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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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.
**Introduction**

*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 non-covalently 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 as a distinct *M. tuberculosis* phenotype, exhibiting pronounced characteristics regarding minimum inhibitory concentrations of antibiotics (8), drug tolerance (9) and gene expression (10).

We here show 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.
Results

*M. tuberculosis* 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 3-6 days of incubation (Figure 1A and 1B). EsxA could not be detected when detergent was used in the broth, on EsxA-deficient *M. tuberculosis* cultivated without detergent and in the control staining without primary antibody (Figure 1A). The effect was more pronounced on bacterial aggregates consisting of two or more bacteria (the prevailing morphological structure in detergent-free cultures) (Figure 1B). Immunogold-labeling of EsxA followed by transmission electron microscopy (TEM) confirmed the presence of EsxA on the bacterial surface, while absent from the EsxA-deficient mutant and Tween-80 broth cultivated wildtype bacteria (Figure 1C and 1D).

Next, we hypothesized that surface retention of EsxA would be affected in *M. tuberculosis* during exposure to lung surfactant, which consists of amphiphilic phospholipids having similar properties as 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 obtained with 1% Curosurf® (Figure 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* 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* cultivated without Tween-80 reduced macrophage viability by 80% compared to uninfected cells within 24 hours, 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 (Figure 2A).

EsxA is lost from the bacterial surface upon contact with cell membranes. To our knowledge, our work is the first demonstration of immunolabelling 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 DIC images. During the course of the infection, numbers of free bacteria decreased, as the amount of cell-associated *M. tuberculosis* increased (Figure 2B). Coinciding with 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 even more pronounced at the later time points, whereas EsxA-positivity did not change...
in the free bacteria (Figure 2B and C). After infection with the EsxA-deficient mutant cultivated without Tween-80, we could not observe any EsxA staining (Figure 2B). Furthermore, we could not observe EsxA in any cellular organelles or structures at any time point. To rule out the possibility of the EsxA antibody not having 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 (Supplementary Figure S1), suggesting that the observed decline in EsxA staining upon attachment and internalization is not a technical artefact. Furthermore, intracellular bacteria could encounter conditions with 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 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 both expressed the fusion proteins, but only EsxA-HA was successfully secreted into the culture supernatant (Figure 3A). Cultivation of M. tuberculosis expressing EsxA-HA without detergent followed by HA-antibody staining reproduced the EsxA staining pattern (Figure 3B). In line with the previous observation, also EsxA-HA staining declined as M. tuberculosis were internalized by macrophages (Figure 3C and 3D).

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 (Figure 4A) and bacteria residing on the macrophage surface (Figure 4B), we observed that the proportion of free bacteria positive for EsxA did not change during 120 min of infection (Figure 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 (Figure 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 as a distinct *M. tuberculosis* phenotype, exhibiting pronounced characteristics regarding minimum inhibitory concentrations 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 extract of *M. marinum* grown on agar plates (12). In line with 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 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 immunolabelling of EsxA. Reinoculating the bacteria from detergent-containing to detergent-free broth, EsxA was detectable 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 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 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) 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 immunolabelling 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 to 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 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 is due to loss of secretion of other ESX-1-substrates that are co-dependent on the secretion of EsxA (17, 18). Besides that, deletion of EsxA could also lead to alterations in capsular integrity, as demonstrated for example for ESX-5-substrates (19), and thereby alter cytotoxicity. EsxA has previously been postulated to have direct membrane-lysing activity (13, 20-22). This was refuted by the finding that contaminating detergent was responsible for membrane lysis (2). However, in the present study, contaminating detergents do not cause the observed cytotoxicity, since potential residual Tween-80 was higher in the non-cytotoxic/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 described already in the 1980’s (23). However, to understand the cytotoxic mechanism of EsxA is beyond the scope of this manuscript.

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, 24) highlight the importance of careful evaluation of cultivation conditions for mycobacteria for the generation of physiologically relevant results.
Materials and Methods

Bacterial strains

*Mycobacterium tuberculosis* strains used in this study and antibiotic concentrations for their selection were the following: H37Rv wildtype and the EsxA-deficient strain H37RvΔesxA both carrying the pFPV2 plasmid for constitutive GFP expression (20 µg/ml kanamycin for pFPV2 and 50 µg/ml hygromycin for H37RvΔesxA), and H37Rv carrying the pSMT1 plasmid for expression of luciferase (100 µg/ml hygromycin). For cultivation of strains, frozen stocks were thawed in 7H9 Middlebrook broth supplemented with 0.2% glycerol, ADC (BD), 0.005% Tween-80 and antibiotics and incubated for 2-3 weeks. These stock cultures were then reseeded 6 days before experiments (except in Figure 1B where samples were taken also at other time points) into the same broth, with 0.05%, 0.005% or without Tween-80, or with different concentrations of Curosurf® (Chiesi Farmaceutici, Italy). 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 earlier publications showing that addition of a hexahistidine tag at the C-terminus of EsxA and at the N-terminus for 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 (25) and for secretion of the EsxA/EsxB complex (26).

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 *E.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 oligos with complementary 5’-overhangs after annealing. Upon phosphorylation of the 5’-termini using T4 polynucleotide kinase, oligos 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 the JetStar Plasmid Purification Mini Kit and the correct sequence of the insert was confirmed by sequencing. The different *M. tuberculosis* strains were transformed as described earlier (27). 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 three weeks. Colonies were grown in 7H9 Middlebrook broth supplemented as above. After cultivation for 2 weeks, stocks were frozen at -
80 °C or bacteria 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-vectors**

For western blotting of bacterial culture filtrates (CF) and culture lysates (CL), expressing EsxA-HA or EsxB-HA were grown in 10 ml Sauton’s broths for 5 days under agitation. Cultures were spun down, supernatants filtered through a 0.22 µm filter for preparation of culture filtrates, followed by ultrafiltration using Amicon Ultra centrifugal filter devices with 3 kDa cut-off (Merck Millipore). For generation of CLs, bacterial pellets were resuspended in 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, and then spun down at 7,000 g for 10 min. Protein concentrations of CF and CL were measured using the DC protein assay (Biorad). Samples were mixed with Laemmli buffer for western blotting and boiled at 95 °C for 10 min. Equal amounts of proteins were separated on a 10-20% SDS gels (Lonza) and transferred to a PVDF Immobilon-FL membrane (Merck Millipore). The membrane was blocked using Odyssey Blocking Buffer (LI-COR Biosciences), 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, ab9110, 1:4000), anti-groEL2 (mouse monoclonal, BEI Resources, NR-13813, 1:2000), IRDye 800CW Goat anti-rabbit IgG H+L (LI-COR Biosciences 926-32211, 1:25 000) and IRDye 680RD Goat anti-mouse IgG H+L (LI-COR Biosciences 926-68070, 1:25 000). 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 (300-600 µl) of bacterial cultures or diluted samples were pipetted onto glass coverslips in 24-well plates and plates were centrifuged (500 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 stained. The antibody staining procedure and confocal microscopy are described below.

**Transmission electron microscopy (TEM) of whole bacteria**

We observed that 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% EM-grade PFA (EMS Diasum) in 0.1 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 onto formvar/carbon-coated 100 mesh copper grids for 5 min. Bacteria were blocked with 1% bovine serum albumin (BSA) in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2, pH 7.4) for 5 min and then immunolabelled 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). Grids were allowed to dry at RT for 4 hours and were observed with a Tecnai 12 TEM microscope (FEI, Netherlands) equipped with an Eagle 4kx4k CCD camera.

**Cells**

Human monocyte-derived macrophages 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, 28). Monocytes were allowed to adhere to cell culture flasks in serum-free DMEM supplemented with 2 mM L-glutamine, 100 Units/ml penicillin and 100 µg/ml streptomycin for 1h, then the medium was changed to the same medium containing 10% non-heat inactivated human serum (obtained from Linköping hospital blood bank and pooled from 5 donors). Cells were allowed to differentiate for 5-8 days with one medium change after 3-4 days. The day before infection, 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 triplicates into black plates and infecting with H37RvΔesxA 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 27G needle (which has been shown to not harm the capsule (24)) before diluting in DMEM without serum or antibiotics (29). 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, 29). The measurement of bacterial numbers is based on a strain carrying the pSMT1 plasmid which allows for the expression of luciferase. For the determination of macrophage cell viability, also the EsxA-deleted strain of H37Rv was used in this study but could not be transformed with the pSMT1 plasmid due to identical antibiotic resistance markers. Macrophages were seeded in triplicates into black plates, infected and 25 µl aliquots of the supernatants transferred to a white plate for the quantification of extracellular *M. tuberculosis*. Then, cells were washed three times with PBS, incubated with 4 µM Calcein-AM (Molecular Probes) in PBS for 30 min at RT and green fluorescence 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,
then scratched and pipetted up and down repeatedly. 25 µl of each lysate were again transferred to a white plate, 200 µl of water were added to the samples of 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 arbitrary light units (ALU) of the intra- and extracellular samples were standardized for dilutions and summed up to obtain ALU for the total well. Medians of 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 fold change. Cell viability was expressed as a percentage compared to 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 pre-treated 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 g, 5 min) in order to remove bacterial aggregates which were apparent in the cultures grown without Tween-80. After addition of the bacterial suspensions to the cells (MOI 5), plates were spun at 500 g for 7 min to synchronize the infection. 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 by Abcam), monoclonal anti-LAM (1:20, CS-35, Colorado State University) and anti-HA tag (rabbit polyclonal, 1:500, ab9110, Abcam). All antibodies were and diluted in blocking buffer. After PBS washes, coverslips were incubated for 30 min at 37 °C with Alexa Fluor 594 or 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 200 M microscope (Carl Zeiss) equipped with an aperture correlation confocal unit (VivaTome, Carl Zeiss) using an alpha-plan fluar 100x/1.45 objective. A description of this microscopic technique can be found in (30). Infected cells were imaged on an Observer.Z1 with a LSM 700 confocal module (Carl Zeiss), equipped with a plan-apochromat 63x/1.40 objective. For all EsxA immunolabeling experiments, negative controls were
prepared using the EsxA-deficient strain H37RvΔesxA-GFP. For immunolabeling of HA-tagged EsxA (Figure 3C and 3D), 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 blinded manner, and all analyses were finished before decoding of 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.
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The following reagent was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: Monoclonal Anti-*Mycobacterium tuberculosis* LAM, Clone CS-35 (produced in vitro), NR-13811. The following reagent was obtained through BEI Resources, NIAID, NIH: Monoclonal Anti-*Mycobacterium tuberculosis* GroEL2 (Gene Rv0440), Clone CS-44 (produced in vitro), NR-13813.

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Author contributions

Conception or design of the work: JR, NI, VL, PJP, ML
Data collection: JR, NI, VL
Data analysis and interpretation: JR, NI, VL, PJP, ML
Drafting the article: JR, NI, ML
Critical revision of the article: JR, NI, VL, PJP, ML
Final approval of the version to be published: JR, NI, VL, PJP, ML
References


Figure 1. EsxA can be detected on the surface of *M. tuberculosis* (Mtb) after cultivation in the absence of detergent or surfactant. (A) and (B) Mtb H37Rv wildtype (left) or the EsxA-deleted strain H37RvΔesxA (right) were cultivated with or without Tween-80 and for time points as indicated. Fixed bacteria were stained with anti-EsxA antibody and an Alexa Fluor 594-conjugated secondary antibody.
Images in (A) are from bacteria cultivated for 6 days. In the samples for ‘antibody control’ images (fourth column), the anti-EsxA antibody was omitted. Images were obtained using a 100x/1.45 objective (scale bars: 5 µm). EsxA-positive single bacteria or aggregates (≥ 2 bacteria) were expressed as a percentage of all single bacteria or aggregates in (B). ‘Day 0’ indicates the initial Mtb stock culture which contained 0.05% Tween-80. Bars and error bars depict means and SEM from three independent experiments (on average 79 bacteria or aggregates analyzed per sample, range 17-441). Significant differences between the time points were tested with 2-way ANOVA comparing all time points to the day 0 time point using Bonferroni post-hoc test for multiple comparisons, and the earliest significant time point for each group is indicated by asterisks. (C and D) Mtb H37Rv or the H37RvΔesxA were fixed after 6 days of cultivation with or without Tween-80, followed by immunogold labeling for EsxA and TEM analysis. Representative images are shown. Arrows indicate EsxA-positive debris and arrowheads indicate immunogold particles in the bacterial cell wall. The negative control (neg ctrl) is Mtb wildtype 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 blinded fashion. Bars and error bars show means and SEM from 30-54 bacteria per sample. Significant differences were tested with 1-way ANOVA, followed by Tukey’s post-hoc test comparing all groups. (E) Mtb H37Rv wildtype were cultured in broth containing 0.05% Tween-80, or without detergents and increasing amount of the bovine lung surfactant Curosurf®. Bacteria were fixed, stained, imaged and analyzed as described in (A) and (B). Bars and error bars show means and SEM from 5 experiments (on average 106 bacteria analyzed per sample, range 38-349). Significant differences were tested with 1-way ANOVA, followed by Dunnet’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.
Figure 2. Superficial EsxA leads to a rapid decrease in macrophage cell viability and is lost from *M. tuberculosis* (*Mt*) during cell interaction. (A) Human monocyte-derived macrophages (hMDMs) were infected with luciferase-expressing Mtb cultivated with (0.05%) or without Tween-80 and with H37RvΔesxA cultivated without Tween-80. Cell viability was measured 1 and 24 h after infection using Calcein-AM (black symbols), and bacterial numbers of the wildtype strain were determined using a luminometry-based method (red symbols). The percentage of Calcein-AM signal (compared to uninfected cells from the same time point, left axis) and the fold change in bacterial numbers (compared to 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 as tested with 2-way ANOVA and Bonferroni’s post-hoc test. (B) hMDMs were infected for the indicated times with GFP-expressing Mtb 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 EsxA staining intensity and the respective bacteria were classified as free bacteria (not associated with any actin or cellular structures) or cell-associated based on the F-actin staining and DIC images. The arrows and arrowheads depict Mtb classified as cell-associated, with the arrow pointing at EsxA-positive and the arrowhead at EsxA-negative bacteria. Parallel samples infected with EsxA-deficient Mtb served as background controls (fourth row). (C) Bacteria in confocal images were quantified as EsxA-positive (filled) or negative (striped) and with regards to their local distribution (free or cell-associated). Bars and error bars show means and SEM from 5 experiments (on average 147 bacteria analyzed per sample, range 62-241). Changes in the proportion of EsxA-positive bacteria as a ratio of total bacteria within the respective population (free and cell-associated) were tested using 2-way ANOVA and Bonferroni’s post-hoc test. Among the extracellular bacteria, the fraction of EsxA positives did not change over time, while for cell-associated bacteria we observed a significant drop after 30, 60 and 120 min of infection as compared to the ‘0 min’-time point (p < 0.001).
Figure 3. Secretion of overexpressed HA-tagged EsxA and detection on M. tuberculosis (Mtb) grown without detergent and during macrophage infection.

(A) Mtb carrying a plasmid for the expression of HA-tagged EsxA or EsxB (or no plasmid) were 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-tag antibody. GroEL2 was included as a control for bacterial autolysis. (B) GFP-expressing Mtb H37Rv and H37Rv+EsxA-HA carrying a plasmid for the expression of HA-tagged EsxA were cultivated without Tween-80 for 6 days, then immobilized on glass coverslips, fixed, immunostained with an anti-HA-tag antibody and a
secondary antibody. (C and D) hMDMs were infected with GFP-expressing Mtb 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), 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 do not exhibit EsxA staining. Scale bars: 5 µm (B) and 10 µm (C-D).
Figure 4. 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 GFP-expressing Mtb H37Rv wildtype. Staining was performed the same way as in Figure 2. (A) To determine the EsxA-positivity of free Mtb, 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 co-localizing with F-actin structures or structures observed in DIC were included in the analysis (here indicated by asterisks). (B) For the analysis of Mtb 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 differential interference contrast (DIC) images for localization of the bacteria on cellular structures, before the respective bacteria were analyzed for EsxA-positivity. Arrowheads depict Mtb positive for EsxA staining. (C) Quantification of EsxA-positivity of Mtb residing between cells (free Mtb) and on the surface of cells (cell-associated Mtb). Differences in EsxA-positivity between the two groups were tested by 2-way ANOVA, and changes over time were tested using 2-way ANOVA with Bonferroni’s post-hoc test comparing the zero-time point to the other time points. All imaging was performed on a confocal microscope using a 63x/1.4 objective. For microscopic analyses, bars and error bars represent means and SEMs from 5 independent experiments (on average 74 bacteria analyzed per sample, range 33-144). * p < 0.05, ** p < 0.01, *** p < 0.001, scale bars: 10 µm.
Supplementary Figure and Figure Legend

Supplementary Figure S1

**Supplementary Figure S1. Control for antibody staining of intracellular *M. tuberculosis* (Mtb).**

hMDMs were infected with GFP-expressing H37Rv or H37RvΔesxA cultivated in Tween 80-free broth and fixed after 60 min of infection. Samples were stained with an anti-LAM antibody and an Alexa Fluor 647-conjugated secondary antibody as well as Alexa Fluor 546 Phalloidin, following the same protocol as for EsxA antibody staining. Scale bars: 10 μm.