Mycobacterial ESX-1 secretion system mediates host cell lysis through bacterium contact-dependent gross membrane disruptions

William H. Conrad[a,1], Morwan M. Osman[a,b,1], Jonathan K. Shanahan[a,c], Frances Chu[d,2], Kevin K. Takaki[a], James Cameron[d,3], Digby Hopkinson-Woolley[e,4], Roland Brosch[f], and Lalita Ramakrishnan[a,d,5]

[a]Department of Medicine, University of Cambridge, Cambridge CB2 0QH, United Kingdom; [b]Molecular and Cellular Biology Graduate Program, University of Washington, Seattle, WA 98105; [c]Wellcome Trust PhD Program in Infection, Immunity, and Inflammation, University of Cambridge, Cambridge CB2 0XY, United Kingdom; [d]Department of Microbiology, University of Washington, Seattle, WA 98105; [e]Department of Biology, Eton College, Windsor SL4 6DW, United Kingdom; and [f]Unit for Integrated Mycobacterial Pathogenomics, Institut Pasteur, 75015 Paris, France

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Mycobacterium tuberculosis **and** ***Mycobacterium marinum*** **are thought to exert virulence, in part, through their ability to lyse host cell membranes. The type VII secretion system ESX-1 [6-kDa early secretory antigenic target (ESAT-6) secretion system 1] is required for both virulence and host cell membrane lysis. Both activities are attributed to the pore-forming activity of the ESX-1–secreted substrate ESAT-6 because multiple studies have reported that recombinant ESAT-6 lyses eukaryotic membranes. We too find ESX-1 of *M. tuberculosis* and *M. marinum* lyses host cell membranes. However, we find that recombinant ESAT-6 does not lyse cell membranes. The lytic activity previously attributed to ESAT-6 is due to residual detergent in the preparations. We report here that ESX-1–dependent cell membrane lysis is contact dependent and accompanied by gross membrane disruptions rather than discrete pores. ESX-1–mediated lysis is also morphologically distinct from the contact-dependent lysis of other bacterial secretion systems. Our findings suggest redirection of research to understand the mechanism of ESX-1–mediated lysis.**

Mycobacterium tuberculosis | *Mycobacterium marinum* | ESAT-6 | ESX-1 secretion system | cell membrane lysis

Tuberculosis is an ancient human disease caused by *Mycobacterium tuberculosis* (Mtb) that continues to be a leading infectious human killer despite the availability of effective chemotherapeutic regimens (1). There has been a decades-long search to better understand and identify the precise mechanisms by which Mtb causes disease. One virulence determinant that has been identified is a highly immunogenic secreted protein called ESAT-6 (6-kDa early secretory antigenic target) (2, 3). ESAT-6 was first identified as a secreted antigen that stimulated T cells (4). Humans and multiple laboratory animal species infected with Mtb uniformly show ESAT-6 reactivity (4–6). ESAT-6's role in virulence received further support when its deletion in *Mycobacterium bovis* reduced virulence in guinea pigs (7).

At the same time as ESAT-6 was being implicated in mycobacterial virulence, parallel work discovered and ascribed a role in virulence to a specialized secretion system now called ESX-1. Comparisons of the genomes of Mtb and of the attenuated vaccine strain *M. bovis* bacillus Calmette–Guérin (BCG) revealed a chromosomal region called region of difference 1 (RD1) that was missing in BCG (8, 9). RD1 was confirmed to play a role in virulence by complementary genetic experiments that showed increased virulence when the region was expressed in BCG and attenuated virulence when it was removed from Mtb (10–12). In silico analysis revealed RD1 to encode ESAT-6 and also part of a previously unidentified secretion system (8, 13). The presence of this secretion system solved the conundrum of how ESAT-6 is secreted despite lacking a canonical signal sequence (13). This newly discovered secretion system was found to contain several other potential secretion substrates, yet it was named ESAT-6 secretion system 1 (ESX-1) (13, 14), highlighting the primary role ascribed to ESAT-6 in mycobacterial pathogenesis (2).

ESAT-6 was first hypothesized to exert virulence by lysing host cell membranes when a transposon insertion mutant in ESAT-6's cotranscribed ESX-1 substrate 10 kDa culture filtrate antigen (CFP-10) was found to have lost cytolytic activity for a cultured pneumocyte cell line (15). When purified ESAT-6 and CFP-10 were examined for disruption of artificial lipid bilayers, it was found that that ESAT-6 but not CFP-10 caused membrane lysis (15). These results were confirmed in a series of reports, all implicating eukaryotic cell membrane lytic activity as ESAT-6's virulence mechanism (16–18). When transposon mutants in the closely related species, *Mycobacterium marinum* (Mm), were screened to identify those defective for hemolysis, all attenuated mutants were within the ESX-1 locus and were defective for ESAT-6 secretion (16). Moreover, the mutants were avirulent and failed to lyse infected macrophages, implicating secreted

Significance

Secreted pore-forming toxins are a common feature of bacterial virulence. *Mycobacterium tuberculosis*, the agent of human tuberculosis, has been reported to possess a pore-forming toxin called 6 kDa early secretory antigenic target (ESAT-6) that is secreted through a specialized secretion system called ESX-1 (ESAT-6 secretion system 1). We report here experiments showing that ESAT-6 does not lyse cells; the lytic activity previously attributed to this secreted protein is due to contaminating detergent in the recombinant protein preparations. Whereas the ESX-1 secretion system does lyse host cell membranes, we find this lysis is dependent on bacterial cell contact with the host membrane and results in tears in the membrane without any pore formation. Understanding the mechanism of this lysis may provide clues to how mycobacteria cause disease.

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[1]W.H.C. and M.M.O. contributed equally to this work.

[2]Present address: Research and Development, InBios International Inc., Seattle, WA 98104.

[3]Present address: Environmental and Fisheries Science Division, National Oceanic and Atmospheric Administration, Seattle, WA 98115.

[4]Present address: Zoology, St. Hilda's College, University of Oxford, Oxford OX4 1DY, United Kingdom.

[5]To whom correspondence should be addressed. Email: lr404@cam.ac.uk.

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ESAT-6 in mediating virulence through host cell cytolysis (16). Meanwhile, purified recombinant ESAT-6 (rESAT-6) was reported to be capable of disrupting liposome membranes (19), lysing red blood cells (RBCs) (18), and killing cultured macrophages (17, 18). Thus, the experimental evidence from both genetic and biochemical approaches firmly supported the idea that ESAT-6 was responsible for ESX-1–mediated bacterial pathogenicity and was due to its cytolytic activity.

The consensus of contemporary research ascribes a critical role for ESX-1–mediated membrane lysis during TB pathogenesis via permeabilization of the mycobacterial phagosome following bacterial entry into host macrophages (20–22). Phagosomal permeabilization has been found to activate host cytosolic sensing pathways that are exploited by the bacteria for growth and virulence (23, 24). By extension, ESAT-6 is held to be directly responsible for phagosomal permeabilization and its downstream pathogenic effects (25, 26).

In this report, we confirm that a functional ESX-1 secretion system is required for host cell membrane lytic activity and virulence. However, we find that ESAT-6 is not sufficient for ESX-1's lysis of host cell membranes. Rather, we find that the various lytic activities attributed to ESAT-6 at neutral pH are the result of a widely used protocol to isolate ESAT-6, which leaves residual detergent in the preparations. We find that detergent-free ESAT-6 preparations can disrupt liposomes under acidic conditions, as previously observed (25, 27, 28). However, this intrinsic low pH-dependent activity of ESAT-6 is not responsible for mycobacterial phagosomal permeabilization in host macrophages.

Results

Mtb ESX-1 Secretion Is Linked to Virulence and Hemolysis. We previously showed that deleting the Mm RD1 orthologous region (*MMAR_5446-MMAR_5455*) results in similar attenuation phenotypes to those reported for Mtb. This strain, Mm−ΔRD1, is attenuated for growth in cultured mammalian macrophages as well as adult and larval zebrafish. The reduced bacterial burdens in the zebrafish are accompanied by increased survival (29, 30). To test whether the Mm and Mtb ESX-1 systems were functionally equivalent, we complemented Mm−ΔRD1 with a cosmid containing either the Mtb ESX-1 locus (Mm−ΔRD1::*rv3861-rv3885*$_{mt}$, referred to as Mm−ΔRD1::WT$_{mt}$) (11) or the Mtb ESX-1 locus bearing a point mutation in the gene encoding ESAT-6 (Mm−ΔRD1::M93T$_{mt}$), which fails to restore virulence in the mouse (31). Similar to the Mtb results, we found that Mm−ΔRD1::WT$_{mt}$ but not Mm−ΔRD1::M93T$_{mt}$ rescued virulence (Fig. 1 *A*–*C*). We observed that Mm−ΔRD1::M93T$_{mt}$ had diminished secretion not only of ESAT-6, but also of CFP-10 (Fig. 1 *D* and *E*), which has been shown to be dependent on ESAT-6 for its secretion in Mtb (32).

ESX-1 function in both Mtb and Mm has been associated with RBC lysis (16, 33). After confirming that wild-type (WT) Mm exhibited similar dose-dependent hemolytic activity for sheep, rabbit, horse, and cow RBCs (Fig. S1), we used sheep RBCs for subsequent experiments. We found that Mm−ΔRD1 was defective for hemolysis (Fig. 1*F*) and furthermore that Mm−ΔRD1::WT$_{mt}$ but not Mm−ΔRD1::M93T$_{mt}$ rescued hemolytic activity (Fig. 1*F*). These findings show the functional equivalence of Mtb and Mm ESX-1 and confirm the requirement of ESX-1 secretion for both virulence and cytolytic activity.

ESX-1–Mediated Hemolysis Is Contact Dependent. Prior work had suggested that ESAT-6 mediated ESX-1 membrane lytic activity through pore-forming activity (15, 18, 28, 34). If so, mycobacterial culture supernatants should produce hemolysis. However, even concentrated culture supernatants from WT bacteria are not hemolytic (Fig. 2*A*). This finding raised the possibility that ESX-1–mediated hemolysis is contact dependent as reported by King et al. (35). Moreover, they showed that only the virulent Mtb strain H37Rv conferred hemolysis; both its avirulent counterpart Mtb H37Ra and BCG were nonhemolytic. At the time of the King et al. (35) publication, it was not known that BCG has the RD1 deletion, which ablates ESX-1 function (8, 9), or that Mtb H37Ra is defective for ESX-1 secretion (36, 37). Reinterpreting the King et al. (35) paper in this new light, our finding that mycobacterial supernatants did not confer lysis strongly suggested to us that ESX-1 mediates exclusively contact-dependent hemolysis without any contact-independent lysis. We tested our hypothesis of contact-dependent hemolysis by placing Mm in direct contact with RBCs or by separating bacteria from RBCs, using the 0.4-μm cell barrier present in commercially available Transwell. Both WT Mm and Mm−ΔRD1::WT$_{mt}$ were capable of hemolysis only when in direct contact with RBCs (Fig. 2*B*). In contrast, *Staphylococcus aureus* retained substantial hemolytic activity when separated by the Transwell barrier, consistent with its known ability to secrete hemolytic pore-forming toxins (38) (Fig. 2*B*). These results demonstrated that the hemolytic activity mediated by both Mtb and Mm ESX-1 requires direct bacterial cell contact with host membranes and accounts for essentially all mycobacterial hemolytic activity (Fig. 2*B*).

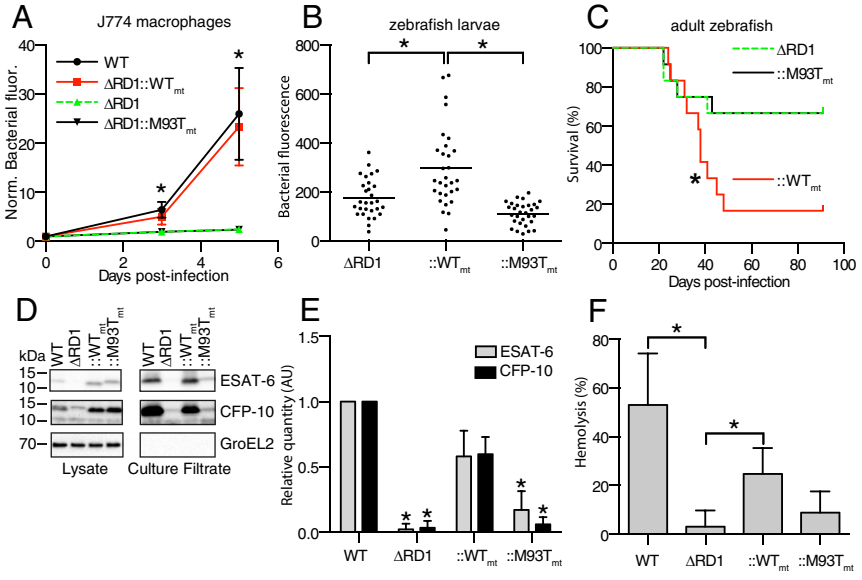

Fig. 1. Mtb ESX-1 secretion is linked to virulence and hemolysis. (*A*) Bacterial growth in the J774 macrophage cell line as assessed by intracellular bacterial fluorescence. $n = 12$ fields per condition. *$P < 0.05$, two-way ANOVA with Dunnett's test. (*B*) Bacterial burdens in 4-day-postinfection (dpi) zebrafish larvae as assessed by bacterial fluorescence. $n = 30$ larvae per condition. *$P < 0.05$, one-way ANOVA with Dunnett's test. (*C*) Survival of adult zebrafish infected with 100 colony-forming units (CFU) of Mm. $n = 12$ fish per condition. *$P < 0.05$, log-rank (Mantel–Cox) test. (*D*) Immunoblot of Mm lysates and culture filtrates, representative of four experimental replicates. (*E*) Protein quantification from *D* by image densitometry, relative to WT culture filtrates. *$P < 0.05$, one-way ANOVA with Dunnett's test relative to ::WT. (*F*) Contact-dependent sheep RBC lysis by 3.0×10^8 CFU Mm. $n = 4$ experimental replicates. (*E* and *F*) *$P < 0.05$, one-way ANOVA with Dunnett's test relative to ΔRD1. Error bars, SD.

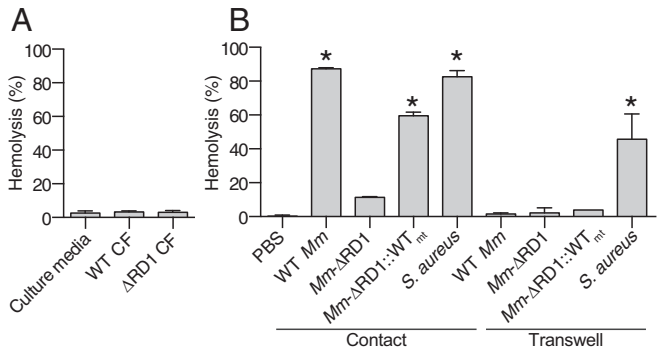

Fig. 2. ESX-1–dependent hemolysis requires direct contact. (*A*) Hemolysis following addition of culture filtrate from WT Mm, Mm–ΔRD1, or uninoculated media. $n = 6$ experimental replicates. (*B*) Hemolysis following addition of the indicated bacterial strains, either in direct contact with RBCs or separated by a Transwell. $n = 3$ experimental replicates. $^*P < 0.05$, one-way ANOVA with Dunnett's test relative to ΔRD1 (contact). Error bars, SD.

ESX-1–Dependent Hemolysis Is Accompanied by Membrane Disruptions at Points of Bacterial Contact Without Apparent Pore Formation or Host Membrane Penetrating Bacterial Structures. We assessed the morphology of RBC lysis by transmission electron microscopy (TEM). Consistent with our findings in the hemolysis assay, the total number of RBCs was reduced and RBC ghosts were more abundant following incubation with WT Mm (Fig. 3 *A*–*C*). Higher-magnification views revealed gross RBC membrane disruptions only in WT Mm samples; the few RBC ghosts seen in the Mm–ΔRD1 condition did not have any membrane disruptions (Fig. 3 *D*–*F*). WT membrane disruptions were predominantly at regions of bacterial contact with RBC membranes (11/12 or 91%) (Fig. 3*D*). Significantly more disruptions were observed at the point of contact with WT Mm (Fig. 3*F*). Mm did not induce discrete pores in the RBCs in contrast to what has been observed for hemolysis caused by classical pore-forming toxins, like *S. aureus* α-hemolysin (Hla) (39) and *Clostridium perfringens* necrotic enteritis B-like toxin (NetB) (40) (Fig. S2).

These findings corroborate the hypothesis that ESX-1 mediates contact-dependent membrane disruption and show that host cell lysis is accompanied by gross cell membrane disruptions at points of bacterial contact rather than by pore formation. Accordingly, we looked for morphological similarities to contact-dependent cell lysis mediated by specialized bacterial secretion systems of other bacteria. We did not observe any structures mediating contact between Mm and host cells such as the needle structures or pili that have been observed for the type III secretion-mediated contact-dependent hemolytic activity of *Yersinia enterocolitica* (41) or *Escherichia coli* (42). Moreover, the gross membrane disruptions we observed are in stark contrast to the normal morphology observed for RBC lysis upon contact with *Shigella* (43). Thus, the mycobacterial ESX-1 secretion system appears to mediate lysis through another distinct mechanism.

Most Membrane Lytic Activities Attributed to ESAT-6 Are Due to Residual Detergent in the Preparations. Our findings were contradicted by prior work suggesting that ESAT-6 alone directly lyses membranes by functioning as a secreted pore-forming protein (15, 18, 28, 34). We were puzzled by this discrepancy particularly because we too had previously found that the widely used, commercially prepared recombinant ESAT-6 [from Biodefense and Emerging Infections (BEI) Research Resources Repository] mediated hemolysis and so did the rESAT-6 we prepared using the published protocol (44). Further analysis of the purification protocol revealed that rESAT-6 showed hemolytic activity only when we included the endotoxin removal step—washing of the column with the zwitterionic detergent, ASB-14. When we omitted this wash step, we found the preparation had no hemolytic activity. This result made us wonder whether the lytic activity ascribed to ESAT-6 could be due to residual detergent. We compared the hemolytic activity of rESAT-6 prepared side by side with and without the detergent wash step and found that omitting the detergent wash step consistently yielded non-hemolytic rESAT-6 protein preparations (Fig. 4*A*). While our experiments were in progress, a paper was published that also found that rESAT-6 lysed RBCs only if the preparations were treated with ASB-14 (34). These authors interpreted the data to mean that detergent modifies the structure of ESAT-6, activating its lytic activity. If true, the hemolytic activity should be dependent on both ESAT-6 and detergent and should be abrogated by proteolyzing detergent-treated ESAT-6. However, we found no decrease in hemolytic activity of our rESAT-6 preparations following Proteinase K treatment (Fig. 4*A*). Similarly, Proteinase K treatment also failed to decrease the lytic activity of rESAT-6 obtained from BEI Resources (Fig. 4*B*). However, Proteinase K treatment completely abrogated the lytic activity of the classic secreted pore-forming toxins Hla and pneumolysin (PLY) (Fig. 4 *C* and *D*). In light of these results, we reviewed the ESAT-6 literature and found that publications reporting ESAT-6 cytolytic activity included detergent in the purification as determined from their described methods (Table 1). In contrast, those that did not use the detergent step reported ESAT-6 to be devoid of cytolytic activity (Table 1). Of note, both we and others (34) have found that recombinant CFP-10 preparations (including from BEI Resources) fail to lyse cells despite being subjected to the identical detergent wash step to that for ESAT-6. This result suggests that any residual detergent was removed during the subsequent wash and dialysis steps in the case of CFP-10 but not ESAT-6 because of tighter binding of detergent to the latter

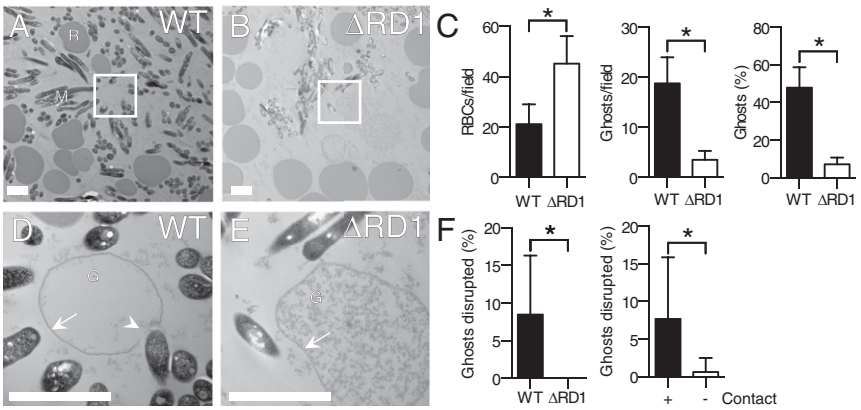

Fig. 3. ESX-1–dependent hemolysis is accompanied by membrane disruptions at points of bacterial contact without apparent pore formation. (*A* and *B*) Transmission electron micrographs of RBC pellets undergoing contact-dependent hemolysis by WT Mm and Mm–ΔRD1, respectively. M, Mm; R, RBCs. Boxes in *A* and *B* indicate the areas magnified in *D* and *E*, respectively. (*C*) Total RBCs, lysed ghosts, and lysed ghosts as percentage of total RBCs per field. $n = 10$ fields. (*D* and *E*) Membrane-disrupted ghost in contact with WT Mm (*D*) and intact ghost in contact with Mm–ΔRD1 (*E*). G, ghost; arrow, intact membrane; arrowhead, disrupted membrane. (*F*) Percentage of WT and Mm–ΔRD1 ghosts disrupted per field (*Left*) and percentage of contact-dependent or -independent WT ghost disruptions per field (*Right*). (Scale bar, 2 μm.) Error bars, SD. $^*P < 0.05$, Student's *t* test.

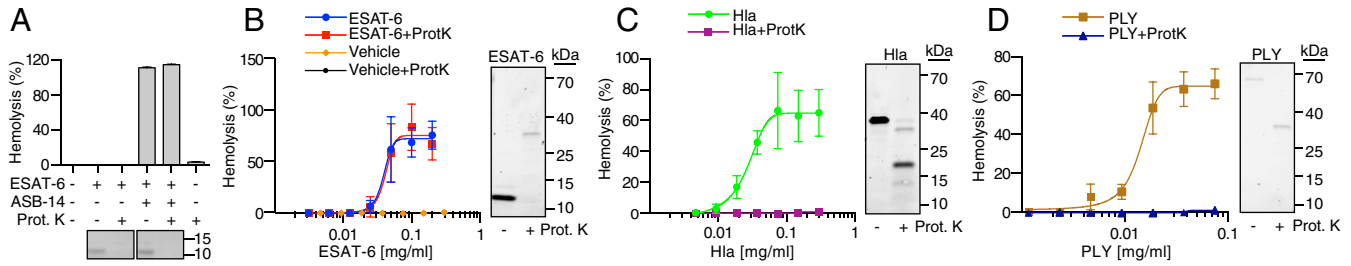

Fig. 4. Recombinant ESAT-6 lyses host membranes via residual contaminating detergent. (*A*) Lysis of RBCs treated with 0.06 mg/mL rESAT-6 prepared with or without ASB-14 and treated with Proteinase K (Prot. K.). $n = 3$ replicates (*Top*) and Coomassie Blue-stained gel of 0.6 μg of corresponding sample (*Bottom*). (*B*) RBC lysis following addition of serially diluted rESAT-6 (BEI) or vehicle ± Prot. K. $n = 3$ experimental replicates (*Left*) and Coomassie Blue-stained gel of 1.2 μg protein sample from each condition (*Right*). (*C* and *D*) RBC lysis following addition of serially diluted *S. aureus* α-hemolysin (Hla, ~38 kDa) (*C, Left*) or *Streptococcus pneumoniae* pneumolysin (PLY, ~70 kDa) (*D, Left*), with Coomassie Blue-stained gels of 1.2 μg protein on *Right* for each panel.

protein. In summary, we conclude that the cytolytic activity attributed to rESAT-6 is due to residual bound detergent.

ESAT-6 Can Disrupt Liposomes at Acidic pH. Whereas we had ruled out that ESAT-6 functions as a lytic protein at neutral pH, detergent-free rESAT-6 is reported to disrupt liposome membranes at pH ≤5 (Table 1) (25). We too found that our detergent-free ESAT-6 preparations lysed liposomes at acidic pH <4.5 (Fig. 5*A*). Also consistent with the prior report (25), rCFP-10 and an rESAT-6/CFP-10 heterodimer prepared from a bicistronic construct did not lyse liposomes (Fig. 5*A*). These findings confirmed that detergent-free ESAT-6 has intrinsic liposome-disrupting activity at acidic pH (25).

ESAT-6's pH-Dependent Membrane Lytic Activity Is Not Required for ESX-1–Mediated Macrophage Phagosomal Permeabilization. ESAT-6's lytic activity at acidic pH is thought to be responsible for ESX-1's ability to permeabilize macrophage phagosomes and allow mycobacteria to access the cytosol (25, 26). If so, then blocking phagosomal acidification with the vacuolar ATPase (vATPase) inhibitor, Bafilomycin (45), should decrease ESX-1–mediated phagosomal permeabilization. To test this, we first confirmed that Bafilomycin inhibited acidification of both WT Mm and Mm−ΔRD1 containing phagosomes in the human macrophage cell line THP1 by staining with LysoTracker, an acidophilic dye that labels lysosomal compartments (46)(Fig. 5 *B* and *C*). Next, we tested the effect of Bafilomycin on mycobacterial phagosomal membrane permeabilization with an assay that uses the fluorescence resonance energy transfer (FRET)-based dye, CCF4-AM (22). CCF4-AM is absorbed into the host cytosol and produces a green fluorescent signal (525 nm) (22). If the mycobacterial phagosome is permeabilized, then the dye is cleaved by the endogenous Mm cell-surface–associated β-lactamase BlaC, which is otherwise inaccessible to the dye. Cleavage causes a loss of FRET and an increase in blue fluorescence (450 nm). We observed similar loss of FRET for WT Mm and Mm−ΔRD1, showing they had similar BlaC activity (Fig. S3). Thus, phagosomal permeabilization can be measured by the shift from green to blue fluorescence within an infected cell (22). We used this dye to measure the phagosome-permeabilizing ability of WT Mm and Mm−ΔRD1 following treatment with Bafilomycin or vehicle. We confirmed prior findings that permeabilization was increased in WT infection compared with Mm−ΔRD1 (22) (Fig. 5 *D* and *E* and Fig. S4). However, we found no reduction in permeabilization in Bafilomycin-treated macrophages (Fig. 5*E*). Thus, acidification is not a prerequisite for permeabilization of the mycobacterial phagosome, suggesting that ESX-1–mediated phagosomal permeabilization does not occur through the acidic pH-dependent membrane lytic activity of ESAT-6. Rather, it appears that ESX-1–mediated phagosomal permeabilization proceeds through the same mechanism as RBC lysis that either is independent of ESAT-6 or requires additional mycobacterial determinants.

Discussion

We show that ESAT-6 does not function as a pore-forming protein or possess intrinsic membrane lytic activity under physiological conditions. We find, however, that ESX-1–mediated cell lysis occurs through a contact-dependent mechanism that causes gross disruptions at points of bacterial contact. Other specialized bacterial secretion systems are known to mediate contact-dependent cytolysis (41, 42). It is tempting to speculate that this might be the case for mycobacteria as well, given a very recent study suggesting that the ESX-1 substrate EspC may form a surface-exposed filamentous structure spanning the mycobacterial cell envelope (47, 48). The ESX-1–mediated membrane disruptions we observe in RBCs look similar to the ESX-1–mediated phagosomal membrane degradation of infected myeloid cells observed by cryoelectron microscopy (20, 21), suggesting that it is relevant to mycobacterial pathogenesis. The intracellular pathogen *Rickettsia* also disrupts phagosomal membranes, and it does so via phospholipase A2 (49) in a manner that appears similar to these ESX-1–mediated membrane disruptions. Whereas mycobacterial phospholipases are not involved in Mtb's phagosomal permeabilization (50), some hemolytic activity is attributed to a mycobacterial sphingomyelinase (33). In addition, a host phospholipase A2 may be involved in phagosomal permeabilization (51). Such findings leave open the possibility that ESX-1 regulates the activities of bacterial and host lipolytic enzymes to disrupt membranes. Alternatively, ESX-1–mediated lysis may occur through a secreted component (including ESAT-6) that needs to be activated by a mycobacterial (or even host) membrane component. Such a scenario would make lysis through a secreted lysin contact dependent and is exemplified in the case of *Serratia marcescens* hemolysin ShlA (52). ShlA is secreted and activated by its partner ShlB only in the presence of

Table 1. Literature reports of recombinant ESAT-6–mediated lysis parsed by detergent wash step use in the preparation

	Detergent used		Detergent not used	
Phenomenon tested	Effect	No effect	Effect	No effect
Lipid bilayer disruption	1*	0	0	0
Host cell lysis	6†	2‡	0	3§
Liposome disruption	0	0	4¶	0

Papers that reported recombinant ESAT-6–mediated lysis or membrane disruption were found through a PubMed Search. Whether detergent was used in the ESAT-6 preparation was determined from the *Materials and Methods* section in the case of ESAT-6 prepared in house or from the manufacturer's website if it was purchased.

*Ref. 15.

†Refs. 17, 18, 34, 60, 63, and 64.

‡Refs. 65 and 66.

§Refs. 34, 59, and 67.

¶Refs. 19, 25, 27, and 28.

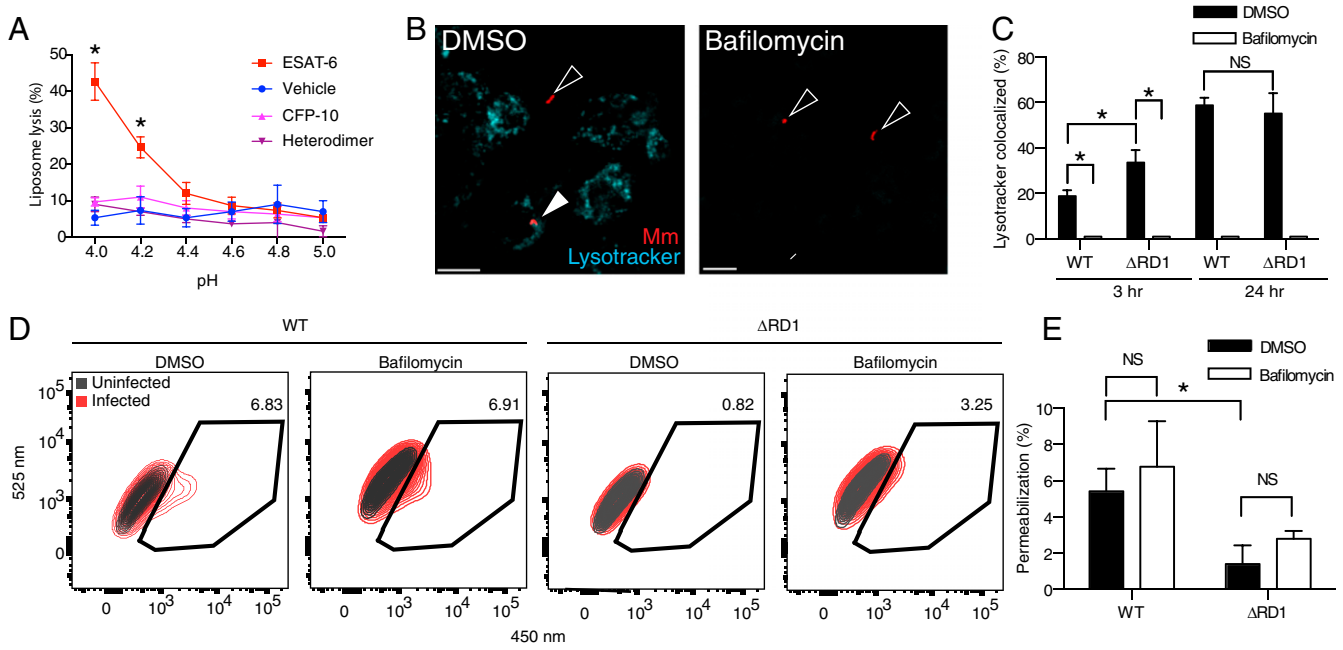

Fig. 5. Recombinant ESAT-6 lyses membranes at acidic pH, whereas mycobacterial ESX-1–mediated lysis proceeds at neutral pH. (*A*) Quantification of pH-dependent liposome lysis by recombinant ESAT-6, but not CFP-10 or ESAT-6/CFP-10 heterodimer as measured by fluorescent ANTS release from DOPC liposomes. $*P < 0.05$, two-way ANOVA with Dunnett's multiple comparisons. (*B*) Representative images of THP-1 macrophages infected with WT Mm 6 h after treatment. (Scale bar, 20 μm.) (*C*) Quantification of colocalization of WT Mm and Mm–ΔRD1 with acidified compartments. $n = 3$ technical replicates. $*P < 0.05$, Mann–Whitney *U* test. (*D*) Flow cytometry of WT- or ΔRD1-infected THP-1 macrophages. Gate highlights permeabilization events in live, infected macrophages. (*E*) Quantification of permeabilization events. $n = 3$ experimental replicates. Error bars, SD. $*P < 0.05$, one-way ANOVA with Tukey's multiple-comparisons test.

phosphatidylethanolamine, a lipid commonly found in biological membranes (52).

Our experiments raise the question of what precise role ESAT-6 plays in mycobacterial virulence. Prior reports that ESAT-6 secretion-defective mutants are defective for host cell lysis (15, 16, 53) are confounded by the finding that several ESX-1 substrates are mutually codependent for secretion (54, 55). However, because it is an immunodominant antigen, it is likely to have a role in virulence. Perhaps it acts downstream of ESX-1–mediated membrane permeabilization to directly influence pathogenesis by regulating immune determinants (56–58). The finding that ESAT-6 binds to liposomal membranes may reflect its ability to bind lipids and thereby eukaryotic cell membranes (19, 59) to initiate or subvert the requisite cell signaling events. In such a scenario, ESX-1–mediated phagosomal permeabilization would enable ESAT-6 to gain contact with the cytosol of the infected macrophage or even neighboring cells to influence their immune program.

Our quest to confirm and expand the model of ESAT-6 as a cytolytic, pore-forming toxin took an unexpected turn when a serendipitous omission in a well-defined and widely used purification protocol led us to reexamine the biological function that had been widely attributed to this mycobacterial virulence determinant. Our experiments led us to reinterpret ESX-1's membrane lytic activity. We hope our findings will lead to a better understanding of this and of ESAT-6's biological function.

Materials and Methods

The materials and methods used are detailed at length in *SI Materials and Methods*. Mycobacterial strains, plasmids, hemolysis and liposome lysis assays, zebrafish and macrophage infections, electron microscopy, production of recombinant proteins and culture filtrates, and the phagosomal permeabilization assay are described therein. Plasmids and mycobacterial strains used are listed in Table S1 and S2.

Zebrafish husbandry and experiments were in compliance with guidelines from the UK Home Office and the US National Institutes of Health and approved by the University of Washington Institutional Animal Care and Use Committee.

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