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Infect and Inject: How *Mycobacterium tuberculosis* Exploits Its Major Virulence-Associated Type VII Secretion System, ESX-1

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ABSTRACT *Mycobacterium tuberculosis* is an ancient master of the art of causing human disease. One important weapon within its fully loaded arsenal is the type VII secretion system. *M. tuberculosis* has five of them: ESAT-6 secretion systems (ESX) 1 to 5. ESX-1 has long been recognized as a major cause of attenuation of the FDA-licensed vaccine *Mycobacterium bovis* BCG, but its importance in disease progression and transmission has recently been elucidated in more detail. This review summarizes the recent advances in (i) the understanding of the ESX-1 structure and components, (ii) our knowledge of ESX-1's role in hijacking macrophage function to set a path for infection and dissemination, and (iii) the development of interventions that utilize ESX-1 for diagnosis, drug interventions, host-directed therapies, and vaccines.

INTRODUCTION

Tuberculosis (TB) is a global health problem caused by the airborne pathogen *Mycobacterium tuberculosis*. Currently, one-third of the world's population is infected with *M. tuberculosis*, and this slow, tenacious bacterium kills 1.6 million people around the world each year, equating to over 4,300 deaths every day (1). Failure to eradicate this age-old disease is the result of an ineffective vaccine and extended, often insufficient, chemotherapy. To date, the only licensed vaccine available is *Mycobacterium bovis* BCG, a live attenuated strain of *M. bovis* discovered in 1919 by Albert Calmette and Camille Guérin following 230 subcultures of the original virulent isolate (2, 3). Distribution of this vaccine to various countries, and more subculturing, led to genetic variations between different BCG strains. However, all strains possess a common deletion that occurred prior to 1919. The deleted region is called region of difference 1 (RD1), and it encodes a key part of the type VII secretion system known as ESAT-6 secretion system 1 (ESX-1) (Fig. 1A); deletion(s)

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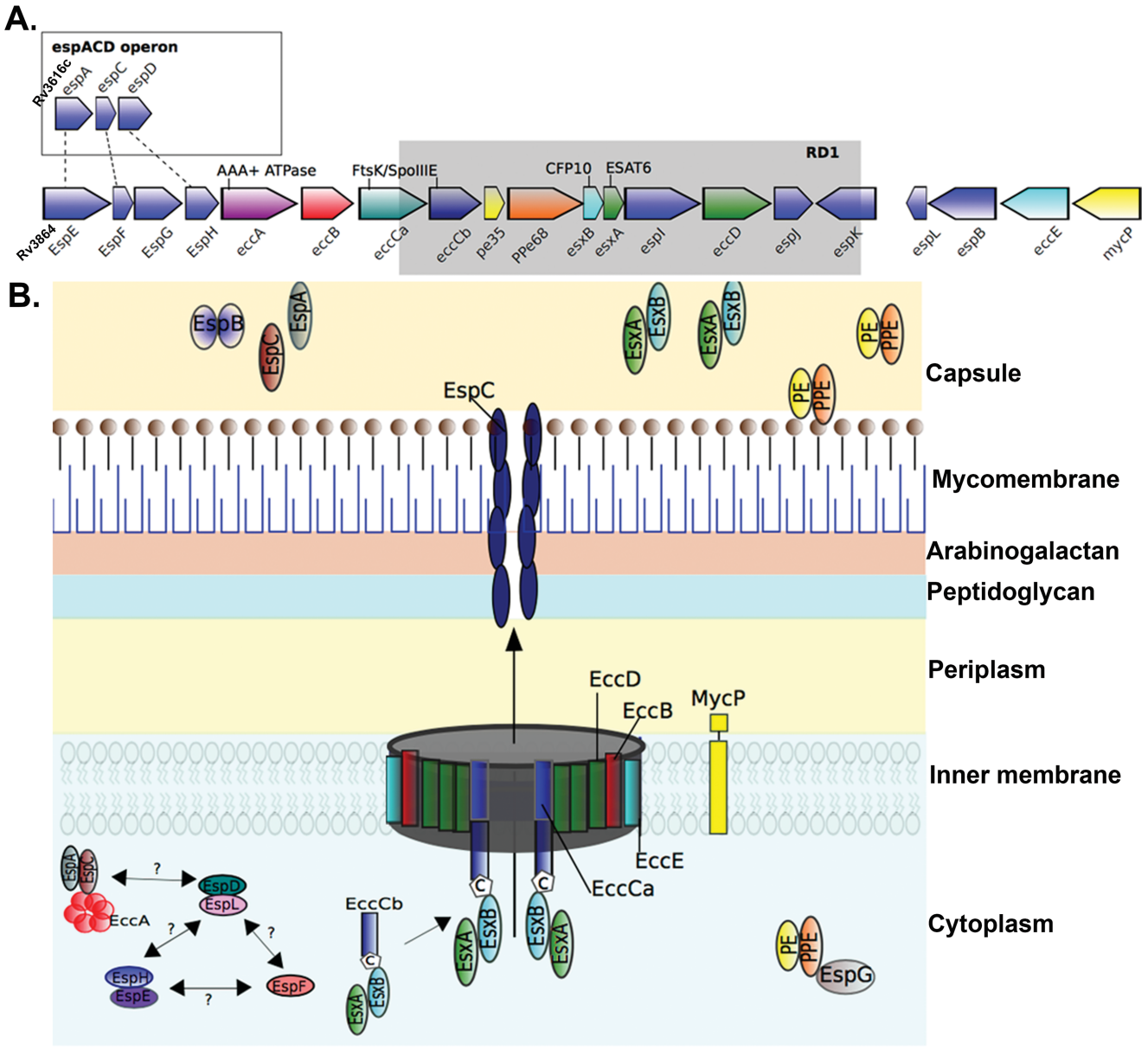

FIGURE 1 Schematic of the ESX-1 secretion system. **(A)** Gene map of the *esx-1* locus and the *espACD* operon in *M. tuberculosis* H37Rv. The *esx-1* locus includes *esx* genes encoding the secreted effector proteins EsxA and EsxB alongside *ecc* genes encoding ESX-conserved proteins and *esp* genes encoding ESX secretion-associated proteins (108). The *espACD* operon is at a locus distinct from the *esx-1* locus but shares sequence homology with *espE*, *espF*, and *espH* of *esx-1* (dashed lines). The spontaneous deletion (Rv3871 to Rv3878) from *esx-1* found in the vaccine strains of *M. bovis* BCG is known as region of difference 1 (RD1) and is indicated by the gray box. **(B)** Model of the ESX-1 secretion system in the mycobacterial cell envelope. In common with all ESX systems, the core structure of the ESX-1 secretion apparatus starts with the inner membrane-spanning conserved components EccB, EccC, EccD, and EccE (109). EccC is an ATP-driven translocase consisting of two subunits (a and b) that are assembled following EccB binding of target substrate, in this case, the heterodimer EsxAB, where EccCb interacts with the carboxyl-terminal signal sequence of EsxB (labeled "C") (21, 110). EsxAB secretion is codependent on the secretion of EspC/EspA, which is also dependent on interaction with the cytosolic ATPase EccA (20, 111). EspC polymerizes during secretion, indicating a role for EccA and EspA as cytosolic chaperones (18), and forms a filamentous structure thought to provide a channel for secretion of ESX-1 substrates (18). Other important ESX-1 substrates include the PE and PPE families of proteins, which form heterodimers and are recruited by the putative cytosolic chaperone EspG to initiate interaction with the core complex of proteins within the inner membrane (112–114). EspB is also secreted by ESX-1 and forms a PE-PPE-like fold, containing a C-terminal domain that is processed by the $MycP_1$ protease during secretion (26, 115). EspD to -F and EspH proteins are cytosolic and were recently shown to be stabilized by the cytosolic chaperone EspL (24–26).

in this particular region are considered the major cause of BCG attenuation (4–6).

Brief History of ESX-1

ESX-1 is considered omnipresent in terms of scientific publications and functionalities, with studies showing its involvement in intercellular conjugation (7), membrane escape (8), and passage through the lung interstitium (9). ESX-1 is a complex, multifunctional type VII secretion system, producing and releasing a plethora of proteins, many of which are required for its own secretion.

Mahairas et al. first observed RD1 (*Rv3871-9*) at the genetic level in 1996 while comparing genetic differences between BCG, its parent *M. bovis,* and its cousin *M. tuberculosis* (4). Following this seminal study, comparative genomics of numerous BCG strains determined that RD1 was the first region of difference that occurred prior to attenuation in 1921 (10). ESX-1 and its four closely related homologues in *M. tuberculosis* were identified as potential secretion systems in an organism that was originally believed to have none, with additional homologues identified in other Gram-positive bacteria (11). Similar to Calmette and Guérin a century earlier, several groups saw the potential for a novel vaccine strategy, postulating that the removal of this particular attenuating region from the backbone of the strains that cause human TB, i.e., *M. tuberculosis*, would result in a vaccine that would work better than BCG. In 2003, the Jacobs, Sherman, and Cole groups were the first to create vaccine candidates based on this hypothesis, with the first two labs knocking out RD1 in *M. tuberculosis* (9, 12) and the latter taking an alternative approach by adding RD1 to BCG (13). Interestingly, RD1 knockdown strain studies in mice revealed intriguing results, wherein the bacterial count was the same but the pathology was dramatically different (9). Protection was observed, but it never achieved the levels found with BCG vaccination in murine models of infection. However, Koch's molecular postulates were fulfilled, telling us that the removal of RD1 resulted in attenuation (14). Interestingly, a recently isolated clinical strain defective in this region has shown similar results, where there is no significant difference in bacterial count but pathology and cytokine response are remarkably different (15).

Although these studies did not lead to novel vaccine candidates, they did provide tools to study *M. tuberculosis* virulence, which may help us design better vaccines and treatment strategies. Recent advances in our understanding of TB disease and ESX-1 are discussed. However, care should be taken to differentiate between cellular functions as they pertain to different organisms; for example, there have been significant advances in *Mycobacterium marinum* studies in its zebrafish host, which have shown that ESX-1 is not virulence related in this particular species of mycobacteria (16).

Vaccine Studies That Taught Us More about Pathogenesis than Protection

No ESX-1 story is complete without the original ESX-1 knockout mutant, *M. bovis* BCG. The tale has been told many times of the 230 subcultures that Albert Calmette and Camille Guérin carried out, culminating in the attenuation of virulent *M. bovis*. The story of BCG begins at the Pasteur Institute in Lille, France, where Guérin began subculturing a virulent strain of *M. bovis* isolated from the udder of a tuberculous cow. The culture was passaged every ~3 weeks on a medium containing potato, glycerine, and ox bile. Mycobacterial cultures are notoriously clumpy, and Calmette and Guérin found that a mycobacterial stew, composed of *M. bovis* growing on potatoes that were cooked in ox bile and then dipped at one end in glycerinated ox bile, led to an emulsified culture, making it easier to standardize inoculating doses. This serendipitously resulted in attenuation of the strain, which led Calmette and Guérin to produce a vaccine utilizing this bacillus (2). By 1919, 230 subcultures had resulted in a strain that failed to produce tuberculous disease when injected into guinea pigs, rabbits, cattle, and horses (17). Following this success, preparations began for the first human clinical trials.

In the early 20th century, ethical standards were somewhat less stringent than today, and the first human "trial" of BCG was undertaken in an unlikely subject, infants. In 1921, an infant under the care of its grandmother, who had TB, was given three doses of oral BCG to prevent likely infection and possible death (3). This initial foray into attenuated TB vaccines was a success, and the child remained healthy. Following this success, a larger trial began 6 months later in which BCG was orally administered to 317 infants at birth. Ultimately, BCG proved to be safe and effective in protecting against childhood TB (3). However, vaccine safety was severely questioned in 1930 following the Lübeck disaster, in which 250 infants were vaccinated in a northern German city with a contaminated BCG strain, resulting in 73 deaths and 135 additional cases of TB. A lengthy investigation attributed the disaster to negligent vaccine preparation, leading to contamination with virulent tubercle bacilli.

Despite this early setback, BCG has subsequently proven to be one of the safest vaccines ever created and has saved the lives of countless individuals, many of them children. However, TB remains an enormous global prob-

lem, bringing attention back to BCG attenuation fundamentals, the loss of RD1, and, in turn, the absence of an ESX-1 secretion system.

THE ESX-1 SECRETION SYSTEM AND ITS PROTEINACEOUS ARMY

Many of the proteins secreted by ESX-1 are immunodominant and are at the forefront of infection and disease. The ESX type VII secretion systems are complex; the current working model of the ESX-1 secretion system is shown schematically in Fig. 1B, with the recent addition of EspC, which forms a filamentous structure spanning the bacterial cell envelope (18).

The main ESX-1 system spans the inner membrane with a channel-like structure composed of conserved ESX components (EccB to -D) that form the membrane core complex. EccB and EccC are ATPases involved in recognizing substrates and providing energy for the secretion of substrates across the mycomembrane (19–21). Interestingly, in ESX-1 and ESX-5, the *eccC* gene, encoding a FtsK/SpoIIIE-type ATPase, has split into two genes that generate the proteins EccCa and EccCb, which interact and form a functional unit for secretion through the ESX-1 system. Although the mycosin protease MycP1 is not an integral part of the central membrane complex, and its protease activity is dispensable for ESX-1-mediated protein secretion, MycP1 is a conserved membrane component associated with the membrane complex, and this association is essential for the stability of the complex (22).

In addition to membrane components, ESX-1 also has cytosolic components, including cytosolic ATPase (EccA), chaperones (EspD to -H), and secreted substrate proteins (EsxAB, PE35-PPE68, EspA to -C, and EspE) called effectors. EccA is a cytosolic AAA (ATPase associated with diverse cellular activities)-type ATPase. Most cytosolic chaperones associated with the ESX-1 system belong to ESX-1 secretion-associated proteins (Esp). EspI (Rv3876) to -L (Rv3880c) are encoded within an operon that generates ESX conserved components (Rv3868 to Rv3883c), and the EspE to -H (Rv3864 to Rv3867) genes are upstream, whereas the EspACD (3616c to 3614c) operon is at a more distant location in the genome (Fig. 1A). EspD itself is secreted by *M. tuberculosis*, although not exclusively in an ESX-1-dependent manner, but it is also required for stabilizing (EspC and EspA) and secretion (EsxA) of ESX-1 substrates (23). More recently, the EspD to -F and EspH proteins were shown to be stabilized by the chaperone EspL (24). Although some Esp proteins serve as cytosolic chaperones, some act as effectors (EspA to -C and EspE) of ESX-1 and are secreted (25, 26). EspC has been shown to form filaments in the membrane (18), whereas EspE localizes to the cell wall. Fusion of EspE with a fluorescent marker protein in *M. marinum* demonstrated that ESX-1 localizes at new poles with active peptidoglycan synthesis following cell division (27). How the Esp proteins encoded within the *esx-1* operon or distally in the genome interact with each other and with other components of the ESX-1 system and contribute to its integrity and functionality are still active areas of study.

The other major effectors secreted by the ESX-1 system are key immunogenic, highly secreted ESX proteins, including the early secreted antigenic target of 6 kDa (ESAT-6, also called EsxA) and the culture filtrate protein of 10 kDa (CFP-10 or EsxB). EsxA and EsxB are secreted as a heterodimer (EsxAB) in a codependent complex (28). Binding of the EsxAB heterodimer to the ESX-1 inner membrane core complex component EccC for secretion involves recognition of the bipartite secretion signal motif, consisting of WXG, located on EsxA, and tyrosine-X-X-X-aspartic acid/glutamic acid (YXXXD/E), located on EsxB (21, 25, 29). In contrast, secretion of the PE35-PPE68 heterodimer is dependent on direct binding to EspG (30). The ESX-1 system is highly complex, and this complexity is not restricted to the machinery and effector molecules; the regulation of ESX-1 itself also appears to be multifactorial and is indirectly regulated by PhoPR (31), WhiB6 (32), EspR (33), Lsr2 (34), and MprAB (35).

ESX-1 Lyses Membranes

The macrophage phagosome is a highly inhospitable environment; however, *M. tuberculosis* survives and replicates within this environment. It has long been debated whether *M. tuberculosis* can escape the phagosome and replicate within the cytosol, and McDonough et al. were some of the first researchers to demonstrate the controversial escape of *M. tuberculosis* from phagosomes to the cytosol using electron microscopy (36). This has since been shown to be ESX-1 mediated via ESAT-6 (37). The ESX-1 system was also found to lyse whole cells, causing macrophage and epithelial cell necrosis in *in vitro* infection experiments (4). Many researchers carried out the obvious experiments, adding ESAT-6 (EsxA) directly to cell cultures to look for membrane lysis, often in terms of measuring the release of cytoplasmic contents. However, no cell lysis was observed, until the missing link of low pH was identified (38, 39). Conrad et al. stated that contact dependence was required for ESAT-6 membrane lysis but then, in the same study, showed that low pH caused this as well (40). The Jacobs laboratory tried this

experiment in 2003 before we knew of the low-pH trigger, and although our study was unsuccessful, we did demonstrate total membrane disruption in a simplified artificial membrane model with ESAT-6 or ESAT-6 and CFP-10 together, but not with CFP-10 alone (9). In addition, cryo-electron micrographs of ESAT-6 and CFP-10 proteins showed differently sized pore-like structures (Fig. 2, inset a) (8).

Phagosome Permeabilization and Its Prospective Roles

The implications of phagosomal escape have been discussed in many reviews covering mycobacterial species and other intracellular pathogens, such as *Salmonella enterica* serovar Typhimurium or *Listeria monocytogenes* (41). The reasoning includes cytoplasmic nutritional availability, antigen presentation to CD8 T cells, and dissemination. In *M. tuberculosis,* it is clear that this escape happens (37) and that it is ESX-1 mediated; however, some studies suggest that such escape is a temporal by-product of ESX-1 lysing the cell membrane to escape from the cell (42).

Nutrient-rich cytoplasm seems an attractive environment for proliferation, providing an advantage to intracellular pathogens that develop mechanisms to permeabilize, and escape from, the phagosome. Interestingly, to counter host-imposed nutrient restrictions, *M. tuberculosis* can synthesize most of the essential nutrients for its growth, but it is also capable of acquiring nutrients from the host. *M. tuberculosis* can obtain carbon, nitrogen, and some amino acids from the host (41, 43, 44), but intriguingly, it is not able to acquire arginine, methionine, or leucine from the host (45–47). In the Jacobs laboratory, we have demonstrated that arginine or methionine auxotrophy is bactericidal to *M. tuberculosis in vitro* and *in vivo*, both in macrophages and in mice (45, 46), whereas leucine auxotrophy is bacteriostatic (45–47). This finding was noteworthy, as mouse plasma has an arginine concentration of ~200 μM under normal conditions, and *M. tuberculosis* possesses arginine transporters (48, 49). *Salmonella* Typhimurium establishes an active arginine recovery system using its arginine transporter ArgT and by promoting accumulation of the host arginine transporter (mCAT1) on phagosomes (50), which does not appear to be the case with *M. tuberculosis*. This amino acid autarky is, as yet, an unexplored metabolic vulnerability. However, it does suggest that if ESX-1 is functional and provides access to the cytosol, *M. tuberculosis* amino acid auxotrophs should acquire their missing nutrient from the cytosol and proliferate and disseminate as observed with other pathogens (41, 51). However, by utilizing nutrients from the host in this manner, these pathogens also alert the host immune system. It is unclear whether *M. tuberculosis*'s ability to biosynthesize most of the amino acids and metabolic shutdown during famine of the nutrients provide an evolutionary advantage by enhancing the ability of *M. tuberculosis* to persist in the host, ultimately evading vaccine and drug treatments. Further studies are needed to better answer these questions and fully elucidate the essentiality and sufficiency of ESX-1 system to lyse phagosomal membranes and gain access to the nutrient-rich cytosol for proliferation and dissemination.

Another implication of phagosome permeabilization by *M. tuberculosis* is egress of the organism to the cytosol for dissemination and pathogenesis of disease. ESAT-6-mediated phagosome disruption activates the cytosolic inflammasome receptor NLRP3, also triggering increased necrosis (52, 53). Augenstreich et al. suggested that ESX-1, along with phthiocerol dimycocerosates, causes phagosomal rupture but leads to an alternative mode of death, specifically, apoptosis (54). Further, ESX-1-mediated phagosome permeabilization has been shown to enhance type I interferon (IFN) secretion as a pathogenic mechanism for promoting bacterial replication and manipulation of host immunity (8, 55). With so many conflicting studies, it is clear that phagosome disruption remains a much-debated topic in the TB field. Engendering further conflict in the field is the question of whether ESX-1 mediates exit from the cell by the formation of ejectosomes, membrane protrusions containing the bacilli that propel themselves by means of an actin tail. These structures have been observed in *M. marinum* (56), but whether they occur in *M. tuberculosis*-infected human macrophages has yet to be determined, particularly as *M. tuberculosis* does not have the required active tail (57).

ESX-1 at the Site of Disease: Disease Progression via Necrosis

Orme suggested that necrosis is itself a means to progression of TB disease (58), and following intracellular replication within alveolar macrophages at the site of disease, cell death often occurs, with *M. tuberculosis* ESX-1 promoting necrosis, not apoptosis (4). Indeed, *M. tuberculosis* actively suppresses macrophage apoptosis, reducing bacterial replication (59) with ESX-1, while ESX-1-mediated necrosis enhances bacterial replication (60). *M. tuberculosis* also mediates cellular necrosis in other host cells, such as alveolar epithelial cells in the epithelial bilayer, which is needed for infection and disease progression (61–63). King believes the interaction of *M. tuberculosis* with alveolar epithelial cells is too

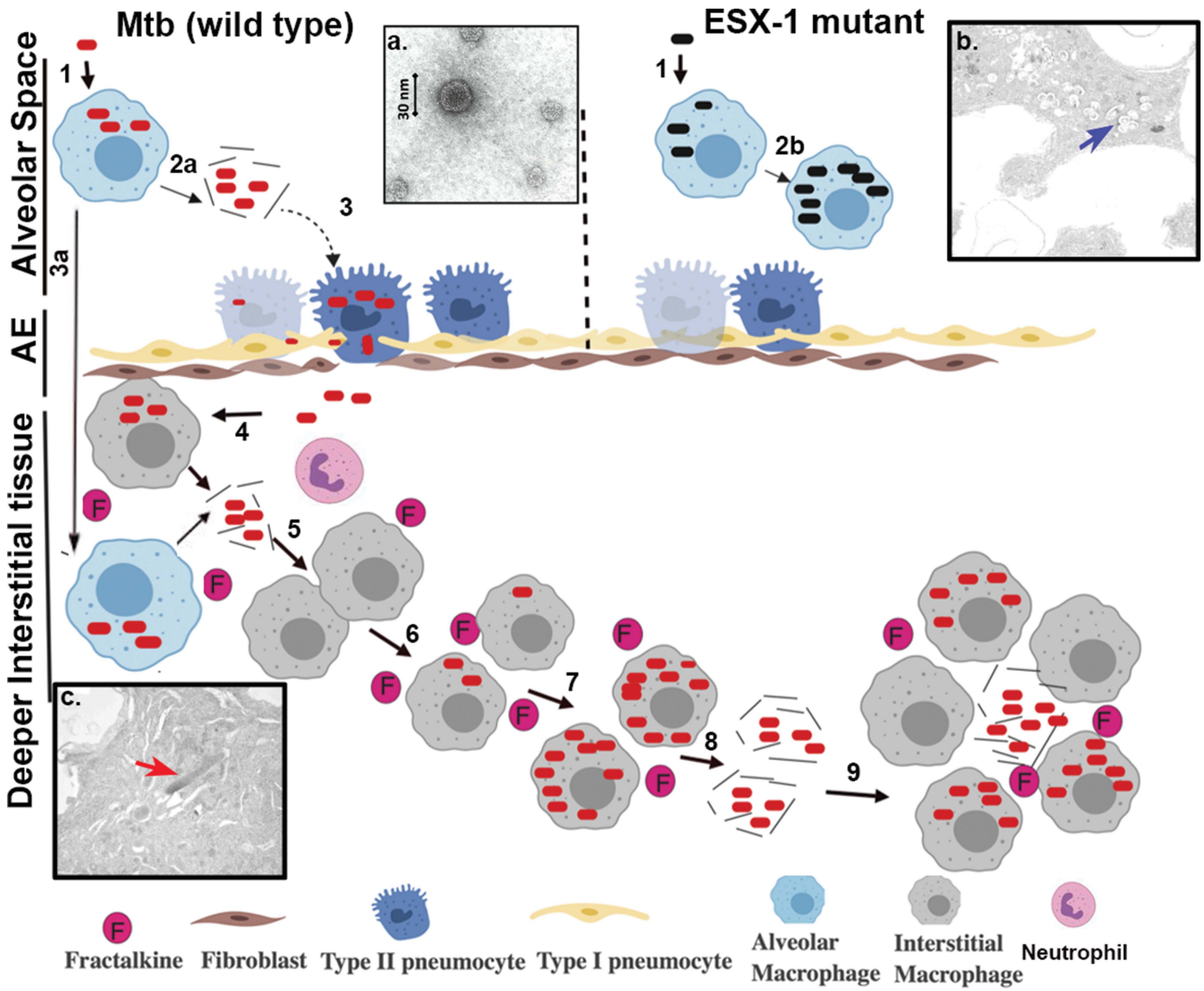

FIGURE 2 ESX-1-related disease progression within the lung. Steps involved in progression of disease are represented with the numbers 1 to 9. (1) Infection of alveolar macrophages with *M. tuberculosis* (wild type) or ESX-1 mutant. (2) Lysis of the phagosomal and cellular macrophage membranes is carried out by EsxA from wild-type *M. tuberculosis* (2a), while the ESX-1 mutant remains trapped in the alveolar macrophages in the alveolar space (2b). (3) Infection of type II pneumocytes in the alveolar epithelium (AE) by *M. tuberculosis*, with resulting ESAT-6-mediated lysis, allowing passage into the interstitial tissue or (3a) translocation of infected alveolar macrophage to lung interstitial tissue. (4) Translocated bacteria are ingested by and replicate within macrophages, which produce cytokines such as fractalkine. (5) Release of bacilli by necrosis of infection-dependent macrophages. (6) Recruitment of neutrophils and naive macrophages by fractalkine and infection of new macrophages and other cells by phagocytosis. (7) Intracellular replication of bacilli in recruited infected macrophages. (8) Continuation of the cycle, leading to egress of *M. tuberculosis* from the host cells into deeper interstitial tissue and dissemination within the lungs. (9) Establishment of granulomas and necrosis. (Insets) (a) Electron micrograph of EsxAB. (b) Electron micrograph of the ESX-1 mutant (blue arrow) trapped within the phagosome of an alveolar macrophage in alveolar space in a murine model. (c) Electron micrograph showing wild-type *M. tuberculosis* (red arrow) following egress to the cytoplasm and interstitial spaces in the murine lung.

often overlooked in TB disease, with most researchers conducting studies using macrophages and often missing these short-lived interactions, i.e., the egress across the alveolar interstitium within epithelial cells (63). Notably, studies have shown that type II pneumocytes are highly susceptible to *M. tuberculosis* infection, with the bacilli translocating into interstitial tissue through the basolateral surfaces of these cells via exocytosis or necrosis (64). In collaboration with our laboratory, King's group performed screens to find mutants of *M. tuberculosis* incapable of lysing alveolar epithelial cells, discovering that ESX-1 was required for epithelial cell lysis (9). We also observed ESX-1-mediated macrophage lysis both *in vitro* and *in vivo* (Fig. 2), with *M. tuberculosis* ESX-1 mutants remaining trapped within alveolar macrophages but wild-type bacilli escaping and egressing to the interstitial tissue (9).

Krishnan et al. predicted in 2010 that *M. tuberculosis*-infected alveolar macrophages may translocate from alveolar spaces to lung interstitium to disseminate *M. tuberculosis* (65), and recent studies support their hypothesis. Cohen et al. recently provided evidence of *M. tuberculosis*-infected alveolar macrophages being relocated to the lung interstitium due to ESX-1 and interleukin 1R (IL-1R) dependence (66). ESX-1 effector protein involved in dissemination of infected alveolar macrophages into the interstitium in mice is still undetermined (9, 66).Once *M. tuberculosis* escapes from the activated macrophage via necrosis, it needs a niche. In this instance, a growth-permissive naive macrophage serves as the perfect host where *M. tuberculosis* can survive and replicate. Therefore, to attract naive macrophages, *M. tuberculosis* has evolved a mechanism involving induction of fractalkine production by *M. tuberculosis*-infected cells.

ESX-1 and the Chemokine Fractalkine

First discovered by Bazan et al. in 1998 (67), fractalkine is a rather unusual chemokine and in a class of its own, having a strange stalk-like structure that can also tether host cells. It has been reported to attract naive macrophages to the lung, and fractalkine production from *M. tuberculosis*-infected cells has been determined to be ESX-1 regulated (68). This study also linked fractalkine levels to the influx of naive macrophages during TB infection using bronchoalveolar lavage samples taken directly from the lungs of TB patients. Other cytokines are likely involved in this influx, such as ESX-1-regulated IL-1R (66, 68, 69).

If *M. tuberculosis* ESX-1 mediates fractalkine production from the infected macrophage, what is the effector protein responsible for triggering its production? This appears to be ESAT-6, which activates the tyrosine kinase Syk (53); Syk functions in an upstream activation pathway to produce fractalkine, and its inhibition can stop *M. tuberculosis* ESX-1-mediated fractalkine production and the resulting monocytic infiltration (68). The fractalkine axis has been proposed as a treatment target via the inhibition of monocyte infiltration and thus inflammation in diseases such as Crohn's (70), and a vaccination approach has successfully been used to protect against respiratory syncytial virus infection in an animal model (71). Early establishment of infection and subsequent bacillary dissemination relies upon the availability of permissive "niche" cells. Therefore, a chemotactic signal would be a requisite for increasing the number of the cells from the approximate one macrophage per every 9 ml of lung volume (72). The question of whether other ESX-1-dependent effectors also are involved in the induction of fractalkine production by *M. tuberculosis*-infected macrophages is an open area of investigation.

DO OTHER BACTERIA HAVE ESX-1 SECRETION SYSTEMS?

Homologues of ESX-1 proteins have been detected in various members of the *Actinobacteria* (other mycobacterial species and *Streptomyces coelicolor*), *Firmicutes* (*Staphylococcus aureus*, *L. monocytogenes*, *Bacillus anthracis*, and *Bacillus subtilis*), and *Gammaproteobacteria*, such as *Helicobacter pylori* (73). These secretion systems contain at least one FtsK-SpoIII ATPase, plus one member of the WXG100 protein family (73). A type VII-like secretion system in *S. aureus* is composed of five membrane proteins (EsaA and EssA to -D), three cytosolic proteins (EsaB, -E, and -G), and five secreted virulence factors (EsxA to -D and EsaD) (74). In *S. aureus*, this specialized type VII secretion system is rapidly induced in response to interaction of the bacteria with host fluids, including blood serum, nasal secretions, and pulmonary surfactant (74). Furthermore, by generating mutants with deletions of genes homologous to the *M. tuberculosis* ESX-1 membrane core complex genes *essA*, *essB*, and *essC* in two *S. aureus* strains, Kneuper et al. demonstrated that these genes play a major role in nasal colonization and in development of pneumonia in cystic fibrosis murine infection models (75). Deletion of this secretion system in *S. aureus* leads to its attenuation via decreased bacterial growth and a subsequent decrease in the number of abscesses in host kidneys, spleen, and liver in mice (76, 77). Conversely, an ESX-1 secretion system found in *L. monocytogenes* is not required for epithelial

cell invasion and intracellular multiplication in macrophages *in vitro*; indeed, in an *in vivo* murine model, the expression of the ESAT-6 homologue EsxA was detrimental to *L. monocytogenes*, resulting in decreased infection (78). Whether deletion of ESX secretion systems from these pathogens can be exploited for live-vaccine design to protect against infection remains to be investigated.

PRACTICAL IMPLICATIONS OF ESX-1

Drug Interventions and Diagnostics

Studies on ESX-1 proteins have been of continuous interest for the development of drug interventions, diagnostic markers, and vaccines. EsxA and EsxB, two of the most highly secreted proteins of *M. tuberculosis*, have formed the basis for a major breakthrough in TB diagnostics, the IFN-γ release assays (IGRAs). The addition of another ESX-1 protein, EspC, which is present in BCG but not secreted, may potentially enhance the sensitivity and specificity of these assays (79). Interestingly, lysis of *M. tuberculosis* phagosomal membranes via EsxA and EsxB is associated with NOD2-RIP2- or cGAS-STING-dependent or -independent activation of type I interferon IFN-α and IFN-β induction (80). Furthermore, Barczak et al. showed that PDIM production and export are required for coordinated secretion of ESX-1 substrates for phagosome permeabilization and induction of type I IFN response (81).

Several studies have shown that induction of type I IFNs can worsen the outcome of TB (80, 82–84). Berry et al. performed whole-blood transcript signature studies on patients with active and latent TB to identify signatures linked to the progression of active disease (85). Recently, Singhania et al. identified type I IFN as a part of the transcriptome signature that can differentiate between patients with active and latent TB (86). These studies suggest that inhibitors of *M. tuberculosis* pertaining to IFN-α and IFN-β induction are good targets for novel immunotherapeutic strategies to combat *M. tuberculosis*. In addition to the effector proteins EsxA and EsxB, potential targets include immunogenic PE (Pro-Glu) and PPE (Pro-Pro-Glu) proteins and Esp proteins. Effectors such as EspE that are localized on the *M. tuberculosis* cell surface are potential therapeutic targets for antibodies, generating antibody-dependent cell cytotoxic responses, with the aim of eliminating extracellular *M. tuberculosis*. In a recent study, IL-2 induced by stimulation of whole blood with ESX-1-secreted PE35 and PPE68, using a technique similar to that used for IGRAs, was capable of discriminating between patients latently infected with *M. tuberculosis* and those with active TB (87).

The use of ESX-1 proteins distinguishes between BCG-vaccinated and nonvaccinated humans, as BCG does not possess or secrete these proteins. Determining the difference between latent TB infection, BCG vaccination, and active TB infection could revolutionize TB treatment strategies, allowing the development of differentiating biomarkers. Better characterizations of the T-cell-stimulatory proteins secreted by ESX-1 will lead to development of improved IGRAs with enhanced prognostic value.

Why Do We Need a New Vaccine?

BCG is good at protecting a range of animals, from badgers to horses. Unfortunately, that same coverage does not translate to adult humans, resulting in a failure to eradicate TB. However, there is a silver lining with BCG, as it is able to elicit some protection against the disease in children (88). However, if we are to rid the world of TB by 2020, which is the aim of the World Health Organization, we need a better vaccine, and luckily, there are several in the clinical pipeline that are related to ESX-1 or other type VII secretion systems.

In 2012, the Tuberculosis Vaccine Initiative led the TB vaccine community to generate a blueprint for TB vaccine development (https://www.who.int/immunization/sage/3_TB_Vaccines_Strategic_Blueprint_draft_Oct2011_nov11.pdf). TB vaccine development is difficult, as there is still no known immune correlate of protection. In 2012, there were 13 candidate TB vaccines undergoing clinical trials. In the 5 years that followed, progress of these candidates through the TB vaccine pipeline slowed or failed altogether, with very few preclinical candidates emerging (89). This impeded progress resulted in the current pipeline of 12 TB vaccine candidates that are currently in phase 1 to 3 clinical trials (Fig. 3). This pipeline consists of a variety of delivery methods (protein/adjuvant, attenuated/killed cells or cell extract, and viral vectors), but the range of antigens and vaccine technologies is actually quite narrow; most rely on eliciting a strong T-helper 1 (Th1) and cell-mediated immune response, known to be important in anti-TB immunity, based on the attenuated *M. tuberculosis* vaccine strain (BCG).

Approaches that focus on ESX-1 and other type VII secretion systems include live, attenuated MTBVAC (90) and VPM1002 (91) and the protein subunit vaccines ID93+GLA-SE (92), H4:IC31 (93), H56:IC31 (94), and M72+AS01E (95). ID93+GLA-SE, H4:IC31, and H56:IC31 have ESAT-6 as one of the antigens secreted by ESX-1, while M72+AS01E has PPE18 secreted by ESX-5 (92–95). MTBVAC was developed from an attenuated *M. tuberculosis* clinical isolate that retained most of the discovered *M. tuberculosis* T-cell epitopes, including the

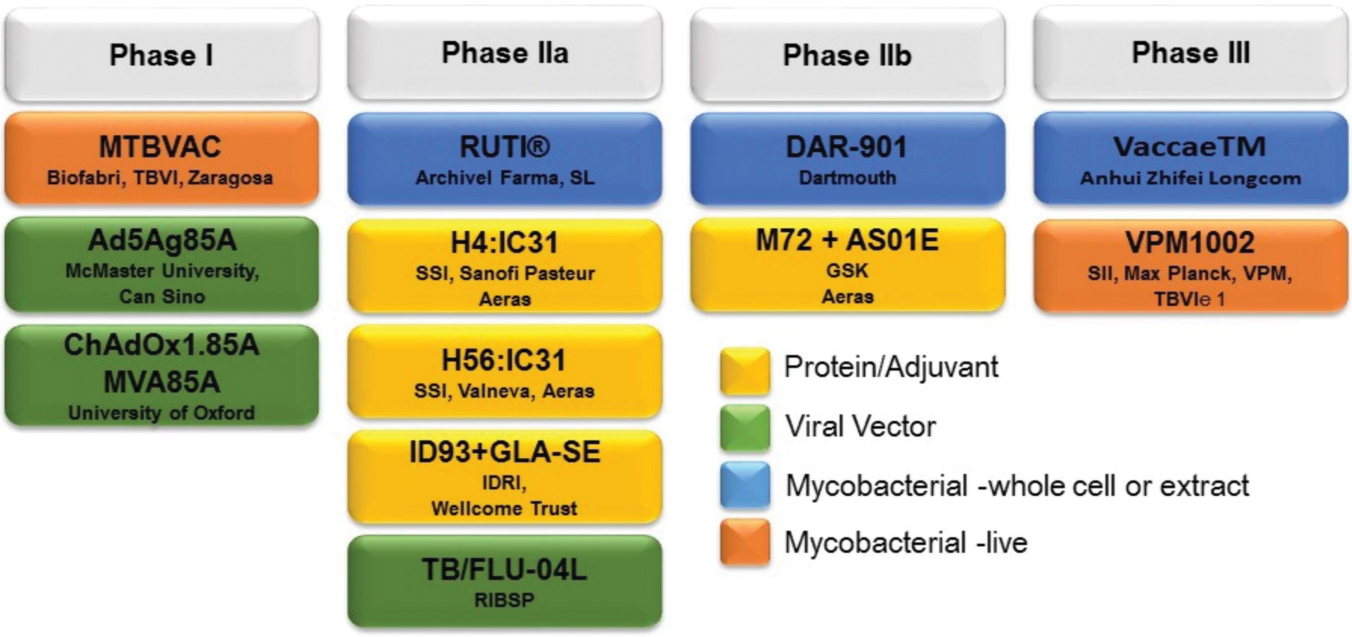

FIGURE 3 TB vaccines in the pipeline, undergoing phase 1 to 3 clinical trials. Current vaccine candidates in the pipeline include protein/adjuvant-based, attenuated/killed or cell extract-based, and viral vector-based vaccines.

immunodominant antigens EsxA and EsxB of the RD1 region deleted from BCG (Fig. 1A). MTBVAC has entered clinical trials as a preventive vaccine in newborns, adolescents, and adults. It is believed that by targeting the virulence-specific epitopes missing from the BCG vaccine, MTBVAC might afford better protection against TB in human hosts (96). VPM1002 is a recombinant BCG strain with the urease gene (*ureC*) replaced by the listeriolysin O gene *hly* from *L. monocytogenes* (91). In phase II clinical trials, VPM1002 afforded safety and immunogenicity to newborn infants as well as adults. Furthermore, the incidence of abscess formation was lower with VPM1002 than BCG (97).

However, it may be possible that *M. tuberculosis* whole-cell vaccines alone cannot confer the desired level of protection and need to be combined with novel approaches involved in enhancing host immune response, such as that taken in the development of the ID93+GLA-SE and M72+AS01E vaccine candidates. ID93+GLA-SE vaccine was rationally designed as a fusion protein of four immunogenic protein targets that are associated with either virulence (Rv3619, Rv3620, and Rv2608) or latency (Rv1813) (92, 98). Rv3619 and Rv3620 are the ESX-5 ESX protein pair (ESXM/N) paralogs called EsxV and EsxW and are uniquely expressed by *M. tuberculosis*, not *M. bovis* or BCG, while Rv2608 (PPE42) and Rv1813 are common to *M. tuberculosis*, *M. bovis*, and BCG (99–101). The immunogenicity of this vaccine candidate has been boosted by using a Toll-like receptor 4 agonist as an adjuvant, resulting in the induction of a humoral response with a preferential increase in IgG1 and IgG3 subclasses and a Th1-type cellular response (102). Similarly, M72+AS01E includes the antigens Rv1196 (PPE18) and Rv0125, along with the adjuvant AS01E, and showed an efficacy of 54% efficacy in phase 2b trials against *M. tuberculosis* (103). Although this candidate includes an alternative agonist and is designed to promote T cell and antibody responses, we do not know if these are the only correlates that will protect.

There are also questions regarding the role of chemokines in the vaccine response. This subclass of proteins is responsible for recruiting host immune cells and has been largely neglected by TB vaccinologists. Perhaps a better chemokine-centered vaccine could be developed that could halt or contain the spread of *M. tuberculosis* upon initial infection. *M. tuberculosis* uses ESX-1 to spread into the lung interstitium from its initial encounter with the alveolar macrophage (66), in granuloma formation in a human lung tissue model (104), and to modulate the infected macrophage to produce the chemokine fractalkine, which calls in permissive macrophages that can lead to *M. tuberculosis* progression (68). Altering this chemotactic call may switch the immune response, favoring the host. It is therefore possible that

ESX systems will lead the way for novel vaccine development. If so, we need to further understand how they work before we can harness their secretory host-controlling powers.

Moreover, multiple strains have been observed in the same patient (105), with mixed infections being massively underrepresented in the majority of diagnostic methods used today (106). Such incomplete diagnosis is, in itself, a huge issue with regard to curbing the spread of TB, potentially resulting in incorrect treatment regimens and enhanced TB rates, but this topic is beyond the scope of this article. In addition, many of the circulating strains causing TB may be other members of the *M. tuberculosis* complex (107), indicating that we may need to look for vaccines that will also protect against other members of the *M. tuberculosis* complex, including *Mycobacterium africanum*, *M. bovis*, etc.

CONCLUDING REMARKS

Without ESX-1, *M. tuberculosis* is highly attenuated. *M. tuberculosis* uses virulence-associated ESX-1 to lyse membranes, egress through cells and lung tissue, and cause tuberculous disease. We need to understand the exact role of each of the plethora of proteins that ESX-1 employs to manipulate and modulate. We urgently need novel strategies to protect against and prevent *M. tuberculosis* infections. Furthering our understanding of this proteinaceous army could enable us to target specific ESX-1 proteins involved in the hijacking of the host pathways and ultimately halt the spread of disease. Perhaps this will open new avenues leading to the development of novel immunotherapeutic strategies for TB and a variety of other bacterial diseases.

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