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Mycobacterial Secretion Systems ESX-1 and ESX-5 Play Distinct Roles in Host Cell Death and Inflammasome Activation

Abdallah M. Abdallah,*,†,‡1 Jovanka Bestebroer,†,‡ Nigel D. L. Savage,‡ Karin de Punder,* Maaike van Zon,* Louis Wilson,‡ Cees J. Korbee,‡ Astrid M. van der Sar,‡ Tom H. M. Ottenhoff,‡ Nicole N. van der Wel,* Wilbert Bitter,‡ and Peter J. Peters*‡§

During infection of humans and animals, pathogenic mycobacteria manipulate the host cell causing severe diseases such as tuberculosis and leprosy. To understand the basis of mycobacterial pathogenicity, it is crucial to identify the molecular virulence mechanisms. In this study, we address the contribution of ESX-1 and ESX-5—two homologous type VII secretion systems of mycobacteria that secrete distinct sets of immune modulators—during the macrophage infection cycle. Using wild-type, ESX-1– and ESX-5–deficient mycobacterial strains, we demonstrate that these secretion systems differentially affect subcellular localization and macrophage cell responses. We show that in contrast to ESX-1, the effector proteins secreted by ESX-5 are not required for the translocation of Mycobacterium tuberculosis or Mycobacterium marinum to the cytosol of host cells. However, the M. marinum ESX-5 mutant does not induce inflammasome activation and IL-1β activation. The ESX-5 system also induces a caspase-independent cell death after translocation has taken place. Importantly, by means of inhibitory agents and small interfering RNA experiments, we reveal that cathepsin B is involved in both the induction of cell death and inflammasome activation upon infection with wild-type mycobacteria. These results reveal distinct roles for two different type VII secretion systems during infection and shed light on how virulent mycobacteria manipulate the host cell in various ways to replicate and spread. The Journal of Immunology, 2011, 187: 4744–4753.

Pathogenic bacteria secrete virulence proteins that can facilitate colonization and persistence in the host (1). There are many different bacterial effector proteins, whereas the secretion systems used for protein delivery are more homogeneous. Six different secretion systems have been described in Gram-negative bacteria, whereas Gram-positive bacteria secrete proteins mainly via the Sec or Tat system. Mycobacteria, although they have been considered as Gram-positive bacteria, do not fit this classification well. They have an uncommon and highly complex cell wall and contain multiple copies of a recently identified novel secretion system known as early secretory Ag target 6 (ESAT-6) system (ESX), or type VII secretion system (T7SS) (2).

A number of species of the Mycobacterium genus are important human and animal pathogens, and proteins secreted via T7SSs contribute to their pathogenesis. The five known mycobacterial T7SSs are encoded by discrete gene clusters termed ESX-1 to ESX-5 (2, 3). ESX-3 is required for iron transport and as such is essential for the viability of Mycobacterium tuberculosis (4, 5), whereas ESX-1 and ESX-5 are involved in the virulence of M. tuberculosis and Mycobacterium marinum (6, 7). ESX-1 and ESX-5 have been found to secrete different effector proteins that modulate host cell functions, although the molecular targets and mechanisms of action are yet to be identified (reviewed in Ref. 2). The substrates or structural components of ESX-1 include among others EsxA (ESAT-6) and EsxB (CFP-10). Within the phagolysosome of macrophages, M. tuberculosis and M. marinum secrete ESX-1 substrates that interfere with the integrity of the phagosomal membrane, leading to “leaky” membranes, phagosomal rupture, and bacterial entry into the cytosol (8, 9). ESX-5 is involved in the secretion of various proteins containing Pro-Pro-Glu, Pro-Glu, and polymorphic GC-rich sequences, and ESX-5 mutants are unable to modulate macrophage cytokine responses (7, 10).

Macrophages are important both for bacterial control and for bacterial multiplication during the early stages of mycobacterial infection. They respond to mycobacterial infection by production of proinflammatory cytokines. IL-1β synthesis is strongly upregulated upon infection with M. tuberculosis (11). IL-1β is first synthesized as inactive pro–IL-1β and requires proteolytic matu-
rion by caspase-1 for cellular release (12). Caspase-1 itself needs activation via inflammasomes, protein complexes that sense cytosolic bacterial products and endogenous danger signals. Both *M. tuberculosis* and *M. marinum* reportedly induce IL-1β secretion through NALP3 inflammasomes in an ESX-1–dependent manner (13–16). Recently, we observed that ESX-5 is also required for IL-1β secretion upon infection with *M. marinum* (7). Mechanisms regulating this event and the precise contribution of ESX-5 remain to be characterized.

Macrophages further respond to mycobacterial infection by induction of cell death. This is an effective innate immune mechanism in which apoptosis, but not necrosis, is associated with reduced bacillary viability. Sequestration and retention of mycobacteria within apoptotic bodies allows for engulfment by newly recruited activated phagocytic cells (17, 18). Sly et al. (19) showed that virulent *M. tuberculosis* inhibits macrophage apoptosis by upregulation of the antiapoptotic Bcl-2 family member Mcl-1, supposedly by maintaining the integrity of the mitochondrial membrane and thereby preventing release of cytochrome c and activation of caspases. Others have reported that virulent strains of *M. tuberculosis* prevent apoptosis and cause necrosis, thereby promoting bacterial survival (20–22). Although the importance of macrophage cell death is evident, the underlying bacterial factors and the transport of these factors have not been identified.

In this study, we examined the effects of ESX-1 and ESX-5 secretion systems on macrophage responses during infection with *M. marinum* and *M. tuberculosis*. Using wild-type and ESX-1– and ESX-5–deficient strains, we demonstrate that these T7SSs differentially affect macrophage cell death and IL-1β secretion. Additionally, we identify cathepsin B as playing a major role in both responses.

**Materials and Methods**

**Reagents**

Z-VAAD, Z-DEVD-FMK, staurosporine (STS), Z-IEETD-FMK, Z-LEHD-FMK, Z-VAD, Z-DEVD-FMK, t-butyldihydroperoxide (TBH), cyclosporin A, cytochalasin D, BAPTA-AM, N-acetyl-L-cysteine (NAC), glutathione, diphenyleneiodonium chloride (DPI), and nigericin were from Sigma-Aldrich. CA-O74-Me ([L-3-carboxybenzamido (2-naphthyl)-carbonyl]t-Bu)-diazomethylketone), and pepstatin A were from Calbiochem. Anti-TLR2 (2392) was from Genentech. Anti-human TNF-α (N-terminus in 50 pmol, Briefer) was from Genetec. Anti-human TNF-α was from R&D Systems.

**Bacterial strains and growth conditions**

Wild-type (WT) *M. marinum* E11 strain, ESX-5 mutant 7C1 with transposon in *mmr2676* (homolog of *Rv1794* (7), 10), complemented ESX-5 mutant with restored ESX-5 secretion (10), ESX-1 mutant fas3.1 or fas30 with transposons in *eccB1* (23), complemented ESX-1 mutant with restored ESX-1 secretion (23), and *M. tuberculosis* CDC1551 strain and the ESX-5 mutant [JHU1783-2086 (24); transposon mutant in *Rv1783* with defective ESX-5 secretion (E.N.G. Houben, J. Bestebroer, J. Luirink, and W. Bitter, manuscript in preparation)] were grown in Middlebrook 7H9 liquid medium or Middlebrook 7H10 agar supplemented with 10% Middlebrook ADC or OADC, respectively (BD Biosciences) and 0.05% Tween 80. For infection experiments, bacteria were grown to logarithmic phase, washed, and diluted in RPMI with glutamax-1 medium (Life Technologies) supplemented with 10% FCS (RPMI/FCS). To remove bacterial clumps, bacterial suspensions were subjected to low-speed centrifugation (300 × g, 10 min) after which the supernatant was passed through a 5-μm filter and used at a multiplicity of infection of 10, unless indicated otherwise.

**Cells**

Human acute promonocytic leukemia THP-1 cells (American Type Culture Collection), mouse macrophage cell line RAW 264.7 (American Type Culture Collection), and human type II alveolar epithelial cells A549 (American Type Culture Collection) were cultured in or RPMI or DMEM (Life Technologies) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Differentiation of THP-1 cells into macrophage-like cells was induced with 10 ng/ml PMA (Sigma) for 24 h. RAW 264.7 and A549 cells were trypsinized (0.5%; Life Technologies), washed with fresh medium without antibiotics, seeded in 24-well culture plates (Costar), and allowed to grow to >70% confluency before infection.

Monocytes were isolated from peripheral blood of healthy donors by magnetic cell sorting using anti-CD14–coated beads. Isolated cells were polarized with 50 U/ml GM-CSF (Novartis Pharma, Arnhem, The Netherlands) to obtain type 1 macrophages, 50 ng/ml M-CSF (R&D Systems) for type 2 macrophages, and 1000 U/ml GM-CSF and 500 U/ml IL-4 for monocyte-derived dendritic cells (25). Cells were seeded 1 d before infection.

**Infections**

For cyto-immunogold electron microscopy, THP-1 cells were seeded at 10 × 10^6 per flask (75 cm²). For cytotoxicity and IL-1β secretion assays, THP-1 cells or type 1 macrophages were seeded at 5 × 10^4 and 3 × 10^5 cells/well in 24-well culture plates, respectively, in 1 ml RPMI/FCS. Cells were infected with bacteria for 1 h at 33°C for *M. marinum* and 37°C for *M. tuberculosis* and 5% CO₂. Supernatant was removed, and infected cells were washed three times and incubated in fresh RPMI/FCS at appropriate temperatures for the indicated times. For cell counting, undifferentiated THP-1 cells were used, and viability was determined by both trypan blue exclusion and acridine orange staining. Cells were preincubated with inhibitory compounds or proteins for 1–2 h before and during infection.

**Electron microscopy**

Cells were fixed with paraformaldehyde and glutaraldehyde for 2 h, similarly as described previously (9). After cutting 50-mm-thick sections at −120°C, sections were labeled with anti-LAMP-1 (H4A3; BD Biosciences) or anti-CD63 (M1544; Sanquin) and protein A conjugated to 10 nm gold (EM Laboratory, Utrecht University). Sections were examined using an FEI CM10 transmission electron microscope.

**Cytotoxicity assays**

Cytotoxicity was monitored via measurements of cytosolic lactate dehydrogenase (LDH) release (OD₅₆₂nm) with CytoTox 96 assay (Promega). Maximum release was achieved by lysis of cells with 1% Triton-X, and supernatants of uninfected cells were used to assess spontaneous release. Percentage cytotoxicity through LDH release (OD₅₆₂nm) was calculated with the formula: [(test LDH release – spontaneous release)/spontaneous release] × 100. Where indicated, apoptosis was induced with 1 μM STS and necrosis with 200 μM THB. The neutralizing ability of the TRL2 Ab was confirmed by challenging differentiated THP-1 cells or TLR2-transfected HEK293 cells with PAM3CSK4 in the presence or absence of the TLR2 Ab (not shown).

**IL-1β secretion**

Cell supernatants were assayed for IL-1β by ELISA (Invitrogen) according to the manufacturer's instructions.

**Small interfering RNA-mediated inhibition of cathepsin B expression**

Differentiated THP-1 cells were seeded overnight in 6-well plates. Transfection was carried out the next day using cethapsin B and control (scrambled) small interfering RNA (siRNA) according to the manufacturer's protocol (Santa Cruz Biotechnology, Santa Cruz, CA). Control siRNA is an mRNA sequence that will not cause the specific degradation of any cellular message. After transfection, cells were infected with *M. marinum* as described earlier. Cell lysates were analyzed by Western blot analysis using anti-cathepsin B (CA10; Abcam). Equal loading was confirmed with anti–β-actin mAb (13E5; Cell Signaling).

**IL-1β and caspase-1 processing**

Type 1 macrophages or THP-1 cells (differentiated for 3 h with 300 ng/ml PMA and extensively washed) were seeded at 1 × 10^6 cells/well in 12-well plates. After 24 h, cells were infected with *M. marinum* for 1 h at a multiplicity of infection of 10. Cells were washed three times, and serum-free RPMI was added for 24 or 48 h. Where appropriate, inhibitory compounds were added during and after infection. Nigericin was added 2 h before sample collection. Supernatants were collected, and proteins were precipitated with methanol–chloroform extraction as described in Ref. 26. Cell lysates were made with RIPA lysis buffer, and protein concentration was determined (Pierce). Samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Blots were probed with anti-human caspase-1 p20 (D57A2; Cell Signaling) for detection of cleaved
caspase-1, anti-human caspase-1 p10 (sc-515; Santa Cruz Biotechnology) for procaspase-1, anti-human IL-1β (MAB201; R&D Systems) for pro–IL-1β and IL-1β, and for equal loading with anti–β-actin mAb (Cell Signaling).

**Production and secretion of ESAT-6**

Mycobacteria were grown to mid-logarithmic phase in Middlebrook 7H9 liquid medium supplemented with 0.2% dextrose and 0.05% Tween 80. Proteins in the cell-free supernatants were precipitated with 10% TCA, and cell pellets were sonicated. Proteins were separated by SDS-PAGE and visualized by immunoblot using mouse anti–ESAT-6 (Hyb76-8) and mouse anti-GroEL2 (CS44; Colorado State University).

**Results**

**ESX-5 deficiency does not affect translocation from phagolysosome to cytosol**

To explore the role of T7SSs in pathogenicity of mycobacterial infection, the subcellular localization of WT and mutant *M. marinum* and *M. tuberculosis* was analyzed in human macrophage-like THP-1 cell line. It has been reported that the ESX-1 secretion system is essential for mycobacterial translocation and virulence (9). Therefore, we determined the subcellular localization of *M. marinum* E11 WT strain, an isogenic ESX-1 secretion mutant with transposon in *eccB1* (23), and isogenic ESX-5 secretion mutant MMAR2676 (homolog Rv1794) (7, 10) in PMA-differentiated THP-1 cells. Moreover, we analyzed *M. tuberculosis* CDC1551 WT strain and an isogenic ESX-5 secretion mutant with transposon in *eccC5* (*CDC1783*; JHU1783-2086) (24). Cells infected with various mycobacteria were analyzed by cryo-immunogold electron microscopy, and quantification confirmed that both *M. marinum* and *M. tuberculosis* WT strains translocated from phagolysosomes into the cytosol (Fig. 1 and Supplemental Fig. 1B). As we have previously described for *M. tuberculosis* (9), an ESX-1 mutant of *M. marinum* did not translocate and was retained in phagolysosomes (Fig. 1A). By contrast, ESX-5 deficiency did not affect translocation either in *M. marinum*, as shown before (7), or in *M. tuberculosis* (Fig. 1 and Supplemental Fig. 1B). Contrary to ESX-1, the ESX-5 mutant of *M. marinum* secretes ESAT-6 (Supplemental Fig. 1A), confirming that ESX-1–dependent secretion is linked to translocation.

When translocation of these mycobacterial species was quantified over time, the *M. marinum* ESX-5 secretion mutant showed a delayed ability to translocate compared with the WT (Fig. 1B). After the initial 24 h, the final percentage of cytosolic bacteria was similar to that of the WT. As previously demonstrated (9), *M. tuberculosis* only starts to translocate at 48 h postinfection. From this time point, the percentage of cytosolic *M. tuberculosis* was similar for WT and the ESX-5 mutant (Fig. 1C) during the entire course of infection. This effect was thus not observed for the *M. tuberculosis* ESX-5 secretion mutant (Fig. 1C). In conclusion, whereas deficiencies in ESX-1 block translocation, deficient ESX-5 does not.

**ESX-1 and ESX-5 are involved in cell death**

While quantifying translocation of mycobacteria, a rapid induction of THP-1 cell lysis was observed for WT *M. marinum*, whereas the

![FIGURE 1. ESX-5 mutants translocate to the cytosol of infected macrophages. THP-1 cells were infected with *M. marinum* WT, ESX-1, or ESX-5 mutants (A, B) and *M. tuberculosis* WT and ESX-5 mutant (C). At different time points, cells were fixed and processed for electron microscopy and cryo-immunogold labeling with lysosomal marker CD63. A. Representative electron micrographs of cells infected with *M. marinum* ESX-1 mutant after 2 d (Mm1, top panels) or ESX-5 mutant (Mm5, bottom panels) after 5 d. Scale bars, 200 nm. B and C. Quantification of cells containing cytosolic *M. marinum* WT, ESX-1, and ESX-5 mutants (B) or *M. tuberculosis* WT and ESX-5 mutant (C). Depicted is the percentage of cytosolic bacteria at 4, 12, 24, and 72 h postinfection. Results depict representative electron micrographs and quantification of the percentage of cytosolic bacteria of three independent experiments. L, lysosome; M, mitochondrion.](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org)
T7SS mutants were less cytotoxic (7). To evaluate the contribution of ESX-5 and ESX-1 to cell death, THP-1 cells were infected with WT *M. marinum*, or the ESX-5 or ESX-1 mutants, and cell death was monitored by measuring release of cytosolic LDH and by trypan blue exclusion and acridine orange staining. Within 24 h, WT *M. marinum* induced cell death in infected cells (Fig. 2A, 2B). By contrast, the ESX-5 secretion mutant and ESX-1 secretion mutant showed impaired cytotoxicity; at 24 h postinfection, the WT induced 40% cell death whereas the ESX-1 and ESX-5 mutants induced 15 and 16%, respectively. A constant reduction in cell death of ∼50% was apparent during the whole 72-h culture for both mutants (Fig. 2A, 2B). ESX-1 (23) and ESX-5 complementations (10) resulted in restored induction of cell death comparable with that of the WT strain (Fig. 2A). In addition to cell death induction in THP-1 cells, ESX-5 cytotoxicity was also examined in other phagocytic cell types, in primary cells as well as in several cell lines (Supplemental Fig. 2). For all infections, compared with the WT, both the ESX-5 and ESX-1 mutants showed significantly reduced induction of cell death of up to 70% in human primary monocytes, monocyte-derived pro- and anti-inflammatory macrophage subsets, dendritic cells, as well as in the murine macrophage cell line RAW264.7 and human type II alveolar epithelial cells A549. As we observed similar effects in different cell types, we continued our experiments using THP-1 cells, unless stated otherwise. Similarly to *M. marinum*, THP-1 cell infections with WT *M. tuberculosis* also induced cell death, which was reduced upon infection with the ESX-5 mutant (Fig. 2C, 2D). At 24 h, the WT induced cell death in 30% of the cells, whereas the mutant was responsible for 16% cell death. Thus, mycobacterial cytotoxicity is dependent on ESX-5 and ESX-1.

Next, we examined whether active internalization of bacteria was required for induction of cell death. Treating THP-1 cells with cytochalasin D, which blocks actin polymerization and thus phagocytosis, made these cells resistant to cytotoxicity after infection with WT *M. marinum* or ESX-1 or ESX-5 mutants (Fig. 2E). As a control, cytochalasin D did not inhibit STS-induced cytotoxicity. Furthermore, heat inactivation of the bacilli also abrogated mycobacterial-induced cytotoxicity in macrophages (data not shown). In summary, viable mycobacteria need to be internalized by host cells to induce ESX-1 and ESX-5, T7SS-dependent cell death.

**A nonapoptotic M. marinum-induced cell death**

To delineate how T7SSs induce host cell death, the role of several known cell death pathways was investigated. Caspases are involved in many apoptotic pathways (27, 28), and herein caspase-3 is central (29). Caspase-1 and its conversion of IL-1β are involved in cell death induced through Type 3 secretion system substrates of many Gram-negative bacteria (30), but caspase-1 is not required for apoptosis (31) and can be involved in necrosis. Furthermore, cell wall-associated and soluble mycobacterial factors have been reported to induce TLR2-dependent cell death (32, 33), and TNF-α is a classic cytotoxic host protein that induces cell death (34–36). Also, different cellular stresses such as the generation of reactive oxygen species (ROS) or increases in cytosolic Ca²⁺ levels have been connected to Mycobacterium-induced death (37, 38). We assessed the role of these pathways during *M. marinum*-induced cell death of macrophages by using specific inhibitory agents or blocking Abs during infection. Even though some of these factors have been connected to *M. tuberculosis*-induced death, they did not play a role in *M. marinum*-induced cytotoxicity of THP-1 cells as evident from the lack of any effect of the inhibitors or blocking Abs used during infection, as shown in Supplemental Fig. 3.

Necrotic cell death has also been reported for *M. tuberculosis*-infected macrophages due to mitochondrial damage. We observed that cell treatment with cyclosporin A—which prevents mitochondrial pore opening (39)—prior to infection with *M. marinum* had a marginally protective effect; the release of cytosolic LDH was reduced by 5–23% (Fig. 3A). *M. marinum*-induced cytotoxicity is thus also partially dependent on mitochondrial depolarization and therefore mostly resembles a nonapoptotic type of cell death.

**Cathepsin B is involved in M. marinum- and M. tuberculosis-induced macrophage cytotoxicity**

Lyosomal proteases that translocate to the cytosol in response to various stimuli can mediate cell death. Previous infection studies

![FIGURE 2. ESX-5 of *M. marinum* and *M. tuberculosis* is required for induction of cell death. A–D, THP-1 cells were infected with *M. marinum* WT, ESX-1, or ESX-5 mutants, ESX-5 complemented strain (ESX-5.C), or ESX-1 complemented strain (ESX-1.C) (A, B) and *M. tuberculosis* WT or ESX-5 mutant (C, D). At several time points, cell death was quantified by measuring cytotoxicity (A, C) through LDH release or cell viability through cell counts (B, D). The results are mean percentages of cytotoxicity ± SD of three experiments, each performed in duplicate. E, Cytotoxicity postinfection with *M. marinum* or 1 μM apoptosis-inducing STS in the presence or absence of 5 μM cytochalasin D.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.4747)
with *M. tuberculosis* strain H37Rv showed that cathepsin B and L inhibitors reduced DNA fragmentation and macrophage cell death, suggesting the involvement of lysosomal proteases in *Mycobacterium*-induced cell death (40, 41). In this study, we investigated a possible role for lysosomal proteases cathepsin B, D, and L in *M. marinum*-induced cell death. As shown in Fig. 3B, treatment of THP-1 cells with the cathepsin L inhibitor z-FY(t-Bu)-dmk or cathepsin D inhibitor pepstatin A did not show any detectable effect on cytotoxicity of THP-1 cells by *M. marinum*. By contrast, inhibition of cathepsin B by CA-074-Me reduced *M. marinum*-infected THP-1 cell cytotoxicity by 55%. The cathepsin B inhibitor was also the only inhibitor used that displayed reduced cytotoxicity upon infection of primary type 1 macrophages with *M. marinum* (Fig. 3C). In addition, we observed the same effects during THP-1 infections with *M. tuberculosis*; inhibition of cathepsin B also partially protected *M. tuberculosis*-infected THP-1 cells (Fig. 3D). Finally, siRNA-mediated silencing of cathepsin B in THP-1 cells was performed. Western blot analysis revealed that the addition of cathepsin B-specific siRNA indeed resulted in a significant reduction of cathepsin B (Fig. 3F). This reduction of cathepsin B partially rescued macrophages from *M. marinum*-induced cell death (Fig. 3E) compared with the addition of control siRNA.

Notably, the contributions of ESX-5 and ESX-1 to cathepsin B-mediated cytotoxicity are different. As observed for WT bacteria, cell death induced by the ESX-5 mutant was also affected by the cathepsin B inhibitor; however, this difference was only marginal for the ESX-1 mutant (Fig. 3B) in which case the bacterium remains in the phagolysosome of THP-1 cells. The same result was also obtained for *M. tuberculosis* WT and the ESX-5 mutant (Fig. 3C). Thus, *M. marinum* and *M. tuberculosis*-induced cytotoxicity in macrophages is partially dependent on the release of lysosomal proteases via ESX-1–mediated translocation from the phagolysosome, and this type of cell death is predominantly due to the proteolytic activity of cathepsin B. ESX-5 mediates cell death through a yet unidentified mechanism.

**ESX-5 is required for inflammasome activation and IL-1β release**

Upon infection, macrophage sensing of bacteria results in IL-1β secretion, and uncovering the bacterial substrates that induce IL-1β production is important to understand how host cells detect infection and possibly constrain disease. Inflammasome activation upon *M. marinum* infection can exacerbate disease (42) indicating a host-detrimental and bacterium-promoting role of the inflammatory pathway during mycobacterial infection (15). Previous studies demonstrated that infection of macrophages with pathogenic mycobacteria results in the induction of IL-1β release, which is dependent on both ESX-1 and ESX-5 (7, 13–15). Fig. 4A shows that indeed, in contrast to WT *M. marinum*, both the isogenic ESX-1 and ESX-5 secretion mutants do not induce IL-1β secretion in human proinflammatory type 1 macrophages. The complemented ESX-1 and ESX-5 mutant showed restored induction of IL-1β secretion. As the ESX-1 effect is most likely due to its inability to translocate from the phagolysosome to the cytosol, we sought to identify how ESX-5 substrates drive IL-1β production.

The secretion of IL-1β is dependent on two distinct signals: induction of pro–IL-1β synthesis and caspase-1 activation. In our system, IL-1β secretion upon infection with *M. marinum* was indeed dependent on caspase-1 activation, as caspase-1 inhibitor z-VAD abrogated IL-1β secretion (Fig. 4B). Therefore, we investigated the effect of ESX-5 on pro–IL-1β synthesis and caspase-1 activation. Cell lysates from type 1 macrophage cells infected with *M. marinum* ESX-5 secretion mutant showed reduced levels of pro–IL-1β expression compared with those of lysates from cells infected with WT or complemented bacteria (Fig. 4C). This difference, however, does not account for the IL-1β discrepancy, as induction of high and equal expression of pro–IL-1β by adding LPS during infection still resulted in strongly reduced secretion of IL-1β in the ESX-5 mutant compared with that in the WT (Fig.
Next, we examined the induction of procaspase-1 and its conversion to caspase-1. Although cell lysates from macrophages infected with WT and ESX-5 mutant contained equal levels of procaspase-1 expression, active caspase-1 was absent for cells infected with the ESX-5 mutant (Fig. 4E). The complemented strain also induced caspase-1 activation, demonstrating ESX-5–specific effects. The differences in caspase-1 activation and IL-1β secretion were still apparent after a prolonged infection of 48 h (Fig. 4F, 4G). In conclusion, optimal activation of caspase-1 and subsequent release of IL-1β by *M. marinum* is dependent on ESX-5 substrates.

**Inflammasome activation by M. marinum is dependent on ROS and potassium efflux**

Caspase-1 conversion is achieved upon assembly and activation of inflammasomes, cytosolic multiprotein complexes converting procaspase-1 into active caspase-1. Inflammasomes can be activated by different signals, and we investigated which triggering mechanisms play a role during *M. marinum* infections. As ROS are known to induce inflammasome activation, we investigated IL-1β secretion upon type 1 macrophage infection with *M. marinum* in the presence of the antioxidant NAC and the NADPH oxidase.
inhibitor DPI. Both inhibitors affected bacterially induced IL-1β release (Fig. 5A). Additionally, generation of active caspase-1 was affected in cells infected in the presence of DPI (Fig. 5B). Thus, ROS play a role in inflammasome activation by *M. marinum*.

Potassium efflux was shown to be important for inflammasome activation by *M. tuberculosis* (14). Involvement of this inflammasome trigger during *M. marinum* infection of type 1 macrophage was investigated by adding KCl to the infection medium. Both IL-1β secretion and caspase-1 activation were inhibited upon increasing concentrations of KCl (Fig. 5C, 5D). Subsequently, we investigated whether K⁺ efflux is sufficient to trigger inflammasome activation and IL-1β release in cells infected with ESX-5 mutant *M. marinum*. Indeed, Fig. 5E shows that IL-1β was secreted by cells infected by the ESX-5 mutant after addition of the potassium ionophore nigericin. This effect was dependent on K⁺ efflux, as addition of extracellular KCl abolished IL-1β secretion. Recovery of IL-1β secretion upon nigericin stimulation supports our finding that pro–IL-1β is sufficiently expressed in the ESX-5 mutant but not secreted effectively. Our results suggest that the loss of IL-1β secretion in the ESX-5 mutant is due to an inability to induce inflammasome activation through ROS generation and K⁺ efflux.

**Induction of IL-1β secretion is dependent on cathepsin B**

Inflammasome activation has recently been reported to be triggered by cathepsin B in the context of infection (43–45). Moreover, sterile stimuli induce inflammasome activation through release of activated cathepsins due to phagosomal rupture (26, 46), a process that we describe to be a determining factor for mycobacterial pathogenicity. Hence, we examined a previously unexplored role for cathepsins in inflammasome activation during mycobacterial infection. For this purpose, THP-1 cell infections were first performed in the presence of the cathepsin B inhibitor CA-074-Me. Compared with untreated cells, cells treated with the inhibitor failed to activate caspase-1 or secrete IL-1β upon infection with *M. marinum* (Fig. 6A, 6B). Inhibitors for other lysosomal proteases cathepsin D (pepstatin A) or cathepsin L [z-FY(t-Bu)-dmk] showed no detectable effect, suggesting a specific requirement for active cathepsin B. The importance of cathepsin B in IL-1β secretion in THP-1 cells was further confirmed through silencing experiments using siRNA (Fig. 6C). Successful but partial knockdown of cathepsin B (Fig. 3F) completely inhibited IL-1β secretion upon infection. In addition to THP-1 cells, inhibition of cathepsin B using CA-074-Me prevented the activation of caspase-1 and secretion of IL-1β in primary type 1 macrophages upon *M. marinum* infection as well (Fig. 6D, 6E). Thus, cathepsin B is instrumental in inflammasome activation (Fig. 7) during mycobacterial infection of macrophages.

**Discussion**

Recently, it has become clear that pathogenic mycobacteria secrete a variety of effector proteins into eukaryotic target cells that undermine the host cellular immune response. For example, delivery of ESX-1 T7SS substrates into phagocytic cells can lead to translocation of the bacteria into the host cytosol (8, 9) and can also modulate host cell responses (7). In this study, we demonstrate that T7SS substrates are linked to cell death and IL-1β conversion in infected phagocytic cells and that ESX-5–secreted substrates are involved in these processes.

There have been reports of mycobacterial infection inducing either apoptosis (35, 47, 48) or necrosis (20, 49, 50). The discrepancy could be caused by the use of different bacterial viru-

**FIGURE 5.** Generation of ROS and potassium efflux are important for inflammasome activation by *M. marinum*. A and B, Release of IL-1β (A) and caspase-1 p20 (B) was analyzed in supernatants of type 1 macrophages infected with *M. marinum* for 24 h in the presence or absence of 2 μM DPI and/or 10 mM NAC. C, IL-1β release was quantified for type 1 macrophages treated with 0–40 mM KCl during infection with *M. marinum* WT for 24 h. D, Presence of pro–IL-1β, IL-1β, or procaspase-1 in cell lysates (125 μg) and caspase-1 p20 in precipitated supernatants was examined in type 1 macrophages infected with *M. marinum* in the presence of 40 mM KCl. β-Actin reflects cell lysate loading control. E, Type 1 macrophages were infected with *M. marinum* ESX-5 mutant in the presence or absence of KCl (0 or 40 nM) for 24 h. Nigericin (Nig) was added for an additional 2 h. IL-1β release was determined in supernatants. The results are representative of at least three experiments.
lence strains, cell types, types of infection, or host genetic susceptibility factors (51) or by the type of assay. In this study, we show that in THP-1 cells, *M. tuberculosis* and *M. marinum* mainly trigger a cathepsin B-dependent (nonprogrammed; without the intricate regulatory mechanisms that are characteristic of apoptosis) necrotic type of cell death. Notably, the T7SSs ESX-1 and ESX-5 play different roles in this process.

It has been shown that ESX-1 substrates facilitate the translocation of pathogenic mycobacteria from phagolysosome into the host cytosol (9), most likely by disrupting the integrity of the phagolysosomal membrane. This is consistent with our data showing that the *M. marinum* ESX-1 mutant remains in the phagolysosome during prolonged infections. Similar to ESX-1, ESX-5 is also required for virulence (7), but its role in pathogenesis and the precise mechanisms of its involvement in mycobacterial infection are yet to be defined fully. Our previous results and the results described in this study show that ESX-5 substrates do not play an important role in phagolysosome translocation, and ESX-5 deficiency seems to affect a later step in the host cell infection cycle. Despite this difference, both mutants affect macrophage cell death. We speculate that substrates of the ESX-1 system induce cell death indirectly by mediating bacterial translocation into the host cell cytosol and concomitant cathepsin B release from the phagosome, whereas ESX-5 substrates play a different role in cell death induction, possibly through the action of secreted effectors as summarized in Fig. 7.

T7SSs are reported to mediate inflammasome activation and IL-1β release during mycobacterial infections. A role for ESX-1 is described in NLRP3 inflammasome activation and IL-1β release (13, 14). As mycobacterial peptidoglycan fragment muramyl dipeptide can activate NALP3 inflammasomes (52) and ESX-1 enables phagosomal escape, Koo et al. (13) have previously suggested that ESX-1 allows entry of muramyl dipeptide or other bacterial products into cytosol to induce inflammasome activation. The ESX-1 substrate ESAT-6 was subsequently reported to have a direct activation effect on inflammasomes (16). By contrast, in this study we have observed that the ESX-5 mutant also affects

**FIGURE 6.** Cathepsin B is involved in inflammasome activation by *M. marinum*. A, B, D, E, THP-1 cells (A, B) or human primary type 1 macrophages (D, E) were infected with *M. marinum* WT or ESX-5 mutant in the presence of 40 μM cathepsin inhibitors. IL-1β secretion (A, D), caspase-1 p20 release (B, E), and pro-caspase-1 expression (E) were determined 24 h postinfection. β-Actin reflects cell lysate loading control. C, Cathepsin B was depleted in THP-1 by means of siRNA for 24 h. Subsequently, untreated, control-treated (siControl), and cathepsin B-depleted (siCathB) cells were infected for 24 h, and IL-1β secretion was determined. Representative Western blot analysis of cathepsin B levels in knockdown cell lysates (50 μg) is shown in Fig. 3F.

**FIGURE 7.** Model of T7SS-induced translocation, necrosis, and IL-1β secretion. Pathogenic mycobacteria are phagocytosed and in time translocate to the cytosol through ESX-1-secreted effector proteins. Phagosomal translocation results in cytosolic release of active cathepsin B and ESX-5 effector proteins. Together, these effectors result in inflammasome activation, IL-1β secretion, and cell death. The effects on cell death and inflammasome activation are probably independent of effector molecules secreted via ESX-1, although the extent of necrosis and IL-1β secretion are (largely) dependent on the ESX-1-dependent bacterial translocation into the cytosol. This illustration was produced using Servier Medical Art.
inflammase activation, whereas secretion of ESAT-6 and translocation into the cytosol are unaffected. As inflammase activation upon infection with *M. marinum* is reported to exacerbate disease without affecting bacterial growth (13), ESX-5 activation of inflammases is most likely beneficial for the bacterium. Therefore, we can conclude that ESX-1 and ESX-5 play distinct roles in inflammase activation. We hypothesize that ESX-1 affects both inflammase activation and onset of cell death indirectly through lysosomal rupture, translocation, and concomitant release of cathepsin B, whereas ESX-5 substrates influence these processes probably more directly through a cytosolic mechanism. We have observed that *M. marinum* further induced inflammase activation through the generation of ROS and KCl efflux, suggesting involvement of NLRP3 inflammase, which was recently also demonstrated by Carlsson et al. (15).

Lysosomal proteases of the cathepsin family have previously been implicated in a number of important cellular processes, such as protein degradation, Ag processing, and the execution of cell death (53, 54). *M. tuberculosis* and *M. marinum* both trigger the disruption of lysosomes, which leads to the release and subsequent activation of the lysosomal protease cathepsin B, triggering a newly discovered cell death pathway as well as an inflammase activation pathway. Inhibition of cathepsin B protected against cell death and blocked caspase-1 activation, indicating that cathepsin B is essential for the caspase-independent cell death triggered by T7SS substrates, as well as for inflammase activation. Although our observation of cathepsin B dependency in *M. tuberculosis*- and *M. marinum*-induced cell death and inflammase activation is novel, involvement of lysosomal proteases in *Mycobacterium*-induced cell death is not unprecedented, as Lee and colleagues (40) previously showed that lysosomal cathepsin inhibitors prevented murine macrophage cell death caused by infection with *M. tuberculosis* H37Rv. By contrast, lysosomal cathepsins have also been reported to be dispensable for macrophage cell death after *M. tuberculosis* infection (41). A role for cathepsin B in inflammase activation has also been observed for some Gram-negative pathogens (43–45).

Zhao and colleagues (55) have proposed that lysosome-dependent cell death involves a positive feedback mechanism between lysosomal membrane permeabilization and mitochondrial damage, resulting in leakage of proteins that can, in turn, increase lysosomal damage and subsequent cell death. It has also been suggested that mitochondrial depolarization constitutes the initial step for lysosomal release of cysteine-cathepsins and subsequent cell death (56). In conjunction with the findings reported in this study, it is reasonable to consider that T7SSs are involved in each of these events. Our experiments therefore support the following model (depicted in Fig. 7): once infection is established and optimal intracellular growth is achieved, mycobacteria translocate from the phagolysosome to the cytosol through the activity of ESX-1 substrates such as ESAT-6. This results in caspase-independent cell death pathways and IL-1β induction. The caspase-independent cell death will progress to necrosis and serve as a mycobacterial spreading and virulence mechanism. Because ESX-5 mutants of *M. tuberculosis* and *M. marinum* translocate from phagolysosomes but still display reduced cytotoxicity and inflammase activation, our data indicate that ESX-5 is involved directly in the induction of the cell death and IL-1β secretion through the action of cytosically secreted effectors. The induction of IL-1β was dependent on cathepsin B and stimulators of NLRP3 inflammases. More work is required to identify the molecular nature of the ESX-5 substrates responsible. One likely candidate is *M. tuberculosis* protein PE_PGRS33, as it induces primary necrosis by localizing to the host cell mitochondria (57).

In conclusion, for a successful infection, two sequential molecular mechanisms trigger two different cellular responses. First, T7SS ESX-1 mediates translocation into the cytosol. This translocation process allows subsequent release of effectors secreted by the secretion system ESX-5 into the cytosol, which triggers cytotoxicity and inflammase activation. Together, the ESX-1 and ESX-5 determine different key events in the pathogenicity of mycobacteria. Greater knowledge of the role of T7SSs in modulating host cell responses will allow further understanding of tuberculosis pathogenesis and may shape the design of future vaccines and better therapeutic drugs (58).

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References


