Retention of EsxA in the Capsule-Like Layer of Mycobacterium tuberculosis Is Associated with Cytotoxicity and Is Counteracted by Lung Surfactant

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BACTERIAL INFECTIONS

Retention of EsxA in the Capsule-Like Layer of *Mycobacterium tuberculosis* Is Associated with Cytotoxicity and Is Counteracted by Lung Surfactant

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ABSTRACT *Mycobacterium tuberculosis*, the pathogen that causes tuberculosis, primarily infects macrophages but withstands the host cell's bactericidal effects. EsxA, also called virulence factor 6-kDa early secretory antigenic target (ESAT-6), is involved in phagosomal rupture and cell death. We provide confocal and electron microscopy data showing that *M. tuberculosis* bacteria grown without detergent retain EsxA on their surface. Lung surfactant has detergent-like properties and effectively strips off this surface-associated EsxA, which advocates a novel mechanism of lung surfactant-mediated defense against pathogens. Upon challenge of human macrophages with these *M. tuberculosis* bacilli, the amount of surface-associated EsxA rapidly declines in a phagocytosis-independent manner. Furthermore, *M. tuberculosis* bacteria cultivated under exclusion of detergent exert potent cytotoxic activity associated with bacterial growth. Together, this study suggests that the surface retention of EsxA contributes to the cytotoxicity of *M. tuberculosis* and highlights how cultivation conditions affect the experimental outcome.

KEYWORDS ESAT-6, EsxA, *Mycobacterium tuberculosis*, cytotoxicity, human macrophage, lung surfactant

Mycobacterium tuberculosis is the causative agent of tuberculosis. One of the most prominent virulence factors of *M. tuberculosis* is EsxA, which is secreted through the type VII secretion system ESX-1 and exerts membrane-lysing activity (1–3), enabling the escape of *M. tuberculosis* from phagosomes (4, 5). Although the molecular mechanisms of EsxA secretion and its role in pathogenesis have been extensively studied, the subcellular localization during macrophage infection has not been determined.

EsxA and other ESX-1-secreted proteins have been found in the mycobacterial capsule (6), a loose noncovalently attached layer surrounding the cell wall (7). Predominantly consisting of polysaccharides, the capsule is shed by *M. tuberculosis* under culture conditions that include detergents, which is the case in most studies (6). *M. tuberculosis* grown without detergent has been described to have a distinct phenotype, exhibiting pronounced characteristics regarding the MICs of antibiotics (8), drug tolerance (9), and gene expression (10).

We show here that cultivation of *M. tuberculosis* in detergent-free broth causes retention of EsxA on the bacterial surface, resulting in a phenotype of *M. tuberculosis* that rapidly induces macrophage cell death. Similarly, lung surfactant, which has detergent-like properties, removes EsxA from the bacterial surface, suggesting a novel role for lung surfactant in antimycobacterial defense.

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RESULTS

***M. tuberculosis* bacteria cultivated without detergent retain EsxA on their surface.** Omitting detergent from the broth to preserve the capsule-like layer (6), we observed that EsxA accumulated at the bacterial surface after 3 to 6 days of incubation (Fig. 1A and B). EsxA could not be detected when detergent was used in the broth, on EsxA-deficient *M. tuberculosis* cultivated without detergent, or in the control staining without primary antibody (Fig. 1A). The effect was more pronounced on bacterial aggregates consisting of two or more bacteria (the prevailing morphological structure in detergent-free cultures) (Fig. 1B). Immunogold labeling of EsxA followed by transmission electron microscopy (TEM) confirmed the presence of EsxA on the bacterial surface, while it was absent from the EsxA-deficient mutant and Tween 80 broth-cultivated wild-type bacteria (Fig. 1C and D).

Next, we hypothesized that the surface retention of EsxA would be affected in *M. tuberculosis* during exposure to lung surfactant, which consists of amphiphilic phospholipids having properties similar to those of detergent. To test this hypothesis, we added Curosurf, a preparation of porcine surfactant for human use, to the culture broth. Curosurf showed a dose-response relationship with EsxA staining on the bacterial surface, with significant inhibition being obtained with 1% Curosurf (Fig. 1E). This shows that lung surfactant strips off EsxA, suggesting a new role of surfactant for defense against *M. tuberculosis* and other capsulated bacteria affecting the lungs.

***M. tuberculosis* bacteria cultivated without detergent exhibit higher cytotoxicity.** We have previously established the correlation between intracellular *M. tuberculosis* growth rates and host cell viability (11) and hypothesized that the accumulation of EsxA on the *M. tuberculosis* surface impacts macrophage viability. Indeed, *M. tuberculosis* bacteria cultivated without Tween 80 reduced macrophage viability by 80% compared to that of uninfected cells within 24 h, coinciding with a 2.8-fold increase in bacterial numbers. On the contrary, *M. tuberculosis* grown with Tween 80 did not affect cell viability during the first 24 h of incubation, and bacterial replication was controlled by the cells during this time period. The EsxA-deficient strain cultivated without Tween 80 did not cause cell death (Fig. 2A).

EsxA is lost from the bacterial surface upon contact with cell membranes. To our knowledge, our work is the first demonstration of immunolabeling of EsxA in the capsule-like layer of *M. tuberculosis*. Using this tool, we next investigated how EsxA is redistributed upon contact with macrophages. The starting time point of infection was synchronized by centrifugation, and at the initial time points, all bacteria were either free (between cells, without contact with any cellular or actin structures) or cell associated, as defined by evaluation of cellular structures by F-actin staining and in the differential interference contrast (DIC) images. During the course of the infection, the numbers of free bacteria decreased as the amount of cell-associated *M. tuberculosis* increased (Fig. 2B). Coinciding with the internalization of the bacteria, we observed a significant drop in EsxA positivity in the cell-associated fraction of bacteria after 30 min of incubation ($P < 0.001$), and this was even more pronounced at the later time points, whereas EsxA positivity did not change in the free bacteria (Fig. 2B and C). After infection with the EsxA-deficient mutant cultivated without Tween 80, we could not observe any EsxA staining (Fig. 2B). Furthermore, we could not observe EsxA in any cellular organelles or structures at any time point. To rule out the possibility that the EsxA antibody did not have sufficient access to intracellular bacteria, we carried out a control staining following the same procedure with an antibody directed against LAM on the mycobacterial surface. This revealed that antibodies indeed have sufficient access to intracellular *M. tuberculosis* (see Fig. S1 in the supplemental material), suggesting that the decline in EsxA staining observed upon attachment and internalization is not a technical artifact. Furthermore, intracellular bacteria could encounter conditions with a lowered pH, leading to a conformational change of EsxA (1), but exposing *M. tuberculosis* to pH 4 and 5 did not impede the detection of EsxA in our system (data not shown). To assess whether EsxA reappears on the surface of replicating *M. tuberculosis* or somewhere within the host cell, we extended the infection time to up to 4 days but

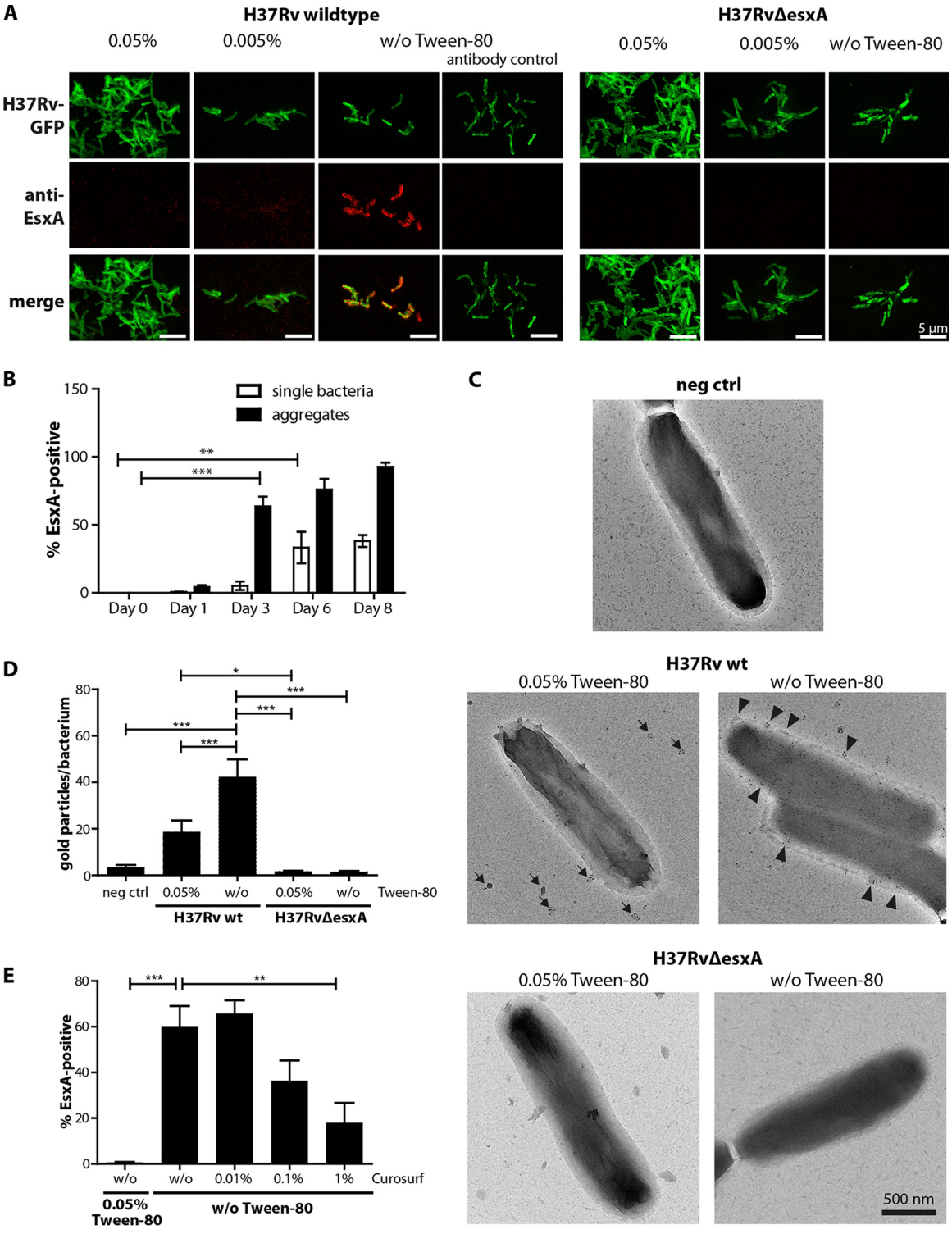

FIG 1 EsxA can be detected on the surface of *M. tuberculosis* after cultivation in the absence of detergent or surfactant. (A and B) *M. tuberculosis* H37Rv wild type (left) or the EsxA-deleted strain H37Rv Δ*esxA* (right) was cultivated with or without Tween 80 and for the indicated times. Fixed bacteria were stained with anti-EsxA antibody and an Alexa Fluor 594-conjugated secondary antibody. The images in panel A are from bacteria cultivated for 6 days. In the samples for the antibody control images (fourth column), the anti-EsxA antibody was omitted. Images were obtained using a 100× (numerical aperture, 1.45) objective. Bars, 5 μm. (B) EsxA-positive single bacteria or aggregates (≥2 bacteria) were expressed as a percentage of all single bacteria or aggregates. Day 0 indicates the initial *M. tuberculosis* stock culture which contained 0.05% Tween 80. Bars and error bars depict means and SEMs from three independent experiments (on average, 79 bacteria or aggregates were analyzed per sample; range, 17 to 441). Significant differences between the time points were tested with 2-way analysis of variance comparing all time points to the day 0 time point using the Bonferroni *post hoc* test for multiple comparisons, and the earliest significant time point for each group is indicated by asterisks. (C and D) *M. tuberculosis* H37Rv or H37Rv

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could not detect any EsxA at the later time points, when bacterial replication and macrophage cell death were obvious (data not shown).

In order to indirectly target EsxA by antibody staining or via its binding partner, EsxB, we created plasmids for the overexpression of hemagglutinin (HA)-tagged EsxA and EsxB. *M. tuberculosis* H37Rv transformed with the plasmids for EsxA-HA or EsxB-HA expressed both the fusion proteins, but only EsxA-HA was successfully secreted into the culture supernatant (Fig. 3A). Cultivation of *M. tuberculosis* expressing EsxA-HA without detergent followed by HA antibody staining reproduced the EsxA staining pattern (Fig. 3B). In line with the previous observation, EsxA-HA staining also declined as the *M. tuberculosis* bacteria were internalized by macrophages (Fig. 3C and D).

Finally, to assess whether the decline in EsxA staining on the *M. tuberculosis* surface is dependent on phagocytosis, we treated macrophages with cytochalasin D, a phagocytosis inhibitor. Evaluating free bacteria without any cellular contact (Fig. 4A) and bacteria residing on the macrophage surface (Fig. 4B), we observed that the proportion of free bacteria positive for EsxA did not change during 120 min of infection (Fig. 4C). In contrast, the percentage of EsxA-positive, cell-associated bacteria decreased over time, and as early as 5 min after infection we observed a significant difference in EsxA staining between cell-associated and free bacteria (Fig. 4C). To exclude the possibility that the saponin treatment used in the staining protocol extracted the cell membrane, including EsxA, the saponin permeabilization step was omitted in cytochalasin D-treated macrophages; however, this did not affect the result (data not shown). Taken together, we found that the decline in EsxA staining on *M. tuberculosis* associated with macrophages was independent of phagocytosis, as it still decreased when phagocytosis was inhibited.

DISCUSSION

In this study, we demonstrate that by exclusion of detergent from broth cultures of *M. tuberculosis*, the secreted virulence factor EsxA is retained on the bacterial surface, which correlates with enhanced cytotoxicity. In most studies, *M. tuberculosis* is cultured in detergent-containing broth to avoid aggregation of the bacilli. *M. tuberculosis* grown without detergent has been described to have a distinct phenotype, exhibiting pronounced characteristics regarding the MICs of antibiotics (8), drug tolerance (9), and gene expression (10). Furthermore, the presence of a mycobacterial capsule containing ESX-1-secreted substrates has been shown on *M. tuberculosis* cultivated without detergents (6), and EsxA has been suggested to be present in surface extracts of *M. marinum* grown on agar plates (12). In line with the findings of our study, very low levels of EsxA on the surface of *M. tuberculosis* cultivated with Tween 80 have been demonstrated (13). However, the heterodimerization partner of EsxA, EsxB (also known as culture filtrate protein 10 [CFP-10]), was associated with the bacterial surface, despite the presence of detergent in the broth (13). The present study was limited to the investigation of EsxA.

To our knowledge, the localization of EsxA on the surface of *M. tuberculosis* or inside macrophages has not been previously demonstrated. A change in cultivation conditions revealed the retention of EsxA on the *M. tuberculosis* surface when cultivated without detergent, as demonstrated by immunolabeling of EsxA. Reinoculating the bacteria from detergent-containing broth to detergent-free broth, EsxA was detectable

FIG 1 Legend (Continued)
Δ*esxA* was fixed after 6 days of cultivation with or without Tween 80, followed by immunogold labeling for EsxA and TEM analysis. (C) Representative images are shown. Arrows, EsxA-positive debris; arrowheads, immunogold particles in the bacterial cell wall. The negative control (neg ctrl) was the *M. tuberculosis* wild type (wt) grown without detergent, where the EsxA antibody was omitted during the labeling procedure. (D) The amount of immunogold particles per bacterium was quantified from the TEM pictures in a blind fashion. Bars and error bars show means and SEMs for 30 to 54 bacteria per sample. Significant differences were tested with a 1-way analysis of variance, followed by Tukey's *post hoc* test comparing all groups. (E) The *M. tuberculosis* H37Rv wild type was cultured in broth containing 0.05% Tween 80 or without detergents and increasing amounts of the bovine lung surfactant Curosurf. Bacteria were fixed, stained, imaged, and analyzed as described in the legend to panels A and B. Bars and error bars show means and SEMs from 5 experiments (on average, 106 bacteria were analyzed per sample; range, 38 to 349). Significant differences were tested with a 1-way analysis of variance, followed by Dunnett's *post hoc* test, comparing all groups to the sample without both Tween 80 and Curosurf. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

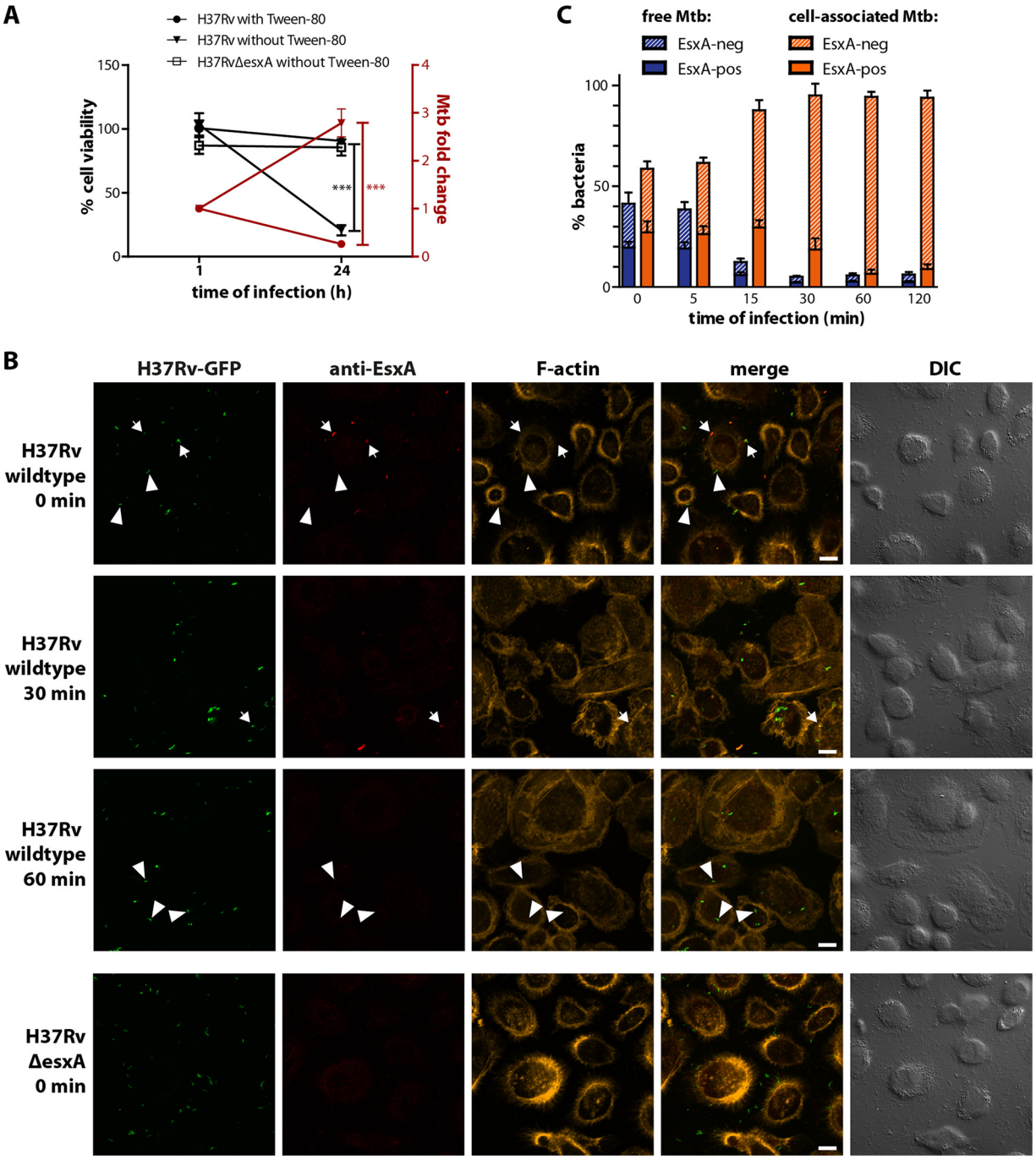

FIG 2 Superficial EsxA leads to a rapid decrease in macrophage cell viability and is lost from *M. tuberculosis* (Mtb) during cell interaction. (A) Human monocyte-derived macrophages (hMDMs) were infected with luciferase-expressing *M. tuberculosis* cultivated with (0.05%) or without Tween 80 and with H37Rv Δ*esxA* cultivated without Tween 80. Cell viability was measured 1 and 24 h after infection using calcein-AM (black symbols), and the bacterial numbers of the wild-type strain were determined using a luminometry-based method (red symbols). The percentage of the calcein-AM signal (compared to the signal for uninfected cells from the same time point; left axis) and the fold change in bacterial numbers (compared to the number at the 1-h time point; right axis) are shown as means and SEMs from four experiments. Significant differences in bacterial growth and cell viability are indicated by asterisks and were tested with 2-way analysis of variance and Bonferroni's *post hoc* test. (B) hMDMs were infected for the indicated times with GFP-expressing *M. tuberculosis* H37Rv that had been cultivated without detergent and subsequently fixed and stained for EsxA (red) and F-actin (orange). Representative confocal and differential interference contrast (DIC) images are shown. Quantification was done by evaluating the EsxA staining intensity, and the respective bacteria were classified as free bacteria (not associated with any actin or cellular structures) or cell-associated bacteria, based on the F-actin staining and DIC images. The arrows and arrowheads depict *M. tuberculosis* bacteria classified as cell associated, with the arrows pointing at EsxA-positive bacteria and the arrowheads pointing at EsxA-negative bacteria. Parallel samples infected with EsxA-deficient *M. tuberculosis* served as background controls (fourth row). (C) The bacteria in confocal images were quantified as EsxA positive (filled symbols) or negative (striped symbols) and with regard to their local distribution (free or cell associated). Bars and error bars show means and SEMs from 5 experiments (on average, 147 bacteria were analyzed per sample; range, 62 to 241). Changes in the proportion of EsxA-positive bacteria as a ratio of the number of total bacteria within the respective

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after 3 days on bacterial aggregates, the predominant morphological structure of *M. tuberculosis* in detergent-free cultures.

During infection of human macrophages, EsxA staining of cell-associated *M. tuberculosis* declined significantly. We could rule out the possibility that this was due to insufficient access of the antibody to the cell-associated *M. tuberculosis* or due to a conformational change upon encounter of a low pH, described earlier for EsxA (1). The antibody used in this study targets amino acids at the N terminus of EsxA (14), a short stretch that was described to protrude from the EsxA-EsxB heterodimer (15) and that is unlikely to directly interact with membranes (16). Still, the N terminus could have become inaccessible for antibody staining due to other reasons. Targeting the C terminus for immunolabeling by using an *M. tuberculosis* strain secreting EsxA with a C-terminal HA tag yielded similar results; also here the HA immunolabeling was lost from most cell-associated bacteria. Finally, we found that the decline in EsxA staining on *M. tuberculosis* associated with macrophages was independent of phagocytosis, as it still decreased when phagocytosis was inhibited. In line with this observation, EsxA has been shown to interact with membranes (1, 3, 16). Therefore, the disappearance of EsxA from *M. tuberculosis* upon cell contact that we observed could represent a translocation of the protein, rendering it inaccessible for antibody staining.

Investigating the cell cytotoxicity of *M. tuberculosis* armored with superficial EsxA revealed that bacteria cultivated in the absence of detergent induced rapid macrophage cell death. This was not due to bacterial aggregation caused by the exclusion of detergent, since the EsxA-deleted strain cultivated without Tween 80, while effectively forming aggregates, did not cause cell death. However, we cannot exclude the possibility that the absence of cytotoxicity of the EsxA-deleted strain was due to the loss of secretion of other ESX-1 substrates that are codependent on the secretion of EsxA (28, 29). Besides that, deletion of EsxA could also lead to alterations in capsular integrity, as demonstrated, for example, for ESX-5 substrates (30), and thereby alter cytotoxicity. EsxA has previously been postulated to have direct membrane-lysing activity (13, 17–19). This was refuted by the finding that contaminating detergent was responsible for membrane lysis (2). However, in the present study, contaminating detergents did not cause the observed cytotoxicity, since potential residual Tween 80 was higher in the noncytotoxic/EsxA-void bacteria. Anyway, our data do not provide evidence from which a putative mechanism for EsxA-mediated cytotoxicity can be derived. The mechanism for membrane damage of EsxA seems to involve contact of the bacterial surface with the host cell membrane rather than a pore-forming mechanism (2), and this aspect of tight contact between *M. tuberculosis* and the phagosomal membrane was already described in the 1980s (20). However, establishment of an understanding of the cytotoxic mechanism of EsxA was beyond the scope of this study.

The physiological relevance of the cytotoxic *M. tuberculosis* phenotype carrying abundant EsxA on its surface is unclear; however, we demonstrate a possible role for surfactant in stripping off surface-bound EsxA. Further studies involving human samples are required to determine the phenotype of transmitted bacilli. In any case, our findings and those of others (6, 10, 21) highlight the importance of careful evaluation of cultivation conditions for mycobacteria for the generation of physiologically relevant results.

MATERIALS AND METHODS

Bacterial strains. The *Mycobacterium tuberculosis* strains used in this study (and the antibiotic concentrations used for their selection) were as follows: the H37Rv wild type and the EsxA-deficient strain H37Rv Δ*esxA*, both carrying the pFPV2 plasmid for constitutive green fluorescent protein (GFP) expression (20 μg/ml kanamycin for pFPV2 and 50 μg/ml hygromycin for H37Rv Δ*esxA*) and H37Rv carrying the pSMT1 plasmid for the expression of luciferase (100 μg/ml hygromycin). For cultivation of strains, frozen

FIG 2 Legend (Continued)
population (free and cell associated) were tested using 2-way analysis of variance and Bonferroni's *post hoc* test. Among the extracellular bacteria, the fraction of EsxA-positive bacteria did not change over time, while for cell-associated bacteria we observed a significant drop after 30, 60, and 120 min of infection compared to that at the 0-min time point ($P < 0.001$).

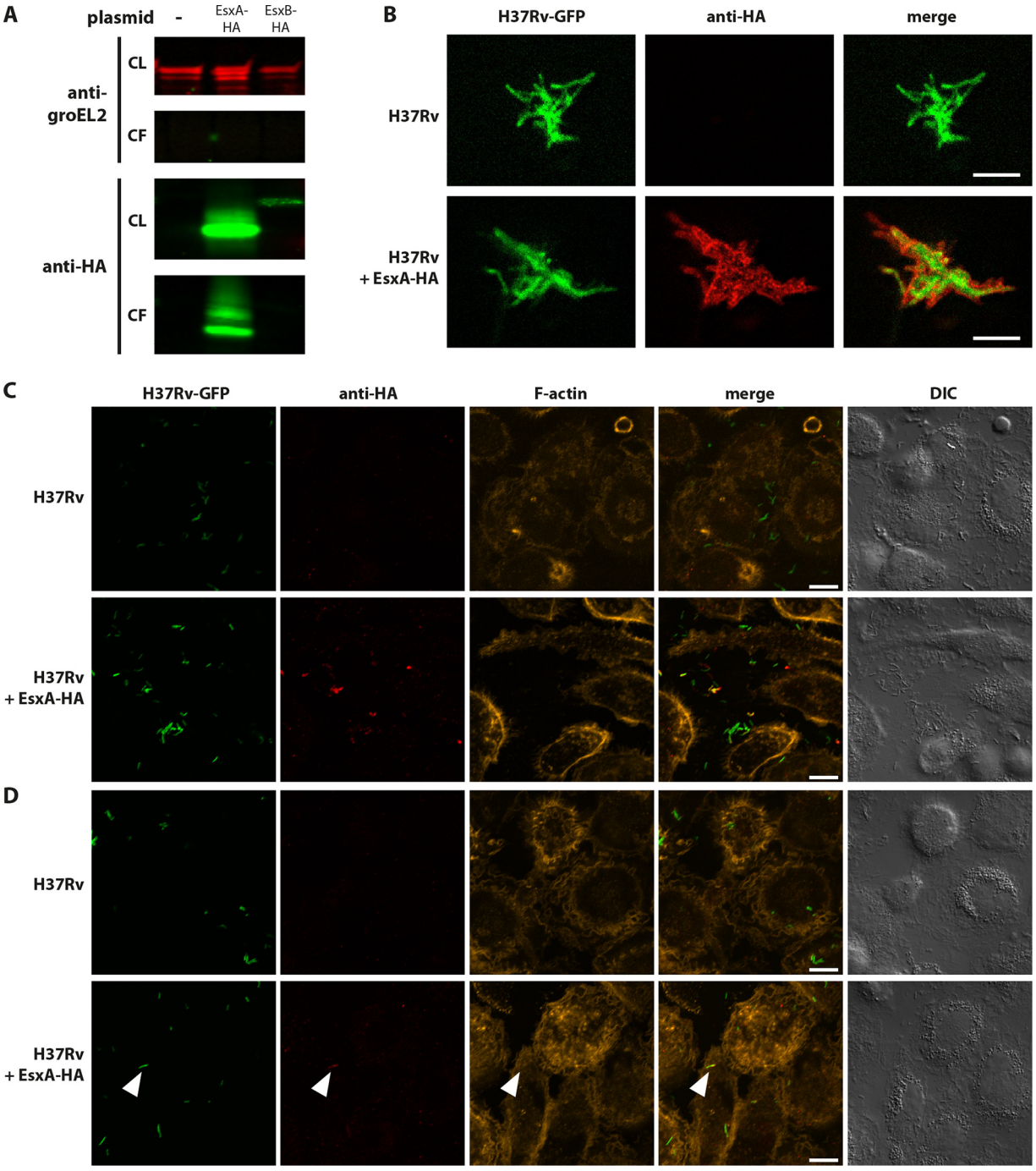

FIG 3 Secretion of overexpressed HA-tagged EsxA and detection on *M. tuberculosis* grown without detergent and during macrophage infection. (A) *M. tuberculosis* carrying a plasmid for the expression of HA-tagged EsxA or EsxB (or no plasmid) was cultivated in Sauton's broth. The culture lysate (CL) and culture filtrate (CF) were analyzed for expression and secretion of tagged fusion proteins by Western blotting with an HA-tagged antibody. GroEL2 was included as a control for bacterial autolysis. (B) GFP-expressing *M. tuberculosis* H37Rv and H37Rv EsxA-HA carrying a plasmid for the expression of HA-tagged EsxA were cultivated without Tween 80 for 6 days and then immobilized on glass coverslips, fixed, and immunostained with an anti-HA-tag antibody and a secondary antibody. (C and D) hMDMs were infected with GFP-expressing *M. tuberculosis* H37Rv or H37Rv EsxA-HA grown in the absence of Tween 80. Samples were fixed at the initial time point (C) and after 30 min of incubation (D) and stained with anti-HA-tag antibody, a secondary antibody, and Alexa Fluor 546-phalloidin for the visualization of F-actin. EsxA-HA staining was detected at the beginning of the infection (C), whereas it was lost after 30 min of incubation (D). The arrowhead depicts an EsxA-HA-positive, cell-associated bacterium, while all other bacteria did not exhibit EsxA staining. Bars, 5 μm (B) and 10 μm (C, D).

stocks were thawed in 7H9 Middlebrook broth supplemented with 0.2% glycerol, albumin-dextrose-catalase (ADC; BD), 0.05% Tween 80, and antibiotics and incubated for 2 to 3 weeks. These stock cultures were then reseeded into the same broth with 0.05% or 0.005% Tween 80, without Tween 80, or with different concentrations of Curosurf (Chiesi Farmaceutici, Italy) 6 days before the experiments (except for

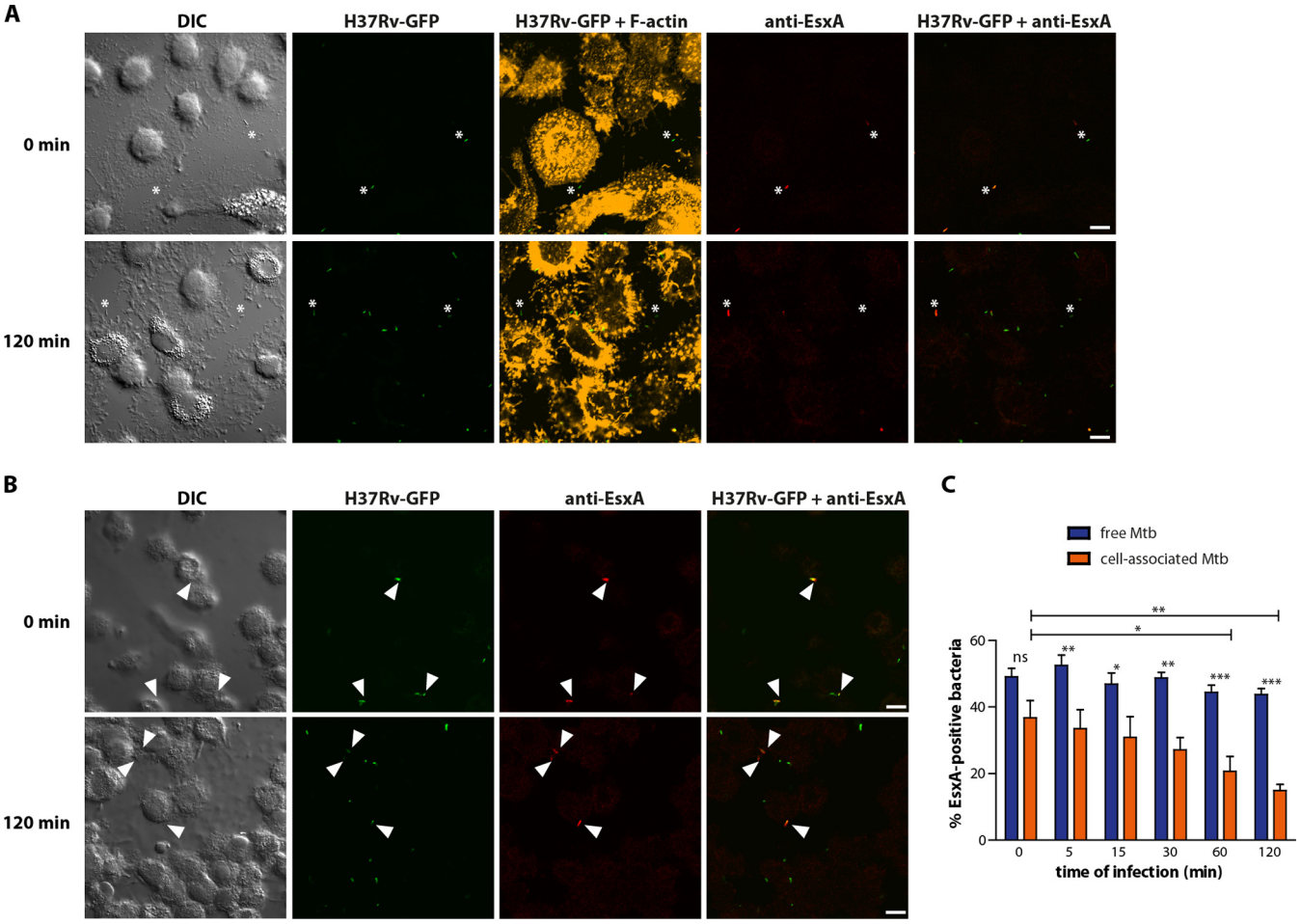

FIG 4 The loss of EsxA from the surface of *M. tuberculosis* (Mtb) during contact with host cell membranes occurs independently of phagocytosis. hMDMs were treated with 10 μM cytochalasin D before infection with the GFP-expressing *M. tuberculosis* H37Rv wild type. Staining was performed in the same way as described in the legend to Fig. 2. (A) To determine the EsxA positivity of free *M. tuberculosis*, images were taken by focusing on the bottom of the sample, detecting free bacteria without any cell contact. This localization was verified by increasing the intensity of the F-actin channel, and only bacteria clearly not colocalizing with F-actin structures or structures observed in differential interference contrast (DIC) images were included in the analysis (here indicated by asterisks). (B) For the analysis of *M. tuberculosis* attached to the cell membrane of macrophages (cell associated) in the same samples, confocal images were taken by focusing on bacteria residing on the surface of the cells. Images were evaluated first by checking the DIC images for localization of the bacteria on cellular structures, before the respective bacteria were analyzed for EsxA positivity. Arrowheads, *M. tuberculosis* positive for EsxA staining. (C) Quantification of EsxA positivity of *M. tuberculosis* residing between cells (free *M. tuberculosis*) and on the surface of cells (cell-associated *M. tuberculosis*). Differences in EsxA positivity between the two groups were tested by 2-way analysis of variance, and changes over time were tested using 2-way analysis of variance with Bonferroni's *post hoc* test, comparing the value at the zero time point to the values at the other time points. All imaging was performed on a confocal microscope using a 63× (numerical aperture, 1.4) objective. For microscopic analyses, bars and error bars represent means and SEMs from 5 independent experiments (on average, 74 bacteria were analyzed per sample; range, 33 to 144). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Bars, 10 μm.

the experiment whose results are presented in Fig. 1B, where samples were also taken at other time points). The carryover of Tween 80 was eliminated by centrifugation of 1 ml of stock culture and resuspension of the pelleted bacteria in 10 ml of fresh broth with 0.05% Tween 80 or without Tween 80.

Generation of *M. tuberculosis* expressing EsxA-HA and EsxB-HA. The HA-tag gene sequence was cloned downstream of the EsxA gene for C-terminal expression but positioned upstream of the EsxB gene for N-terminal tagging. This was done in accordance with findings described in earlier publications showing that addition of a hexahistidine tag at the C terminus of EsxA and at the N terminus of EsxB does not impede the secretion of the fusion protein (15) and that the C terminus of EsxA is not involved in host cell binding, whereas the C terminus of EsxB is essential for binding to the surface of monocytes (22) and for secretion of the EsxA/EsxB complex (23).

To generate vectors allowing for the expression of HA-tagged EsxA or EsxB in *M. tuberculosis*, the sequences for *esxA* (Rv3875) or *esxB* (Rv3874) with a C- or N-terminal HA tag, respectively, were cloned downstream of the Phsp60 promoter into the backbone of the *Escherichia coli-M. tuberculosis* shuttle plasmid pFPV2 carrying a hygromycin resistance gene. Inserts were generated using synthesized DNA oligonucleotides (Sigma-Aldrich) that were designed to yield double-stranded DNA oligonucleotides with complementary 5′ overhangs after annealing. Upon phosphorylation of the 5′ termini using T4

polynucleotide kinase, the oligonucleotides were ligated and amplified by PCR. These inserts were then enzyme digested and ligated with the vector that had been digested with the same enzymes and dephosphorylated. After transformation of *E. coli* DH5α, plasmids were purified using a JetStar plasmid purification minikit, and the correct sequence of the insert was confirmed by sequencing. The different *M. tuberculosis* strains were transformed as described earlier (24). Transformed mycobacteria were plated on 7H10 Middlebrook agar containing ADC and antibiotics (50 μg/ml hygromycin for EsxA-HA and EsxB-HA), and colonies typically appeared after 3 weeks. Colonies were grown in 7H9 Middlebrook broth supplemented as described above. After cultivation for 2 weeks, stocks were frozen at −80°C or the bacteria were reseeded 1:10 into Sauton's broth (for the generation of culture filtrate) or into Middlebrook broth (for microscopy and infection experiments).

Preparation of culture filtrates and lysates and Western blotting of *M. tuberculosis* transformed with HA-tagged vectors. For Western blotting of bacterial culture filtrates (CF) and culture lysates (CL), bacteria expressing EsxA-HA or EsxB-HA were grown in 10 ml Sauton's broth for 5 days under agitation. The cultures were spun down and the supernatants were filtered through a 0.22-μm-pore-size filter for preparation of culture filtrates, followed by ultrafiltration using Amicon Ultra centrifugal filter devices with a 3-kDa cutoff (Merck Millipore). For the generation of CLs, bacterial pellets were resuspended in phosphate-buffered saline (PBS) supplemented with protease inhibitors, bead beated three times at maximum speed in a FastPrep FP120 cell disruptor (Thermo Savant), cooled on ice in between the bead beatings, and then spun down at 7,000 $\times$ g for 10 min. The protein concentrations of CF and CL were measured using a DC protein assay (Bio-Rad). Samples were mixed with Laemmli buffer for Western blotting and boiled at 95°C for 10 min. Equal amounts of proteins were separated on 10 to 20% SDS gels (Lonza) and transferred to a polyvinylidene difluoride Immobilon-FL membrane (Merck Millipore). The membrane was blocked using Odyssey blocking buffer (LI-COR Biosciences) and incubated overnight with primary antibodies at 4°C and with secondary antibodies for 1 h at room temperature (RT). Both primary and secondary antibodies were diluted in Odyssey blocking buffer (LI-COR Biosciences). Antibodies were used at the following dilutions: anti-HA tag (rabbit polyclonal; 1:4,000; ab9110; Abcam), anti-groEL2 (mouse monoclonal; 1:2,000; NR-13813; BEI Resources), IRDye 800CW goat anti-rabbit IgG H+L (1:25,000; 926-32211; LI-COR Biosciences), and IRDye 680RD goat anti-mouse IgG H+L (1:25,000; 926-68070; LI-COR Biosciences). The Western blot membranes were finally imaged on an Odyssey CLx system (LI-COR Biosciences).

Immobilization of bacteria for immunofluorescence staining. In order to immobilize *M. tuberculosis* prior to fixation and antibody staining, aliquots (300 to 600 μl) of bacterial cultures or diluted samples were pipetted onto glass coverslips in 24-well plates and the plates were centrifuged (500 $\times$ g, 7 min) to sediment the bacteria. Fixation was carried out for 30 min at RT by addition of paraformaldehyde (PFA) to yield a final concentration of 4%. Samples were washed with PBS and kept at 4°C until they were stained. The antibody staining procedure and confocal microscopy are described below.

Transmission electron microscopy (TEM) of whole bacteria. We observed that the dilution of bacterial cultures in PBS or broth diminished the EsxA positivity of the bacilli (unpublished observation); therefore, we fixed the crude *M. tuberculosis* culture without any washes. To do so, bacterial cultures grown for 6 days were fixed by addition of an equal volume of 8% electron microscopy-grade PFA (EMS Diasum) in 0.2 M phosphate buffer overnight, and the sedimented bacteria were resuspended in 0.5% PFA in 0.1 M phosphate buffer. Samples were taken directly from the fixed culture without pelleting and attached to Formvar/carbon-coated 100-mesh copper grids for 5 min. The bacteria were blocked with 1% bovine serum albumin (BSA) in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 7.4) for 5 min and then immunolabeled for EsxA using a monoclonal EsxA antibody (1:10 dilution; clone 11G4; Abcam) and bridged with a polyclonal rabbit anti-mouse immunoglobulin antibody (1:200 dilution; Z025902-2; Dako) to allow probing with 10-nm-diameter colloidal gold-conjugated protein A (1:50 dilution; PAG-10; CMC, UMC Utrecht). The grids were allowed to dry at RT for 4 h and were observed with a Tecnai 12 transmission electron microscope (FEI, Netherlands) equipped with an Eagle 4k $\times$ 4k charge-coupled-device camera.

Cells. Human monocyte-derived macrophages (hMDMs) were prepared from buffy coats purchased from the blood bank at Linköping University Hospital. Donors gave written informed consent for research use of the blood. Mononuclear cells were obtained by gradient centrifugation as described earlier (11, 25). Monocytes were allowed to adhere to cell culture flasks in serum-free Dulbecco modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin for 1 h, and then the medium was changed to the same medium containing 10% non-heat-inactivated human serum (obtained from the Linköping University Hospital blood bank and pooled from 5 donors). Cells were allowed to differentiate for 5 to 8 days with one medium change after 3 to 4 days. On the day before infection, the cells were reseeded into 96-well plates (for determination of bacterial growth and host cell death) or on glass coverslips (for microscopy).

Quantification of macrophage cell death and bacterial growth. Infection for luciferase and cell viability measurement was carried out in 96-well plates, seeding 100,000 cells per well in triplicate into black plates and infecting the cells with H37Rv Δ*esxA* or luciferase-expressing H37Rv at a multiplicity of infection (MOI) of 10. For infection, bacteria were prepared by washing twice with PBS and passaging through a 27-gauge needle (which has been shown to not harm the capsule [21]) before diluting in DMEM without serum or antibiotics (26). For the 24-h time point, the medium was changed after 1 h of incubation to serum-containing DMEM.

For the simultaneous determination of intracellular and extracellular *M. tuberculosis* growth and macrophage cell viability, we employed a 96-well-based assay described earlier (11, 26). The measure-

ment of bacterial numbers is based on a strain carrying the pSMT1 plasmid, which allows the expression of luciferase. For the determination of macrophage cell viability, the EsxA-deleted strain of H37Rv was also used in this study but could not be transformed with the pSMT1 plasmid due to identical antibiotic resistance markers. Macrophages were seeded in triplicate into black plates and infected, and 25-μl aliquots of the supernatants were transferred to a white plate for the quantification of extracellular *M. tuberculosis*. Then, the cells were washed three times with PBS and incubated with 4 μM calcein-AM (Molecular Probes) in PBS for 30 min at RT, and the green fluorescence was measured in a plate reader (GloMax-Multi+ detection system with Instinct Software; Promega). In order to obtain the intracellular bacterial numbers, cells were lysed hypotonically with 70 μl ultrapure water for 10 min and then scratched and pipetted up and down repeatedly. Twenty-five microliters of each lysate was again transferred to a white plate, 200 μl of water was added to the samples of the extra- and intracellular fractions, and flash luminescence was detected in the same plate reader equipped with an injector after injection of 25 μl of the luciferase substrate decanal (1%). The numbers of arbitrary light units (ALU) of the intra- and extracellular samples were standardized for dilutions and summed to obtain the total number of ALU for the well. Medians for the triplicates were normalized to the values obtained at the earliest time point (1 h) from the same macrophage donor, in order to express the change in bacterial numbers as the fold change. Cell viability was expressed as a percentage of the number of viable uninfected cells at the same time point.

Infection of cells for confocal microscopy. For microscopy, 250,000 cells/well were seeded on glass coverslips in 24-well plates. Where indicated, cells were pretreated for 60 min with 10 μM cytochalasin D (Sigma-Aldrich). Inhibitors were also included during the infection. Bacteria were prepared as described above, and the bacterial suspension was subjected to an additional low-speed centrifugation step (300 $\times$ g, 5 min) in order to remove the bacterial aggregates which were apparent in the cultures grown without Tween 80. After addition of the bacterial suspensions to the cells (MOI 5), the plates were spun at 500 $\times$ g for 7 min to synchronize the infection. The cells were then incubated for 5, 15, 30, 60, or 120 min before fixation, whereas the 0-min-time point samples were fixed directly after centrifugation by addition of PFA (final concentration, 4%) and incubation for 30 min at RT.

Antibody staining and microscopy analysis. Bacteria immobilized on glass coverslips or infected macrophages were permeabilized and blocked for 30 min at RT with 2% BSA, 10% goat serum, and 0.1% saponin in PBS, and saponin was omitted when only bacteria were stained. Saponin *per se* did not alter the staining intensity of EsxA in a direct comparison (data not shown). Incubation with primary antibodies was carried out overnight at 4°C. The antibodies used were monoclonal anti-EsxA antibody (1:400; anti-ESAT6 antibody [11G4]; ab26246; Abcam), monoclonal anti-LAM (1:20; CS-35; Colorado State University), and anti-HA tag (rabbit polyclonal; 1:500; ab9110; Abcam). All antibodies were diluted in blocking buffer. After PBS washes, coverslips were incubated for 30 min at 37°C with Alexa Fluor 594- or Alexa Fluor 647-conjugated goat anti-mouse IgG (depending on the microscope used for imaging), diluted 1:400 in blocking buffer. In the infected macrophages, F-actin was labeled using Alexa Fluor 546-phalloidin (Molecular Probes), diluted 1:40 in PBS. After further washes, coverslips were mounted using fluorescence mounting medium (Dako).

Bacteria immobilized to glass coverslips were imaged on an Axiovert 200M microscope (Carl Zeiss) equipped with an aperture correlation confocal unit (VivaTome; Carl Zeiss) using an Alpha-Plan Fluar 100$\times$ (numerical aperture, 1.45) objective. A description of this microscopic technique can be found elsewhere (27). Infected cells were imaged on an Observer Z1 confocal laser scanning microscope with an LSM 700 confocal module (Carl Zeiss) equipped with a Plan-Apochromat 63$\times$ (numerical aperture, 1.40) objective. For all EsxA immunolabeling experiments, negative controls were prepared using the EsxA-deficient strain H37Rv Δ*esxA*-GFP. For immunolabeling of HA-tagged EsxA (Fig. 3C and D), parallel samples with the GFP-expressing strain not carrying the EsxA-HA plasmid served as negative staining controls. Quantitative analysis of EsxA positivity was carried out in a blind manner, and all analyses were finished before decoding of the samples.

Ethics statement. Buffy coats were purchased from the blood bank facility at Linköping University Hospital, at which the blood was collected from healthy donors, who had given written consent for research use (besides medical use) of the donated blood in accordance with the Declaration of Helsinki. Since blood donation is classified as a negligible risk to the donors and since only deidentified samples were delivered to the researchers, the use of the samples does not require a specific ethical approval according to paragraph 4 of the Swedish law (2003:460) on Ethical Conduct in Human Research.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00803-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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