15). NLRP3 is activated by *M. tuberculosis* ESX-1 and the activation triggers downstream activation of caspase-1, which then facilitates secretion of cytokines interleukin 1 beta (IL-1beta) and interleukin 18 (IL-18) (19). These cytokine productions are protective against *M. tuberculosis* infections (20, 21). NLRP3 activation can also lead to the induction of a programmed necrotic death in *M. tuberculosis*-infected macrophages (15). *M. tuberculosis* mutants carrying a defective ESAT-6 that are unable to disrupt the phagosome cannot activate NLRP3 (as measured by IL-18 production) and cannot induce necrosis (15). This programmed cell death is not known to have any negative impact on the viability of *M. tuberculosis*. This kind of death is necrotic and highly inflammatory, which contributes to significant tissue damage. Indeed, macrophage necrosis is suggested to play a key role in all major stages of tuberculosis (22). It is presently unclear how NLRP3 senses phagosome disruption. NLRP3 is known to sense cell swelling and hyperosmotic stress (23, 24). It remains to be shown whether ESAT-6 promotes cell swelling and alters cellular osmolarity through its phagosome-disrupting activity. This scenario is possible since it has been shown that ESX-1 can cause residual effects on plasma membrane upon contact with macrophages (25).

Second, cytosolic access can activate a cytosolic surveillance pathway that triggers induction of type I interferon production. This induction by *M. tuberculosis* is dependent on ESX-1 (26). The phagosome-disrupting effect of ESX-1 provides cytosolic access of *M. tuberculosis* materials such as extracellular DNA, which acts as a specific activating ligand for the STING-TBK1-IRF3 signaling axis for type I interferon production (27). The cytosolic DNA sensor that is responsible for direct recognition of *M. tuberculosis* DNA and for activating STING has recently been identified by three independent groups to be cyclic GMP-AMP synthase (28–30). Interestingly, mice deficient in IRF3 are resistant to long-term *M. tuberculosis* infection (27). This observation is consistent with the idea that phagosome disruption is a pattern of pathogenesis. According to the pattern of pathogenesis model, phagosome disruption is sensed by a host mechanism that initiates a pathogenic response. In our case of *M. tuberculosis* infection in macrophage, cytosolic localization of *M. tuberculosis* DNA as a result of phagosome disruption triggers the STING-IRF3-type I interferon pathway responsible for pathogenesis. Indeed, prolonged induction of type I interferon by intranasal poly-L-lysine and carboxymethyl cellulose exacerbates tuberculosis in mice (31). Limiting excessive type I induction by IL-1beta confers host resistance against tuberculosis in mice (32). But *M. tuberculosis* has an ability to inhibit IL-1beta production, through a yet-to-be-defined ESX-1-dependent mechanism to suppress the AIM2 inflammasome that normally activates caspase-1 and triggers IL-1beta production upon recognizing cytosolic DNA (33). Consistent with these observations, excessive induction of type I interferon and reduced IL-1beta responses in tuberculosis patients are associated with tuberculosis exacerbation (32). Furthermore, transcriptional signature dominated by type I interferon signaling correlates strongly with the radiological extent of disease in patients with active tuberculosis and the signature reverts back to the levels seen in healthy controls after anti-*M. tuberculosis* treatment (34). Taken together, phagosome disruption as a result of ESX-1 is responsible for the pathogenesis of tuberculosis, at least in part through induction of type I interferon production by cytosolic *M. tuberculosis* DNA.

Third, cytosolic access enables peptidoglycan recognition by the cytosolic receptor NOD2 (35). *M. tuberculosis* induces the NOD2 pathway in an ESX-1-dependent fashion (36). This induction triggers type I interferon expression (36). Additionally, the NOD2 pathway also plays an important role in apoptosis (35). A role for NOD2 in apoptosis is consistent with the demonstration of a key role for ESX-1 in apoptosis induction by Aguilo et al. (37). Although the authors do not show that the ESAT-6-induced apoptosis is mediated by the NOD2 pathway, they do show that induction of apoptosis

requires p38 MAPK activity, which is known to mediate signaling from the NOD2 pathway (38).

The fourth consequence of ESX-1-induced phagosome disruption is impairment of autophagic flux and fusions with lysosome. Autophagy is a cellular mechanism for degradation of intracellular protein aggregate. A selective form of autophagy known as xenophagy targets damaged organelles or intracellular pathogens. Indeed, autophagy has been shown to target *M. tuberculosis* for degradation in mouse macrophages (39). The initial step of autophagy involves the processing of microtubule-associated protein 1 light chain 3 (LC3) into the smaller isoform 2 (LC3-II). The processed LC3-II is then associated physically with the so-called isolation membrane, which is used eventually to engulf its targeting intracellular materials to create a closed membrane compartment known as autophagosome. Processed LC3-II recognizes polyubiquitin chains that mark intracellular contents destined for autophagy. In the case of damaged mitochondria or *M. tuberculosis*-disrupted phagosomes, the polyubiquitin chain is produced by the ubiquitin ligase parkin and then is recognized by the autophagy receptors NDP52 and p62 for LC3-II binding (40, 41). ESX-1 is necessary for polyubiquitin chain labeling and recruitments of NDP52, p62, and LC3-II on *M. tuberculosis*-containing phagosomes (42). It has been further shown that recognition of cytosolic DNA by the STING-dependent pathway is required for the polyubiquitination of *M. tuberculosis*-containing phagosome for autophagy (42). To complete the autophagy process, the autophagosome must fuse with lysosome for degradation of the phagosomal content. It is this step where autophagy is interrupted by ESX-1 (43). Indeed, ESX-1 genes are found to be critical for blocking phagosome maturation (44, 45). It remains unclear whether ESX-1 can directly permeabilize the outer autophagosomal double membrane to block fusion with lysosomes. *M. tuberculosis*-secreted effectors previously shown to block phagosome acidification and fusions with endosome or lysosome include PtpA, SapM, and EsxH (46–48). It is probable that ESX-1 could provide access of these effectors into the cytosol and impair the autophagosome-lysosome fusion through its membrane-permeabilizing effect.

IS *M. TUBERCULOSIS* AN AMYLOID DISEASE MEDIATED BY ESX-1?

In their investigation about the role for ESX-1 in tuberculosis pathogenesis, Hsu et al. study how an ESX-1-secreted product mediates the lysis of macrophage. The authors first notice an earlier report from Ralph Isberg's group, which shows that host cell lysis caused by *Legionella pneumophila* is mediated by lethal ion fluxes as a result of the pore-forming ability of a protein secretion system and that exogenous glycine can prevent such ion fluxes as well as host lysis (49). To test whether *M. tuberculosis* may process similar pore-forming ability, Hsu et al. find that pretreatment of glycine prevents the *M. tuberculosis*-promoted macrophage lysis (2). Hsu et al. then show further that purified ESAT-6 induces ion fluxes across artificial bilayer membrane and eventual total destruction of the artificial membrane (2). Therefore, their results strongly indicate that ESAT-6 can form pores on membrane.

To further understand how ESAT-6 forms pores, Hsu et al. also observed that ESAT-6 forms structures similarly observed in amyloidogenic intermediate soluble protein (2). This intermediate structure is believed to be the precursor of insoluble amyloid fibril. In humans, amyloid aggregate is associated with several pathological conditions, including Alzheimer's disease, Parkinson diseases, and type 2 diabetes mellitus. Current consensus on amyloid diseases suggests that amyloidogenic prefibril intermediate is the most toxic entity and that aggregation of the amyloidogenic intermediate into insoluble form is a detoxification mechanism (50). The toxic species in amyloid diseases are small oligomers with pore-forming activity composed by the amyloidogenic intermediate (51). By analogy, it is possible that the amyloidogenic nature of ESAT-6 can similarly form small oligomer with pore-forming capability. According to this possibility, *M. tuberculosis* expressing an ESAT-6 mutant defective for amyloid aggregate should not possess pore-forming activity and, as a result, should not produce phenotypes associated with phagosome disruption. Such ESAT-6 mutants that are defective for forming amyloid aggregate have been described biochemically by the laboratory of David Eisenberg (52). When introduced in *M. tuberculosis*, these ESAT-6 mutants can no longer induce macrophage lysis, induce NLRP3 inflammasome, and damage phagosome (15). These observations are therefore consistent with the idea that the amyloidogenic nature of ESAT-6 is critical for disrupting the phagosomal membrane during macrophage infections. Nevertheless, this idea must be confirmed further by a demonstration of the existence of amyloid-like ESAT-6 aggregate, as well as by identification of ESAT-6 amyloid-forming inhibitors that block ESAT-6's cellular effects. In addition, ESAT-6 will not be able to form amyloid within macrophages if it cannot be present without binding to CFP-10. Thus, the

amyloid hypothesis is critically dependent on showing that ESAT-6 exists in macrophages without CFP-10, which remains to be shown.

There has been some new exciting progress in Alzheimer's disease research using the approach of targeting amyloid in treating Alzheimer's disease (53). If ESAT-6 does indeed form toxic amyloid-like intermediate, the opportunity to disrupt such behavior may represent new opportunities for therapeutic intervention.

REGULATIONS OF ESX-1

ESX-1 secretion is regulated by at least two two-component sensing regulatory systems. The first one is PhoPR. A point mutation in PhoP causes drastic reduction of ESAT-6 secretion (54, 55). ESAT-6 secretion is dependent on EspA secretion (56). Expression of *espA* in turn is regulated positively by PhoPR as well as the PhoPR-regulated transcriptional factor EspR (57). Accordingly, ESAT-6 secretion can be triggered by PhoPR, since it induces *espR* and *espA* expressions. Interestingly, the transcriptional factor EspR is also a substrate of ESX-1 and is secreted by ESX-1. Raghavan et al. suggest that the secretion of the positive regulator EspR represents a negative feedback loop for the ESX-1 secretion (58). EspR is exported when ESX-1 secretion is activated. If its intracellular level falls below a threshold necessary to drive *espA* expression to replace the secreted EspA, there will not be enough EspA and ESX-1 secretion will shut down (58). However, ESX-1 secretion can resume when EspA accumulates as a result of signaling from PhoPR.

Tan et al. identify acidic pH and increase in chloride concentration as two synergistic cues sensed by PhoPR (59). These two cues correlate with phagosome maturation and immune activation. In response to these immune stresses PhoPR signals *M. tuberculosis* to turn on ESX-1 secretion. Phagosome maturation is promoted by interferon gamma (IFN-γ) (60). In mice macrophages, immune activation by IFN-γ induces effective *M. tuberculosis*-killing activities (61, 62). But IFN-γ fails to do so in human macrophages infected with *M. tuberculosis*. Instead, it promotes necrosis in *M. tuberculosis*-infected human macrophages without harming *M. tuberculosis* (63). We have recently reported that IFN-γ promotes necrosis in *M. tuberculosis*-infected human macrophages and this process is dependent on the ESX-1 system (64). It seems plausible, although not demonstrated, that the necrosis promoted by IFN-γ is mediated by the ability of IFN-γ to promote phagosome maturation, which then activates PhoPR to promote ESX-1 secretion and ultimately cause necrosis.

Another two-component system that regulates ESX-1 is MprAB, which senses envelope stress such as protein misfolding in extracytoplasmic space. The absence of MprAB blocks secretions of ESAT-6 and EspA, but not secretion of CFP-10 (57). In the *mprAB* mutant, protein levels of EspA and ESAT-6 are unchanged although transcription of *espA* increases (57). It has been proposed that the absence of MprAB causes increased degradation resulting in defective secretion (57).

ESX-1 secretion is also regulated by ATP levels in *M. tuberculosis*. Zhang et al. show that low ATP levels shut down ESX-1 secretion system (65). Bedquiline inhibits ATP production in *Mycobacterium* species and completely blocks ESX-1 secretion (65). Sensing of low ATP levels is mediated by EspI, which is encoded by a gene within the RD1/ESX-1 locus. Mutations within the ATP-binding motif in EspI prevent turning off ESX-1 secretion, even in the presence of bedquiline (65).

ESPB: AN ESX-1 SUBSTRATE THAT TARGETS INNATE IMMUNE MECHANISMS

ESX-1 also secretes other proteins in addition to ESAT-6. One example is the 60-kDa protein EspB encoded by a gene within the RD1 locus. Chen et al. report that the *espB* mutant of *M. tuberculosis* is defective for cytotoxicity against the human macrophage cell line THP-1 (66). Interestingly, purified EspB is capable of binding to phosphatidic acid and phosphatidylserine (66). Phosphatidylserine is an "eat me" signal on the cell surface of apoptotic cells for clearance by macrophages through a mechanism known as efferocytosis. This mechanism is very effective in killing *M. tuberculosis* that resides within apoptotic cells (67). EspB could therefore block efferocytosis through disrupting phosphatidylserine signaling, although this idea awaits confirmation (66). In addition, EspB can inhibit autophagosome formation induced by INF-γ in murine macrophages (68). This result is consistent with the phosphatidic acid binding ability of EspB, because phosphatidic acid is an important intracellular signal for autophagy (69). Taken together, EspB can affect membrane-mediated innate immune mechanisms through binding of phosphatidic acid and phosphatidylserine. Two recent structural studies suggest that exported EspB forms an oligomeric structure with a central pore (70, 71). Since EspB can interact with membrane, it is very possible that the oligomeric EspB can form a membrane pore that facilitates phagosome permeabilization within infected macrophages.

INTERVENTIONS BY TARGET ESX-1 SECRETION

Since *M. tuberculosis* mutants carrying a defect in ESX-1 lose the ability to cause diseases in multiple animal models, drugs that target ESX-1 may lead to potential antituberculosis drugs. Several groups have performed compound library screens and have succeeded in identifying several ESX-1 inhibitors (72). Using a *M. tuberculosis* strain carrying a fluorescence reporter under an acid-inducible PhoPR promoter, Johnson et al. screen for inhibitors that block the PhoPR regulation (73). They identify ethozolamide as an ESX-1 inhibitor. Interestingly, the compound inhibits *M. tuberculosis* carbonic anhydrase activity, which has not previously been linked to PhoPR signaling (73).

Rybniker et al. screen for inhibitors that prevent fibroblast killing by *M. tuberculosis* (72). Their identified inhibitors were able to block ESAT-6 secretion, avert the block in phagosome maturation, reduce bacterial loads, and prevent *M. tuberculosis*-induced lysis. All these effects are consistent with the known roles for ESX-1 during macrophage interactions. Additionally, the screen identifies an inhibitor that targets the histidine kinase MprB and another one that affects *M. tuberculosis* metal-ion homeostasis. This observation has led the authors to uncover zinc stress as a previously unrecognized activating signal for ESX-1 (72). Just as in the previous study, Rybniker's study identifies inhibitors that target bacterial activities that have not previously been linked to regulating ESX-1.

SUMMARY

In this article, we have described several cellular pathological effects caused by the *M. tuberculosis* ESX-1. The effects include induction of necrosis, NOD2 signaling, type I interferon production, and autophagy. We then attempted to suggest that these pathological effects are mediated by the cytosolic access of *M. tuberculosis*-derived materials as a result of the phagosome-disrupting activity of the major ESX-1 substrate ESAT-6. Such activity of ESAT-6 is most likely due to its pore-forming activity at the membrane. The amyloidogenic characteristic of ESAT-6, first observed by Hsu et al., is reviewed here as a potential mechanism of membrane pore formation. In addition to ESAT-6, the ESX-1 substrate EspB interferes with membrane-mediated innate immune mechanisms such as efferocytosis and autophagy, most likely through its ability to bind phospholipids. Overall, the *M. tuberculosis* ESX-1 secretion system appears to be a specialized system for the deployment of host membrane-target proteins, whose primary function is to interrupt key steps in innate immune mechanisms against pathogens. Inhibitors that block the ESX-1 system or block host factors critical for ESX-1 toxicity have been identified and should represent attractive potential new antituberculosis drugs.

REFERENCES

1. **Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, Small PM.** 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* **284:**1520–1523 http://dx.doi.org/10.1126/science.284.5419.1520.

2. **Hsu T, Hingley-Wilson SM, Chen B, Chen M, Dai AZ, Morin PM, Marks CB, Padiyar J, Goulding C, Gingery M, Eisenberg D, Russell RG, Derrick SC, Collins FM, Morris SL, King CH, Jacobs WR Jr.** 2003. The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. *Proc Natl Acad Sci USA* **100:**12420–12425 http://dx.doi.org/10.1073/pnas.1635213100.

3. **Lewis KN, Liao R, Guinn KM, Hickey MJ, Smith S, Behr MA, Sherman DR.** 2003. Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette-Guérin attenuation. *J Infect Dis* **187:**117–123 http://dx.doi.org/10.1086/345862.

4. **Pym AS, Brodin P, Brosch R, Huerre M, Cole ST.** 2002. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Mol Microbiol* **46:**709–717 http://dx.doi.org/10.1046/j.1365-2958.2002.03237.x.

5. **Russell DG, Cardona PJ, Kim MJ, Allain S, Altare F.** 2009. Foamy macrophages and the progression of the human tuberculosis granuloma. *Nat Immunol* **10:**943–948 http://dx.doi.org/10.1038/ni.1781.

6. **Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CM, Appelmelk BJ, Bitter W.** 2007. Type VII secretion–mycobacteria show the way. *Nat Rev Microbiol* **5:**883–891 http://dx.doi.org/10.1038/nrmicro1773.

7. **Armstrong JA, Hart PD.** 1971. Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. *J Exp Med* **134:**713–740 http://dx.doi.org/10.1084/jem.134.3.713.

8. **van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, Pierson J, Brenner M, Peters PJ.** 2007. *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* **129:**1287–1298 http://dx.doi.org/10.1016/j.cell.2007.05.059.

9. **Houben D, Demangel C, van Ingen J, Perez J, Baldeón L, Abdallah AM, Caleechurn L, Bottai D, van Zon M, de Punder K, van der Laan T, Kant A, Bossers-de Vries R, Willemsen P, Bitter W, van Soolingen D, Brosch R, van der Wel N, Peters PJ.** 2012. ESX-1-mediated translocation to the cytosol controls virulence of mycobacteria. *Cell Microbiol* **14:**1287–1298 http://dx.doi.org/10.1111/j.1462-5822.2012.01799.x.

10. **McDonough KA, Kress Y, Bloom BR.** 1993. Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. *Infect Immun* **61:**2763–2773.

11. **Huynh KK, Eskelinen EL, Scott CC, Malevanets A, Saftig P, Grinstein S.** 2007. LAMP proteins are required for fusion of lysosomes with phagosomes. *EMBO J* **26:**313–324 http://dx.doi.org/10.1038/sj.emboj.7601511.

12. **Leake ES, Myrvik QN, Wright MJ.** 1984. Phagosomal membranes of *Mycobacterium bovis* BCG-immune alveolar macrophages are resistant to disruption by *Mycobacterium tuberculosis* H37Rv. *Infect Immun* **45:**443–446.

13. **Myrvik QN, Leake ES, Wright MJ.** 1984. Disruption of phagosomal membranes of normal alveolar macrophages by the H37Rv strain of *Mycobacterium tuberculosis*. A correlate of virulence. *Am Rev Respir Dis* **129:**322–328.

14. Paz I, Sachse M, Dupont N, Mounier J, Cederfur C, Enninga J, Leffler H, Poirier F, Prevost MC, Lafont F, Sansonetti P. 2010. Galectin-3, a marker for vacuole lysis by invasive pathogens. *Cell Microbiol* **12:**530–544 http://dx.doi.org/10.1111/j.1462-5822.2009.01415.x.

15. Wong KW, Jacobs WR Jr. 2011. Critical role for NLRP3 in necrotic death triggered by *Mycobacterium tuberculosis*. *Cell Microbiol* **13:**1371–1384 http://dx.doi.org/10.1111/j.1462-5822.2011.01625.x.

16. Simeone R, Bobard A, Lippmann J, Bitter W, Majlessi L, Brosch R, Enninga J. 2012. Phagosomal rupture by *Mycobacterium tuberculosis* results in toxicity and host cell death. *PLoS Pathog* **8:**e1002507 http://dx.doi.org/10.1371/journal.ppat.1002507.

17. Simeone R, Sayes F, Song O, Gröschel MI, Brodin P, Brosch R, Majlessi L. 2015. Cytosolic access of *Mycobacterium tuberculosis*: critical impact of phagosomal acidification control and demonstration of occurrence in vivo. *PLoS Pathog* **11:**e1004650 http://dx.doi.org/10.1371/journal.ppat.1004650.

18. Vance RE, Isberg RR, Portnoy DA. 2009. Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. *Cell Host Microbe* **6:**10–21 http://dx.doi.org/10.1016/j.chom.2009.06.007.

19. Mishra BB, Moura-Alves P, Sonawane A, Hacohen N, Griffiths G, Moita LF, Anes E. 2010. *Mycobacterium tuberculosis* protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. *Cell Microbiol* **12:**1046–1063 http://dx.doi.org/10.1111/j.1462-5822.2010.01450.x.

20. Schneider BE, Korbel D, Hagens K, Koch M, Raupach B, Enders J, Kaufmann SH, Mittrücker HW, Schaible UE. 2010. A role for IL-18 in protective immunity against *Mycobacterium tuberculosis*. *Eur J Immunol* **40:**396–405 http://dx.doi.org/10.1002/eji.200939583.

21. Mayer-Barber KD, Barber DL, Shenderov K, White SD, Wilson MS, Cheever A, Kugler D, Hieny S, Caspar P, Núñez G, Schlueter D, Flavell RA, Sutterwala FS, Sher A. 2010. Caspase-1 independent IL-1beta production is critical for host resistance to *Mycobacterium tuberculosis* and does not require TLR signaling in vivo. *J Immunol* **184:**3326–3330 http://dx.doi.org/10.4049/jimmunol.0904189.

22. Orme IM. 2014. A new unifying theory of the pathogenesis of tuberculosis. *Tuberculosis (Edinb)* **94:**8–14 http://dx.doi.org/10.1016/j.tube.2013.07.004.

23. Compan V, Baroja-Mazo A, López-Castejón G, Gomez AI, Martínez CM, Angosto D, Montero MT, Herranz AS, Bazán E, Reimers D, Mulero V, Pelegrín P. 2012. Cell volume regulation modulates NLRP3 inflammasome activation. *Immunity* **37:**487–500 http://dx.doi.org/10.1016/j.immuni.2012.06.013.

24. Ip WK, Medzhitov R. 2015. Macrophages monitor tissue osmolarity and induce inflammatory response through NLRP3 and NLRC4 inflammasome activation. *Nat Commun* **6:**6931. http://dx.doi.org/10.1038/ncomms7931.

25. King CH, Mundayoor S, Crawford JT, Shinnick TM. 1993. Expression of contact-dependent cytolytic activity by *Mycobacterium tuberculosis* and isolation of the genomic locus that encodes the activity. *Infect Immun* **61:**2708–2712.

26. Stanley SA, Johndrow JE, Manzanillo P, Cox JS. 2007. The Type I IFN response to infection with *Mycobacterium tuberculosis* requires ESX-1-mediated secretion and contributes to pathogenesis. *J Immunol* **178:**3143–3152 http://dx.doi.org/10.4049/jimmunol.178.5.3143.

27. Manzanillo PS, Shiloh MU, Portnoy DA, Cox JS. 2012. *Mycobacterium tuberculosis* activates the DNA-dependent cytosolic surveillance pathway within macrophages. *Cell Host Microbe* **11:**469–480 http://dx.doi.org/10.1016/j.chom.2012.03.007.

28. Collins AC, Cai H, Li T, Franco LH, Li XD, Nair VR, Scharn CR, Stamm CE, Levine B, Chen ZJ, Shiloh MU. 2015. Cyclic GMP-AMP synthase is an innate immune DNA sensor for *Mycobacterium tuberculosis*. *Cell Host Microbe* **17:**820–828 http://dx.doi.org/10.1016/j.chom.2015.05.005.

29. Wassermann R, Gulen MF, Sala C, Perin SG, Lou Y, Rybniker J, Schmid-Burgk JL, Schmidt T, Hornung V, Cole ST, Ablasser A. 2015. *Mycobacterium tuberculosis* differentially activates cGAS- and inflammasome-dependent intracellular immune responses through ESX-1. *Cell Host Microbe* **17:**799–810 http://dx.doi.org/10.1016/j.chom.2015.05.003.

30. Watson RO, Bell SL, MacDuff DA, Kimmey JM, Diner EJ, Olivas J, Vance RE, Stallings CL, Virgin HW, Cox JS. 2015. The cytosolic sensor cGAS detects *Mycobacterium tuberculosis* DNA to induce type I interferons and activate autophagy. *Cell Host Microbe* **17:**811–819 http://dx.doi.org/10.1016/j.chom.2015.05.004.

31. Antonelli LR, Gigliotti Rothfuchs A, Gonçalves R, Roffê E, Cheever AW, Bafica A, Salazar AM, Feng CG, Sher A. 2010. Intranasal poly-IC treatment exacerbates tuberculosis in mice through the pulmonary recruitment of a pathogen-permissive monocyte/macrophage population. *J Clin Invest* **120:**1674–1682 http://dx.doi.org/10.1172/JCI40817.

32. Mayer-Barber KD, Andrade BB, Oland SD, Amaral EP, Barber DL, Gonzales J, Derrick SC, Shi R, Kumar NP, Wei W, Yuan X, Zhang G, Cai Y, Babu S, Catalfamo M, Salazar AM, Via LE, Barry CE III, Sher A. 2014. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature* **511:**99–103 http://dx.doi.org/10.1038/nature13489.

33. Shah S, Bohsali A, Ahlbrand SE, Srinivasan L, Rathinam VA, Vogel SN, Fitzgerald KA, Sutterwala FS, Briken V. 2013. Cutting edge: mycobacterium tuberculosis but not nonvirulent mycobacteria inhibits IFN-β and AIM2 inflammasome-dependent IL-1β production via its ESX-1 secretion system. *J Immunol* **191:**3514–3518 http://dx.doi.org/10.4049/jimmunol.1301331.

34. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, Wilkinson KA, Banchereau R, Skinner J, Wilkinson RJ, Quinn C, Blankenship D, Dhawan R, Cush JJ, Mejias A, Ramilo O, Kon OM, Pascual V, Banchereau J, Chaussabel D, O'Garra A. 2010. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature* **466:**973–977 http://dx.doi.org/10.1038/nature09247.

35. Inohara N, Nuñez G. 2003. NODs: intracellular proteins involved in inflammation and apoptosis. *Nat Rev Immunol* **3:**371–382 http://dx.doi.org/10.1038/nri1086.

36. Pandey AK, Yang Y, Jiang Z, Fortune SM, Coulombe F, Behr MA, Fitzgerald KA, Sassetti CM, Kelliher MA. 2009. NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to *Mycobacterium tuberculosis*. *PLoS Pathog* **5:**e1000500 http://dx.doi.org/10.1371/journal.ppat.1000500.

37. Aguilo JI, Alonso H, Uranga S, Marinova D, Arbués A, de Martino A, Anel A, Monzon M, Badiola J, Pardo J, Brosch R, Martin C. 2013. ESX-1-induced apoptosis is involved in cell-to-cell spread of *Mycobacterium tuberculosis*. *Cell Microbiol* **15:**1994–2005 http://dx.doi.org/10.1111/cmi.12169.

38. Yao Q. 2013. Nucleotide-binding oligomerization domain containing 2: structure, function, and diseases. *Semin Arthritis Rheum* **43:**125–130 http://dx.doi.org/10.1016/j.semarthrit.2012.12.005.

39. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. 2004. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* **119:**753–766 http://dx.doi.org/10.1016/j.cell.2004.11.038.

40. Manzanillo PS, Ayres JS, Watson RO, Collins AC, Souza G, Rae CS, Schneider DS, Nakamura K, Shiloh MU, Cox JS. 2013. The ubiquitin ligase parkin mediates resistance to intracellular pathogens. *Nature* **501:**512–516 http://dx.doi.org/10.1038/nature12566.

41. Geisler S, Holmström KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, Springer W. 2010. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* **12:**119–131 http://dx.doi.org/10.1038/ncb2012.

42. Watson RO, Manzanillo PS, Cox JS. 2012. Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. *Cell* **150:**803–815 http://dx.doi.org/10.1016/j.cell.2012.06.040.

43. Romagnoli A, Etna MP, Giacomini E, Pardini M, Remoli ME, Corazzari M, Falasca L, Goletti D, Gafa V, Simeone R, Delogu G, Piacentini M, Brosch R, Fimia GM, Coccia EM. 2012. ESX-1 dependent impairment of autophagic flux by *Mycobacterium tuberculosis* in human dendritic cells. *Autophagy* **8:**1357–1370 http://dx.doi.org/10.4161/auto.20881.

44. Brodin P, Poquet Y, Levillain F, Peguillet I, Larrouy-Maumus G, Gilleron M, Ewann F, Christophe T, Fenistein D, Jang J, Jang MS, Park SJ, Rauzier J, Carralot JP, Shrimpton R, Genovesio A, Gonzalo-Asensio JA, Puzo G, Martin C, Brosch R, Stewart GR, Gicquel B, Neyrolles O. 2010. High content phenotypic cell-based visual screen identifies *Mycobacterium tuberculosis* acyltrehalose-containing glycolipids involved in phagosome remodeling. *PLoS Pathog* **6:**e1001100 http://dx.doi.org/10.1371/journal.ppat.1001100.

45. MacGurn JA, Cox JS. 2007. A genetic screen for *Mycobacterium tuberculosis* mutants defective for phagosome maturation arrest identifies components of the ESX-1 secretion system. *Infect Immun* **75:**2668–2678 http://dx.doi.org/10.1128/IAI.01872-06.

46. Mehra A, Zahra A, Thompson V, Sirisaengtaksin N, Wells A, Porto M, Köster S, Penberthy K, Kubota Y, Dricot A, Rogan D, Vidal M, Hill DE, Bean AJ, Philips JA. 2013. *Mycobacterium tuberculosis* type VII secreted effector EsxH targets host ESCRT to impair trafficking. *PLoS Pathog* **9:**e1003734 http://dx.doi.org/10.1371/journal.ppat.1003734.

47. Wong D, Bach H, Sun J, Hmama Z, Av-Gay Y. 2011. *Mycobacterium tuberculosis* protein tyrosine phosphatase (PtpA) excludes host vacuolar-H+-ATPase to inhibit phagosome acidification. *Proc Natl Acad Sci USA* **108:**19371–19376 http://dx.doi.org/10.1073/pnas.1109201108.

48. Vergne I, Chua J, Lee HH, Lucas M, Belisle J, Deretic V. 2005. Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **102:**4033–4038 http://dx.doi.org/10.1073/pnas.0409716102.

49. Kirby JE, Vogel JP, Andrews HL, Isberg RR. 1998. Evidence for pore-forming ability by *Legionella pneumophila*. *Mol Microbiol* **27:**323–336 http://dx.doi.org/10.1046/j.1365-2958.1998.00680.x.

50. Lansbury PT Jr. 1999. Evolution of amyloid: what normal protein folding may tell us about fibrillogenesis and disease. *Proc Natl Acad Sci USA* **96:**3342–3344 http://dx.doi.org/10.1073/pnas.96.7.3342.

51. Demuro A, Mina E, Kayed R, Milton SC, Parker I, Glabe CG. 2005. Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers. *J Biol Chem* **280:**17294–17300 http://dx.doi.org/10.1074/jbc.M500997200.

52. Wang L, Maji SK, Sawaya MR, Eisenberg D, Riek R. 2008. Bacterial inclusion bodies contain amyloid-like structure. *PLoS Biol* **6:**e195 http://dx.doi.org/10.1371/journal.pbio.0060195.

53. Reardon S. 2015. Antibody drugs for Alzheimer's show glimmers of promise. *Nature* **523:**509–510 http://dx.doi.org/10.1038/nature.2015.18031.

54. Frigui W, Bottai D, Majlessi L, Monot M, Josselin E, Brodin P, Garnier T, Gicquel B, Martin C, Leclerc C, Cole ST, Brosch R. 2008. Control of *M. tuberculosis* ESAT-6 secretion and specific T cell recognition by PhoP. *PLoS Pathog* **4:**e33 http://dx.doi.org/10.1371/journal.ppat.0040033.

55. Lee JS, Krause R, Schreiber J, Mollenkopf HJ, Kowall J, Stein R, Jeon BY, Kwak JY, Song MK, Patron JP, Jorg S, Roh K, Cho SN, Kaufmann SH. 2008. Mutation in the transcriptional regulator PhoP contributes to avirulence of *Mycobacterium tuberculosis* H37Ra strain. *Cell Host Microbe* **3:**97–103 http://dx.doi.org/10.1016/j.chom.2008.01.002.

56. Fortune SM, Jaeger A, Sarracino DA, Chase MR, Sassetti CM, Sherman DR, Bloom BR, Rubin EJ. 2005. Mutually dependent secretion of proteins required for mycobacterial virulence. *Proc Natl Acad Sci USA* **102:**10676–10681 http://dx.doi.org/10.1073/pnas.0504922102.

57. Cao G, Howard ST, Zhang P, Wang X, Chen XL, Samten B, Pang X. 2015. EspR, a regulator of the ESX-1 secretion system in *Mycobacterium tuberculosis*, is directly regulated by the two-component systems MprAB and PhoPR. *Microbiology* **161:**477–489 http://dx.doi.org/10.1099/mic.0.000023.

58. Raghavan S, Manzanillo P, Chan K, Dovey C, Cox JS. 2008. Secreted transcription factor controls *Mycobacterium tuberculosis* virulence. *Nature* **454:**717–721 http://dx.doi.org/10.1038/nature07219.

59. Tan S, Sukumar N, Abramovitch RB, Parish T, Russell DG. 2013. *Mycobacterium tuberculosis* responds to chloride and pH as synergistic cues to the immune status of its host cell. *PLoS Pathog* **9:**e1003282 http://dx.doi.org/10.1371/journal.ppat.1003282.

60. Via LE, Fratti RA, McFalone M, Pagan-Ramos E, Deretic D, Deretic V. 1998. Effects of cytokines on mycobacterial phagosome maturation. *J Cell Sci* **111:**897–905.

61. Denis M. 1991. Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. *Cell Immunol* **132:**150–157 http://dx.doi.org/10.1016/0008-8749(91)90014-3.

62. Herbst S, Schaible UE, Schneider BE. 2011. Interferon gamma activated macrophages kill mycobacteria by nitric oxide induced apoptosis. *PLoS One* **6:**e19105 http://dx.doi.org/10.1371/journal.pone.0019105.

63. Warwick-Davies J, Dhillon J, O'Brien L, Andrew PW, Lowrie DB. 1994. Apparent killing of *Mycobacterium tuberculosis* by cytokine-activated human monocytes can be an artefact of a cytotoxic effect on the monocytes. *Clin Exp Immunol* **96:**214–217 http://dx.doi.org/10.1111/j.1365-2249.1994.tb06544.x.

64. Wong KW, Jacobs WR Jr. 2013. *Mycobacterium tuberculosis* exploits human interferon γ to stimulate macrophage extracellular trap formation and necrosis. *J Infect Dis* **208:**109–119 http://dx.doi.org/10.1093/infdis/jit097.

65. Zhang M, Chen JM, Sala C, Rybniker J, Dhar N, Cole ST. 2014. EspI regulates the ESX-1 secretion system in response to ATP levels in *Mycobacterium tuberculosis*. *Mol Microbiol* **93:**1057–1065 http://dx.doi.org/10.1111/mmi.12718.

66. Chen JM, Zhang M, Rybniker J, Boy-Röttger S, Dhar N, Pojer F, Cole ST. 2013. *Mycobacterium tuberculosis* EspB binds phospholipids and mediates EsxA-independent virulence. *Mol Microbiol* **89:**1154–1166 http://dx.doi.org/10.1111/mmi.12336.

67. Martin CJ, Booty MG, Rosebrock TR, Nunes-Alves C, Desjardins DM, Keren I, Fortune SM, Remold HG, Behar SM. 2012. Efferocytosis is an innate antibacterial mechanism. *Cell Host Microbe* **12:**289–300 http://dx.doi.org/10.1016/j.chom.2012.06.010.

68. Huang D, Bao L. 2016. *Mycobacterium tuberculosis* EspB protein suppresses interferon-γ-induced autophagy in murine macrophages. *J Microbiol Immunol Infect* **49:**859–865. 10.1016/j.jmii.2014.11.008.

69. Bruntz RC, Lindsley CW, Brown HA. 2014. Phospholipase D signaling pathways and phosphatidic acid as therapeutic targets in cancer. *Pharmacol Rev* **66:**1033–1079 http://dx.doi.org/10.1124/pr.114.009217.

70. Korotkova N, Piton J, Wagner JM, Boy-Röttger S, Japaridze A, Evans TJ, Cole ST, Pojer F, Korotkov KV. 2015. Structure of EspB, a secreted substrate of the ESX-1 secretion system of *Mycobacterium tuberculosis*. *J Struct Biol* **191:**236–244 http://dx.doi.org/10.1016/j.jsb.2015.06.003.

71. Solomonson M, Setiaputra D, Makepeace KA, Lameignere E, Petrotchenko EV, Conrady DG, Bergeron JR, Vuckovic M, DiMaio F, Borchers CH, Yip CK, Strynadka NC. 2015. Structure of EspB from the ESX-1 type VII secretion system and insights into its export mechanism. *Structure* **23:**571–583 http://dx.doi.org/10.1016/j.str.2015.01.002.

72. Rybniker J, Chen JM, Sala C, Hartkoorn RC, Vocat A, Benjak A, Boy-Röttger S, Zhang M, Székely R, Greff Z, Orfi L, Szabadkai I, Pató J, Kéri G, Cole ST. 2014. Anticytolytic screen identifies inhibitors of mycobacterial virulence protein secretion. *Cell Host Microbe* **16:**538–548 http://dx.doi.org/10.1016/j.chom.2014.09.008.

73. Johnson BK, Colvin CJ, Needle DB, Mba Medie F, Champion PA, Abramovitch RB. 2015. The carbonic anhydrase inhibitor ethoxzolamide inhibits the *Mycobacterium tuberculosis* PhoPR regulon and Esx-1 secretion and attenuates virulence. *Antimicrob Agents Chemother* **59:**4436–4445 http://dx.doi.org/10.1128/AAC.00719-15.