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The ESX-5 Secretion System of *Mycobacterium marinum* Modulates the Macrophage Response[1]

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The ESX-5 secretion system of pathogenic mycobacteria is responsible for the secretion of various PPE and PE-PGRS proteins. To better understand the role of ESX-5 effector proteins in virulence, we analyzed the interactions of *Mycobacterium marinum* ESX-5 mutant with human macrophages (Mϕ). Both wild-type bacteria and the ESX-5 mutant were internalized and the ESX-5 mutation did not affect the escape of mycobacteria from phagolysosomes into the cytosol, as was shown by electron microscopy. However, the ESX-5 mutation strongly effected expression of surface Ags and cytokine secretion. Whereas wild-type *M. marinum* actively suppressed the induction of appreciable levels of IL-12p40, TNF-α, and IL-6, infection with the ESX-5 mutant resulted in strongly induced production of these proinflammatory cytokines. By contrast, infection with *M. marinum* wild-type strain resulted in a significant induction of IL-1β production as compared with the ESX-5 mutant. These results show that ESX-5 plays an essential role in the modulation of immune cytokine secretion by human Mϕ. Subsequently, we show that an intact ESX-5 secretion system actively suppresses TLR signaling-dependent innate immune cytokine secretion. Together, our results show that ESX-5 substrates, directly or indirectly, strongly modulate the human Mϕ response at various critical steps. ***The Journal of Immunology,*** **2008, 181: 7166–7175.**

Most pathogenic mycobacteria are intracellular pathogens that reside and multiply within macrophages (Mϕ)[4] of their host. These intracellular pathogens have evolved sophisticated mechanisms to manipulate host cell organelles and membrane trafficking (1). They modify natural biological processes of host cells to create an environment that is favorable to their survival. During early stages of infection, the extent of mycobacterial survival and proliferation is mainly determined by the efficacy of the innate immune response (2). For instance, Mϕ express a variety of antimicrobial responses to control intracellular bacilli, such as bactericidal peptides and reactive oxygen and nitrogen intermediates. In addition, infected Mϕ initiate or enhance adaptive T cell immunity by Ag presentation and the induction of cellular immune responses (3, 4). Among these cellular immune responses, production of proinflammatory cytokines plays a crucial role (5–9). These cytokines include TNF-α, which plays a key role in granuloma formation (10, 11), IL-6 (12), and IL-12, a pivotal cytokine in the host defense against mycobacteria (8, 13–15). One of the mechanisms used by mycobacteria to modify Mϕ functional properties is their ability to manipulate the secretion of proinflammatory cytokines (16, 17). Exactly how pathogenic mycobacteria are able to subvert cytokine responses, however, is still unclear.

Subversion of eukaryotic host responses by bacterial pathogens often requires specialized secretion systems that deliver effector proteins near or directly into host cells. Transport of proteins across bacterial membranes is a complex process requiring multicomponent machineries spanning the bacterial cell wall. Recently, a novel secretion pathway has been identified in mycobacteria, which has been classified as type VII secretion system (18). The best-characterized type VII secretion system is encoded by the ESX-1 locus. This locus includes the RD1 region, which is deleted in the bacillus Calmette-Guérin vaccine strain. ESX-1 is involved in the secretion of the potent T cell Ags ESAT-6 and CFP-10 (19–22), which are substrates known to modulate TLR2 signaling and cytokine inhibition (23). Thus this secretion system plays a major role in the virulence of different pathogenic mycobacteria, such as *Mycobacterium tuberculosis* and *M. marinum* (reviewed in Refs. 18, 24). An active ESX-1 system has been shown recently to affect the intracellular routing of pathogenic mycobacteria because it is required for the escape of *M. tuberculosis* from the phagolysosome into the cytosol (25). In the same study it was shown that this cytosolic location predates and is important for induced cell death of infected Mϕ (25).

ESX-1 is, however, not the only type VII secretion system in mycobacteria, pathogenic mycobacteria may contain as many as five different type VII secretion systems. Recently, we have shown

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[4] Abbreviations used in this paper: Mϕ, macrophage; MOI, multiplicity of infection; DC, dendritic cell; MoDC, monocyte-derived DC; PGRS, polymorphic GC-rich repetitive sequence; PI, propidium iodide; wt, wild type.

that one of these systems, i.e., ESX-5, is responsible for the transport of all extracellular proline-glutamic acid proteins encoded by genes with polymorphic GC-rich repetitive sequence (PGRS), called PE-PGRS proteins, which are recognized by a PGRS-specific antiserum and three different proline-proline-glutamic acid proteins encoded with major polymorphic tandem repeat (PPE-MPTR) (A. M. Abdallah, T. Verboom, N. C. Gey van Pittius, M. Parra, M. J. Brennan, B. J. Appelmelk, and W. Bitter, submitted for publication) (26). The proline-glutamic acid and proline-proline-glutamic acid protein families are unique to mycobacteria and both are highly expanded in several pathogenic species, such as *M. tuberculosis* and *M. marinum* (27). Although their exact function is unknown, a role in virulence (28, 29), antigenic variation or immune evasion has been predicted (30–33). In this study we characterize the effects of *M. marinum* ESX-5 system on Mϕ function. Our results show that ESX-5 effector molecules clearly manipulate the induction of different Mϕ cytokines following multiple TLR-dependent signaling pathways.

Materials and Methods

Bacterial strains and growth conditions

Wild-type (wt) *M. marinum* E11 strain and mutant 7C1 were routinely grown in Middlebrook 7H9 liquid medium or Middlebrook 7H10 agar supplemented with 10% Middlebrook ADC or OADC albumin-dextrose or oleic acid-albumin-dextrose catalase, respectively (BD Biosciences), and 0.05% Tween 80. For infection experiments, *M. marinum* cultures were grown to logarithmic phase (OD_{600} = 0.5–0.8) in 7H9 medium, washed, and diluted in RPMI 1640 with 10% FCS. To eliminate clumps of bacteria, the *M. marinum* suspensions were subjected to low-speed centrifugation at 300 × *g* for 10 min after which the supernatant was passed through a 5-μm filter and used at a multiplicity of infection (MOI) of 10 bacteria per cell, unless indicated otherwise. The resulting infection mixtures contained a low number of dead bacteria (<4%), as was determined by microscopic counting and CFU plating. Antibiotics were added at concentrations of 25 μg/ml kanamycin and 50 μg/ml hygromycin.

Cells lines and culture conditions

For localization and phagosome isolation experiments, the human acute pro-monocytic leukemia THP-1 cell line was used. Cells were cultured in RPMI 1640 with glutamax-1 medium (Life Technologies) supplemented with 10% FCS, streptomycin, and penicillin. Monocytic differentiation into Mϕ-like cells was induced with PMA (Sigma-Aldrich) at 10 ng/ml final concentration (overnight incubation).

Isolation and culture of human Mϕ and dendritic cells (DC)

Monocytes were isolated to high purity from peripheral blood of healthy donors by magnetic cell sorting using anti-CD14-coated beads; isolated cells were polarized with 5 ng/ml recombinant human GM-CSF to obtain type 1 Mϕ (Mϕ-1) or with 50 ng/ml recombinant human M-CSF to generate type 2 Mϕ (Mϕ-2) as previously described (34). Monocyte-derived DC (MoDC) were generated with 100 ng/ml GM-CSF and 500 U/ml IL-4 as previously described (35).

Infection of Mϕ and electron microscopy

THP-1 cells were used between passages 13 and 20. For phagosome isolation, cells were seeded in 7.5 cm diameter flasks. For apoptosis assays, cells were seeded at 2 and 5 × 10^4 cells per well in 96-well flat-bottom tissue culture plates or 5 and 2 × 10^5 cells/well in 24-well plates containing round glass coverslips, respectively. Human Mϕ-1 and Mϕ-2 were cultured in duplicate at density of 3 × 10^5 per well in 24-well culture plates for cytokines analysis or 9 × 10^5 per well in 6-well culture plates for cell death and apoptosis analysis as described (34, 36). Mononuclear cells were infected with bacteria and incubated for 1 h at 33°C and 5% CO_2. The supernatant was removed and the infected cells were washed three times with medium to remove extracellular bacteria. Subsequently, the cells were incubated in fresh medium with FCS at 33°C for indicated time periods. For electron microscopy, cells were fixed with paraformaldehyde and gluteraldehyde for 2 h, similar as previously described (37). After sectioning 50-nm thick sections at −120°C, sections were immunogold-labeled with LAMP-1 (clone H4A3 from BD Biosciences) or CD63 (M1544 from Sanquin) and protein A-conjugated to 10 nm gold (Electron Microscopy, Utrecht University, Utrecht, The Netherlands). Sections were examined using a FEI CM10 transmission electron microscope. Quantification was done according to stereological methods (37).

Cell death assays

The percentage of apoptotic cells was determined using Annexin V staining of membrane alterations and propidium iodide (PI) staining of dead cells. For Annexin V-PE binding, cells were collected, washed twice with cold PBS, and resuspended in Annexin V-PE binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$). An aliquot of 100 μl (~1 × 10^5 cells) was removed and mixed with 5 μl of Annexin V-PE and 2 μl of PI. The mixture was vortexed and incubated for 15 min at room temperature in the dark. The cells were washed once with binding buffer and resuspended in 500 μl of binding buffer for analysis by flow cytometry.

Cytokine measurements

Cultured human Mϕ (Mϕ-1 and Mϕ-2) supernatants were assayed for cytokine levels using specific ELISAs for IL-10 and IL-12p40 (sensitivity: 20 pg/ml; purchased from BioSource International). The concentration of IL-1β, IL-6, and TNF-α (sensitivity: 10, 7, and 18 pg/ml, respectively) was measured by Fluorescent Bead Immunoassay (Bio-Plex human cytokine assay; Bio-Rad).

Mycobacterial phagosomes isolation and analysis

Isolation of mycobacterial phagosomes was performed as previously described (26). Briefly, infected THP-1 cells (7–10 × 10^7 with MOI of 25 for 48 h) were homogenized in DGE buffer (10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, 0.25 M sucrose (pH 7.4)) by passing through a 23-gauge needle. After removal of the cell nuclei by low-speed centrifugation at 250 × *g* for 10 min, the resulting postnuclear supernatant was transferred to a fresh Eppendorf tube and sedimented at 35,000 × *g* for 30 min. The resulting supernatant corresponds to the Mϕ cytosol, whereas the pellet corresponds to the phagosomes containing the mycobacteria. The bacteria-free phagosomes were recovered, by treating them with 1% Triton X-100 for 15 min at room temperature and sedimenting the intact mycobacteria at 35,000 × *g* for 30 min. The resulting supernatant corresponds to the phagosomes and the pellet to the mycobacteria. All protein mixtures were separated by SDS-PAGE using 10–12% polyacrylamide gels. Proteins were visualized by immunoblotting using Abs directed against PE-PGRS (A. M. Abdallah et al., submitted for publication), GroEL (from Colorado State University, Fort Collins, CO, and the National Institutes of Health, Bethesda, MD, under contract NO1 AI-75320), and LAMP-1 (H4A3, obtained from the Hybridoma Bank, University of Iowa, Iowa City, IA).

Results

ESX-5 is not required for escape of M. marinum *into the cytosol*

The intracellular mycobacterial pathogens *M. marinum* and *M. tuberculosis* have recently been shown to escape from the phagolysosome into the cytosol of host cells (25, 38, 39), and deletion of effector molecules of the ESX-1 pathway are shown to abrogate this translocation (25). To gain further insight into this process, we examined the effect of the ESX-5 pathway on the translocation of *M. marinum* into the cytosol. To address this effect, we compared the intracellular localization of the *M. marinum* wt and the ESX-5 mutant by cryo-immunogold electron microscopy as previously described (40). As a control we also included *M. bovis* bacillus Calmette-Guérin, which does not escape into the cytosol. THP-1 cells and MoDC infected with the different mycobacterial strains were fixed and processed for electron microscopy and cryo-immunogold labeling. In agreement with our previous results, all *M. bovis* bacillus Calmette-Guérin bacteria resided in LAMP-1- or CD63-labeled phagosomes (data not shown). After 24 h of infection, a substantial proportion of *M. marinum* wt bacteria were localized in the cytosol of the infected Mϕ and MoDC (Fig. 1*B*), as was shown previously (38). These bacteria were not surrounded by an extra membrane (Fig. 1*B′*) or associated with the phagosome/lysosome transmembrane proteins LAMP-1 or CD63. The ESX-5 mutant of *M. marinum* was also located in the cytosol of

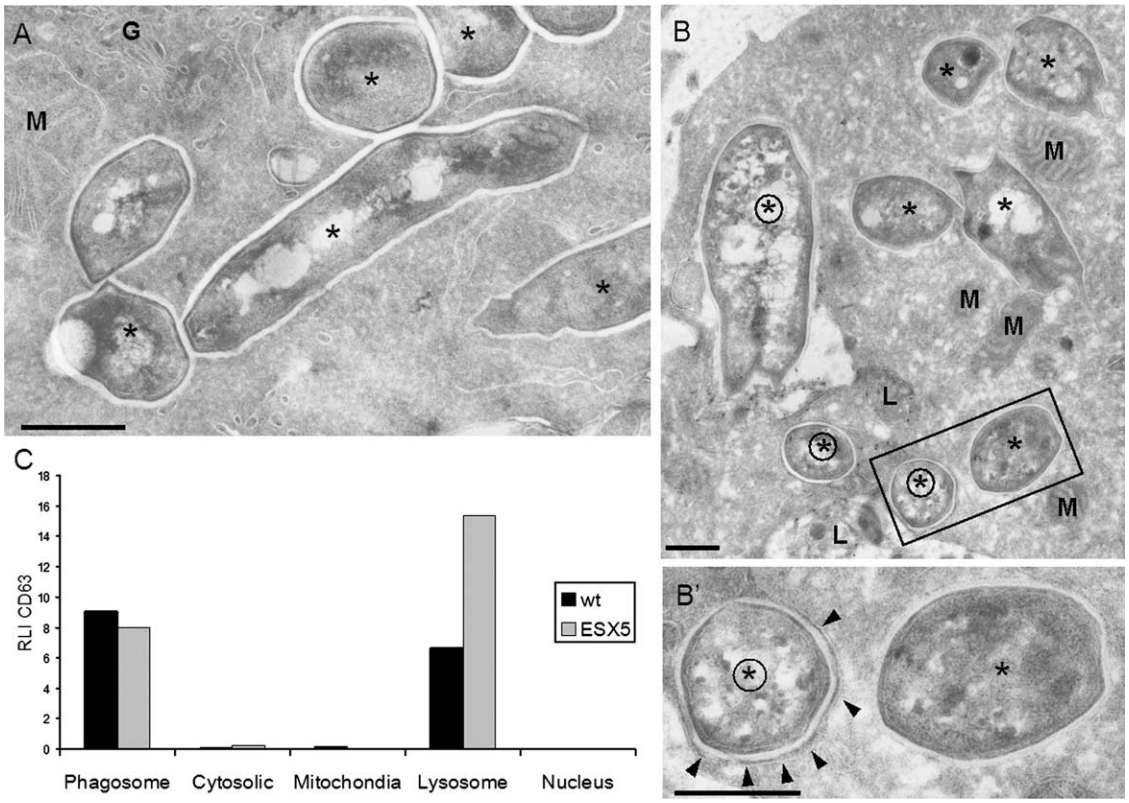

FIGURE 1. *M. marinum* wt E11 strain and ESX-5 mutant translocate from the phagolysosome to the cytosol of THP-1 cells. Representative electron micrograph (EM) image of a LAMP-1-labeled THP-1 cell infected with ESX-5 mutant for 24 h (*A*), and a THP-1 cells infected with *M. marinum* wt E11 strain for 24 h (*B*) with enlargement of phagosomal membrane (indicated with arrowheads) and cytosolic bacterium (*B′*). Scale bar represents 300 nm. Asterisk indicates cytosolic mycobacteria and encircled asterisk indicates phagosomal mycobacteria. G, golgi; L, lysosomes; M, mitochondria; N nucleus. *C*, Quantification of CD63 labeling was performed on THP-1 cells, infected for 24 h with either the wt strain or the ESX-5 mutant, by determining the relative labeling index (RLI) based on the number of gold particles per square micromillimeter on the phagosomal membrane.

the infected Mϕ (Fig. 1*A*) and MoDC (data not shown), which indicates that this mutation does not affect routing of the bacteria. To analyze this affect, we quantified the phagosomal localization of both wt and ESX-5 mutant bacteria by determining the relative CD63 labeling index (37) on the phagosomal membranes, around cytosolic bacteria and on different cellular compartments (Fig. 1*C*). CD63 labeling was only observed for phagosomes and lysosomes. Furthermore, from the quantitative analysis it can be concluded that disruption of the ESX-5 secretion complex does not affect CD63 labeling of the phagosomal membrane. Thus the ESX-5 effector molecules have no effect on the routing of these bacteria in infected host cells.

Next, we tested whether the ESX-5 effector molecules are secreted in human Mϕ. To test this response, THP-1 cells differentiated into Mϕ-like cells in the presence of PMA were infected with *M. marinum* E11 and differentially disrupted. The various subcellular fractions were analyzed by immunoblot for the presence of PE-PGRS proteins (A. M. Abdallah et al., submitted for publication), the major class of ESX-5 substrates. In addition, immunoblots containing these fractions were incubated with Abs directed against the bacterial cytoplasmic protein GroEL and the late endosome/lysosome marker LAMP-1 as controls (Fig. 2, *lanes 1–5*). After 24 h of infection, most of the PE-PGRS proteins were found associated with the bacteria, which could represent intrabacillary or surface-located PE-PGRS molecules. However, after 48 h of infection, a substantial amount of PE-PGRS proteins was present in the cytosol fraction of the Mϕ (at 48 h, Fig. 2*A*, *lane 2*). The cytoplasmic control protein GroEL of *M. marinum* was exclusively present in the bacteria-containing fraction (Fig. 2*B*, *lane 4*), which indicates that the presence of PE-PGRS proteins in the cytosol of the infected cell was not due to bacterial lysis inside the Mϕ. The other control protein, LAMP-1, was identified mainly in the Triton X-100 soluble fraction, representing the contents of Mϕ-derived vesicles as expected (Fig. 2*B*, *lane 3*). In conclusion, this experiment shows that PE-PGRS proteins are indeed secreted inside Mϕ and in particular after 48 h of infection and in the fraction representing the Mϕ cytosol.

Dose response of M. marinum-*induced Mϕ cell death*

To determine the MOI at which infected cells showed minimal signs of cell death after 24 h postinfection, we assessed the relationship between intracellular bacillary load and Mϕ viability. To this purpose, we used freshly isolated human peripheral blood monocytes differentiated into Mϕ-1 and Mϕ-2 subsets as previously described (34, 36). Briefly, Mϕ-1 are considered proinflammatory with high expression of MHC class II and costimulatory molecules such as CD80 and CD86. Mϕ-1 secrete IL-23 and IL-12p70 in the presence of IFN-γ. Mϕ-2 subverted Mϕ-1 immunity and thus may promote chronic infection. Mϕ-2 secrete IL-10 but produce neither IL-23 nor IL-12p70 and have low expression of MHC class II and CD80/CD86. Both Mϕ subsets were challenged with *M. marinum* wt E11 strain or ESX-5 mutants over an MOI ranging from 5 to 80.

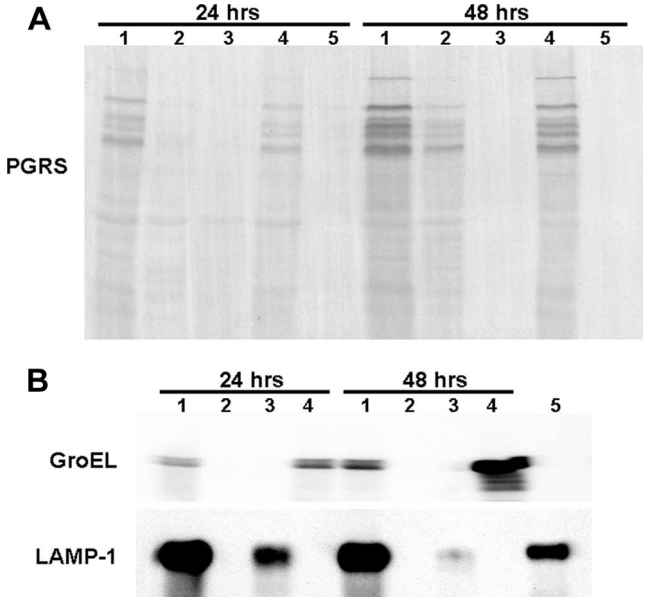

FIGURE 2. Intracellular secretion of PE-PGRS by *M. marinum*. Immunoblot analysis of THP-1 cells infected for 24 or 48 h with *M. marinum* wt E11 strain. Infected cells were homogenized and cell debris was removed by low-speed centrifugation. Subsequently, mycobacteria-containing phagosomes and other vesicles (*lane 1*) were separated from the cell cytosol (*lane 2*) by sedimentation. Isolated phagosomes were lysed by Triton X-100 treatment (*lane 3*) and bacteria were collected by sedimentation (*lane 4*). As a control we also analyzed uninfected THP-1 cells (*lane 5*). The different fractions were analyzed for the presence of PE-PGRS proteins (*A*), for the presence of the cytoplasmic mycobacterial protein GroEL, and for the presence of the late endosome/lysosome marker protein LAMP-1 (*B*).

Mϕ viability was assessed by flow cytometry following PI and Annexin V staining. Both strains caused low levels of Mϕ cell damage at low MOI, but a steep increase in PI-positive cells by 24 h was observed with MOI values above 10 (Fig. 3, data shown for Mϕ-1). Early phases of apoptosis were not detected by Annexin V-positive (data not shown); therefore cell death

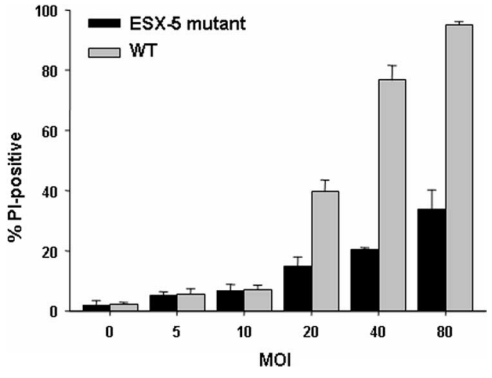

FIGURE 3. High MOI challenge with *M. marinum* rapidly induces cell death in Mϕ-1 cells, whereas the ESX-5 mutant showed a more reduced effect. Mϕ-2 showed the same results (data not shown). Mϕ subsets were infected with *M. marinum* wt E11 strain or its isogenic ESX-5 mutant over a range of MOI for 24 h and stained with PI and Annexin V for analysis by flow cytometry to assess cell viability. A total of 10,000 events were analyzed per sample. Results are expressed as the mean percentage of PI-positive cells ± SE for two donors. Similar results were obtained in two individual experiments.

was determined to be necrotic rather than apoptotic. In addition, *M. marinum* wt cells induced a considerably higher percentage of dead cells as compared with the ESX-5 mutant (Fig. 3). Therefore, the subsequent experiments were designed with an MOI of 10 to analyze cell surface phenotypes and to measure cytokine response of infected Mϕ-1 and Mϕ-2 cells.

ESX-5 effector molecules modify phenotypic maturation of Mϕ

The effect of ESX-5 effector molecules on the cell surface expression of markers involved in Ag presentation and T cell activation was analyzed. Mϕ-1, Mϕ-2, and MoDC were infected with *M. marinum* wt and the ESX-5 mutant and subsequently surface expression of MHC class II DR (HLA-DR), CD80 (B7-1), and CD86 (B7-2) was examined (Fig. 4). Mϕ-1 cells infected with either *M. marinum* wt or ESX-5 mutant showed no changes in the surface expression of the costimulatory molecules CD80, CD86, or HLA-DR, whereas the addition of LPS as a control resulted in the induction of especially CD86 (Fig. 4*A*). However, infection of the Mϕ-2 cells with the ESX-5 mutant showed marked differences when compared with cells infected with wt bacteria. Whereas *M. marinum* wt suppressed the expression of CD80, CD86, and HLA-DR in Mϕ-2 cells, cells infected with ESX-5 mutant showed even an induction of these surface markers, somewhat comparable to the LPS stimulus (Fig. 4). Subsequently, we also tested the effect of the ESX-5 mutation on the expression of these surface markers in MoDC. In DC, the ESX-5 mutant showed a strong increase in CD80, CD86 expression and significant up-regulation of HLA-DR, again comparable to the effect of LPS. Similar to the effects observed in Mϕ-2, *M. marinum* wt-infected MoDC showed partial down-regulation of HLA-DR, CD80, and CD86 expression, respectively, as compared with ESX-5 mutant-infected MoDC (Fig. 4). Down-regulation of HLA-DR, CD80, and CD86 in Mϕ-2 and MoDC by *M. marinum* is in line with previous results describing an attenuated capacity for MHC class II-restricted cells in Ag presentation and costimulation of Th cells following mycobacterial infection (41–43). Therefore, we conclude that ESX-5 effector molecules may diminish the capacity of Mϕ-2 and MoDC for presentation of mycobacterial Ags.

ESX-5 alters the innate immune responses of infected Mϕ

Mϕ secrete a number of proinflammatory cytokines that are essential in eliciting a protective immune response against intracellular pathogens such as mycobacteria. To determine whether ESX-5 secreted proteins are involved in the manipulation of these cytokine responses, we investigated the response of human Mϕ to infection with wt *M. marinum* or with its isogenic ESX-5 mutant. Mϕ-1 cells infected with wt *M. marinum* produced low concentrations of IL-12p40 (Fig. 5*A*), as has been shown previously also for Mϕ infected with *M. tuberculosis* (13, 14). Surprisingly, Mϕ-1 infected with the ESX-5 mutant readily secreted high amounts of IL-12p40, in levels comparable with cells induced by 10 ng/ml LPS (Fig. 5*A*). IL-12p40 can form either IL-12p70 or IL-23, depending on which molecule it pairs with (IL-12p35 or IL-23p19, respectively). It has been shown previously that Mϕ-1 cells mainly produce IL-23 upon infection with *M. tuberculosis*, whereas the production of IL-12p70 is dependent on the presence of exogenous IFN-γ (34). Therefore, we also tested the expression of IL-12p40 in the presence of IFN-γ. For cells infected with *M. marinum* wt, the presence of exogenous IFN-γ hardly affected the level of IL-12p40 production (Fig. 5*A*), whereas the ESX-5 mutant induced even higher levels of IL-12p40 in the presence of exogenous IFN-γ (Fig. 5*A*). A similar effect was observed for the

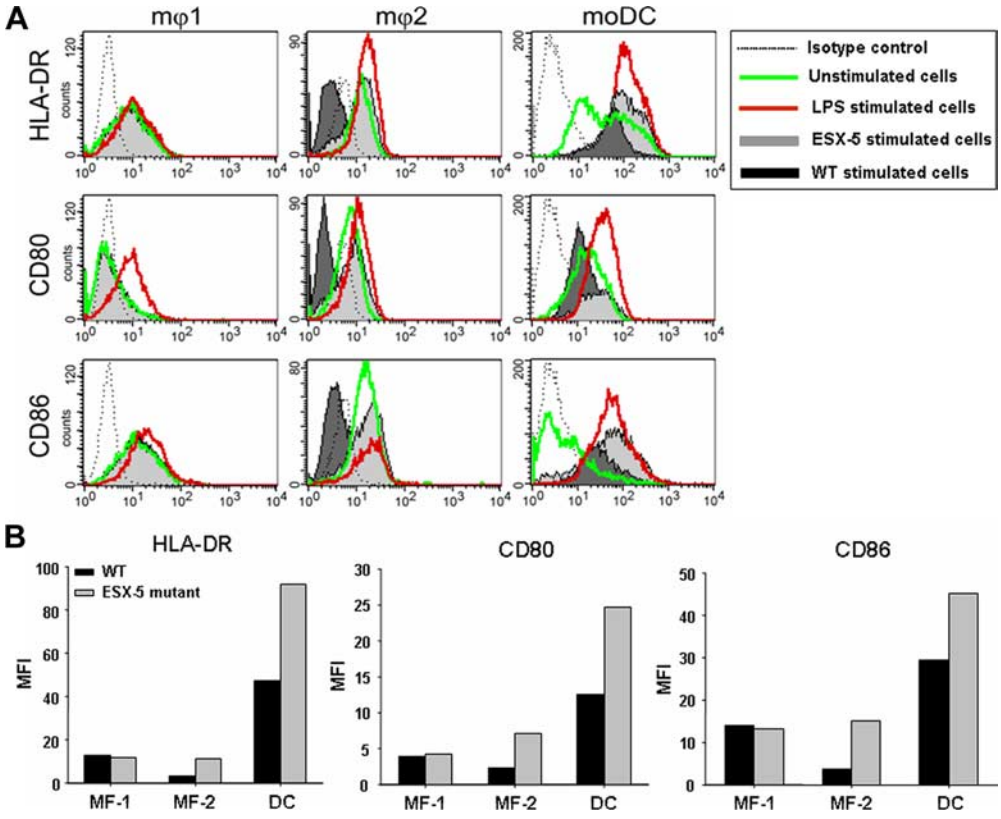

FIGURE 4. Expression of HLA and costimulatory molecules CD80 and CD86 on Mϕ-1, Mϕ-2, and MoDC infected with *M. marinum* wt E11 strain or ESX-5 mutant. *A*, Cells were infected with MOI of 10 for 24 h, and the cell surface phenotypes were analyzed by FACS. A total of 10,000 events were analyzed per sample. As a control the cells were also stimulated with LPS. *B*, Mean fluorescence intensity (MFI) has been calculated. FACS profiles are representative of one experiment, which was repeated three times, using Mϕ from different blood donors.

induction of IL-6 and TNF-α, i.e., both these proinflammatory cytokines were strongly enhanced in Mϕ-1 infected with the ESX-5 mutant strain as compared with the parent strain (Fig. 5*A*).

To determine whether the observed effect on cytokine induction is an active process, we compared live and heat-killed bacteria. Heat-killed ESX-5 mutant elicited high amounts of IL-12p40 secretion, similar to the live ESX-5 mutant. However, Mϕ-1 infected with heat-killed *M. marinum* wt cells elicited high amounts of IL-12p40, comparable to the ESX-5 mutant (Fig. 5*B*). Together, these results show that the ESX-5 mutant is unable to actively manipulate the production of various proinflammatory cytokines by Mϕ-1.

Mϕ-2 cells did not secrete significant amounts of IL-12p40, IL-6, or TNF-α upon mycobacterial infection (Fig. 5*C*), as was also shown previously for *M. tuberculosis* (34). Because IL-10 is a potent down-regulator of the immune response and known as an attenuator of IL-12p40 production in Mϕ (13, 44), we measured the amount of IL-10 elicited by wt bacteria and the ESX-5 mutant from both Mϕ subsets. However, no IL-10 was detected in any of the conditions (data not shown).

Recognition of *M. tuberculosis* by human monocytes leads to IL-1β production (13). Surprisingly, whereas IL-1β production was indeed enhanced in Mϕ-1 infected with wt *M. marinum*, no significant induction was detected in Mϕ-1 infected with the ESX-5 mutant (Fig. 5*A*). A similar result was obtained for Mϕ-2-infected cells (Fig. 5*C*). Secretion of IL-1β is dependent on live bacilli because Mϕ-1 cells infected with heat-killed wt bacteria showed strongly reduced extracellular IL-1β levels as compared with cell infected with live wt bacteria (Fig. 5*B*).

To determine whether an active process was responsible for the suppression of IL-12p40 production in cells infected with wt *M. marinum*, we mixed wt bacilli with either the ESX-5 mutant or with LPS. We found that the presence of wt bacteria strongly suppressed the release of IL-12p40, normally induced by the ESX-5 mutant and LPS (Fig. 6*A*). In addition, wt *M. marinum* also suppressed the release of IL-6 and TNF-α, although for these cytokines the effect was less dramatic (Fig. 6, *B* and *C*). Finally, the presence of wt E11 always resulted in the induction of significant amounts of IL-1β, irrespective of the presence of the ESX-5 mutant (Fig. 6*D*). From these experiments we conclude that ESX-5 effector proteins actively manipulate the cytokine responses of Mϕ-1 cells.

ESX-5 pathway inhibits TLR signaling

TLRs play an important role in the detection of pathogen-associated molecules. However, TLRs also may be targeted by mycobacteria as a mode of immune evasion. Mycobacterial suppression of TLR signaling would be in accordance with the observed suppression of the LPS-mediated induction of IL-12p40 by wt *M. marinum* (Fig. 6). Therefore, we tested the possibility that ESX-5 effector proteins exert an attenuating effect on TLR signaling. To address this possibility, we stimulated Mϕ-1 cells with various prototypical TLR ligands in the presence or absence of *M. marinum* E11 wt or ESX-5 mutant bacilli. The TLR ligands LTA (50 ng/ml), LPS (10 ng/ml), Zymosan A (100 ng/ml), and CL075 (1 ng/ml) stimulate TLR2, TLR4, TLR2/6, and TLR8/7, respectively. All these TLRs signal through the adapter molecule MyD88. Release of IL-12p40 was used as a readout of TLR activation. In line with the data in Fig. 6*A*, infection with wt bacteria resulted in the suppression of IL-12p40 production triggered by all of the different TLR ligands (Fig. 7*A*), whereas the ESX-5 mutant had an additive

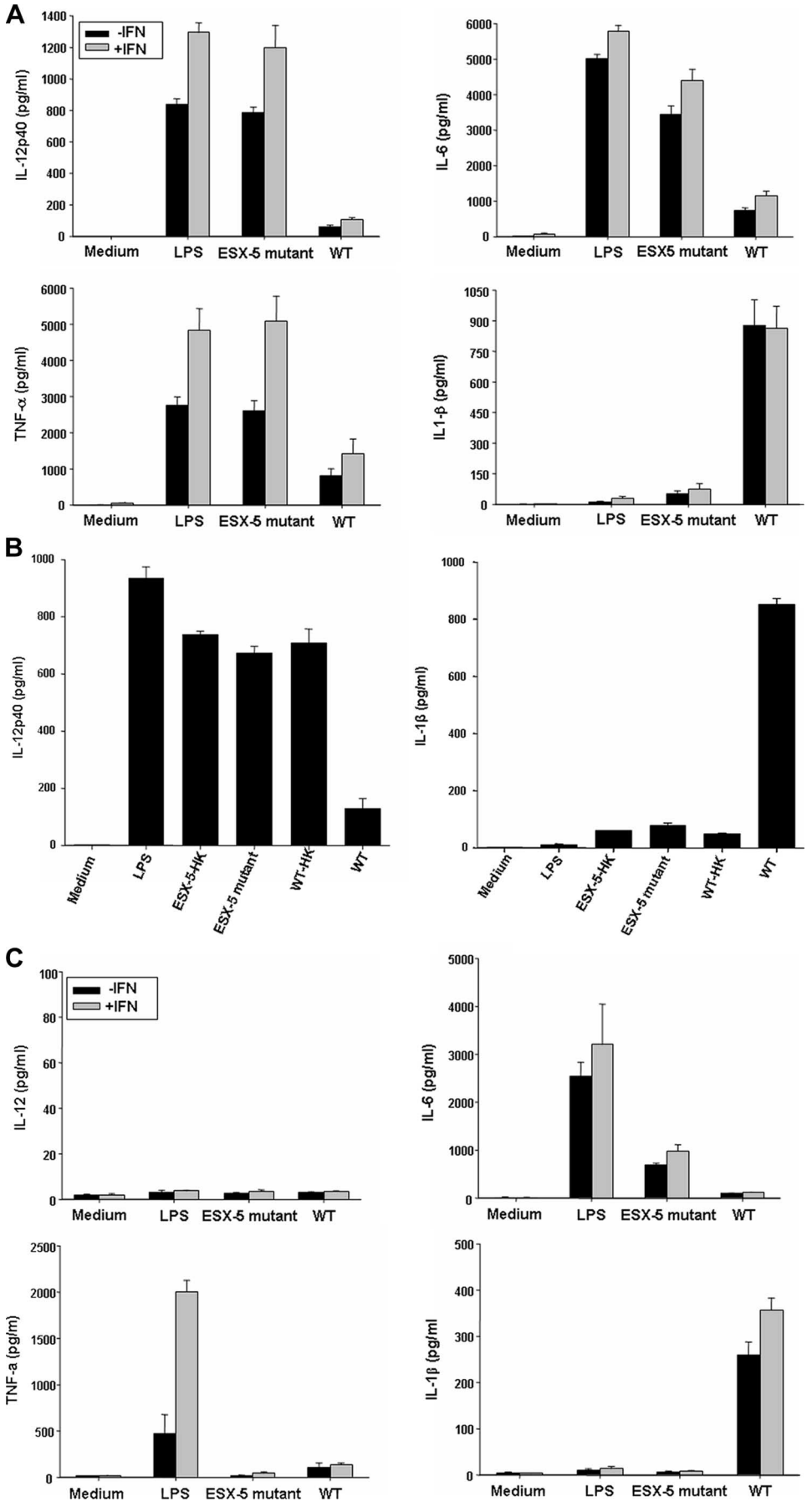

FIGURE 5. ESX-5 effector proteins manipulate the Mϕ inflammatory response. Human monocyte-derived Mϕ-1 (*A* and *B*) and Mϕ-2 (*C*) were infected with *M. marinum* wt and the ESX-5 mutant, or treated with LPS as a control. As a control, heat-killed cells (HK) of both bacterial strains were used to stimulate the cells. Culture supernatants were collected after 24 h of infection and analyzed for the presence of proinflammatory cytokines IL-12p40 by ELISA or for IL-6, TNF-α, and IL-1β by Fluorescent Bead Immunoassay. Each sample was assayed in triplicate and error bar represents mean $\pm$ SE from at least three experiments. Similar results cytokine profiles were obtained with cells from at least five independent donors.

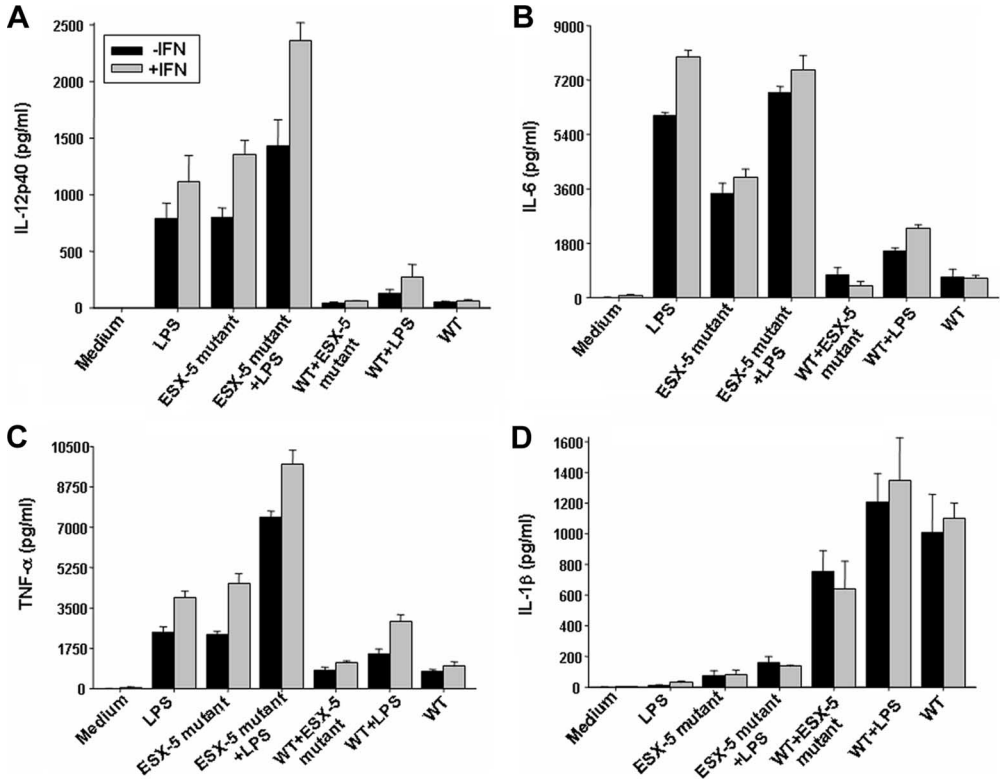

FIGURE 6. ESX-5 inhibits the heterologous induction of type 1 cytokines. *M. marinum* wt bacteria were mixed with the ESX-5 mutant or with LPS to measure active suppression of cytokine induction. Mϕ-1 was infected and culture supernatants were collected and the concentration of IL-12p40 (*A*), IL-6 (*B*), TNF-α (*C*), and IL-1β (*D*) were measured by ELISA and by Fluorescent Bead Immunoassay as mentioned previously. Results represent mean $\pm$ SE of two separate experiments.

effect on IL-12p40 production. The addition of IFN-γ had no effect on mycobacterial suppression of TLR signaling (data not shown). We also analyzed whether ESX-5 effector proteins could affect the secretion of IL-1β, IL-6, and TNF-α. Akin to the IL-12p40 results, only the wt bacteria were able to suppress the release of IL-6 secretion triggered by the different TLR

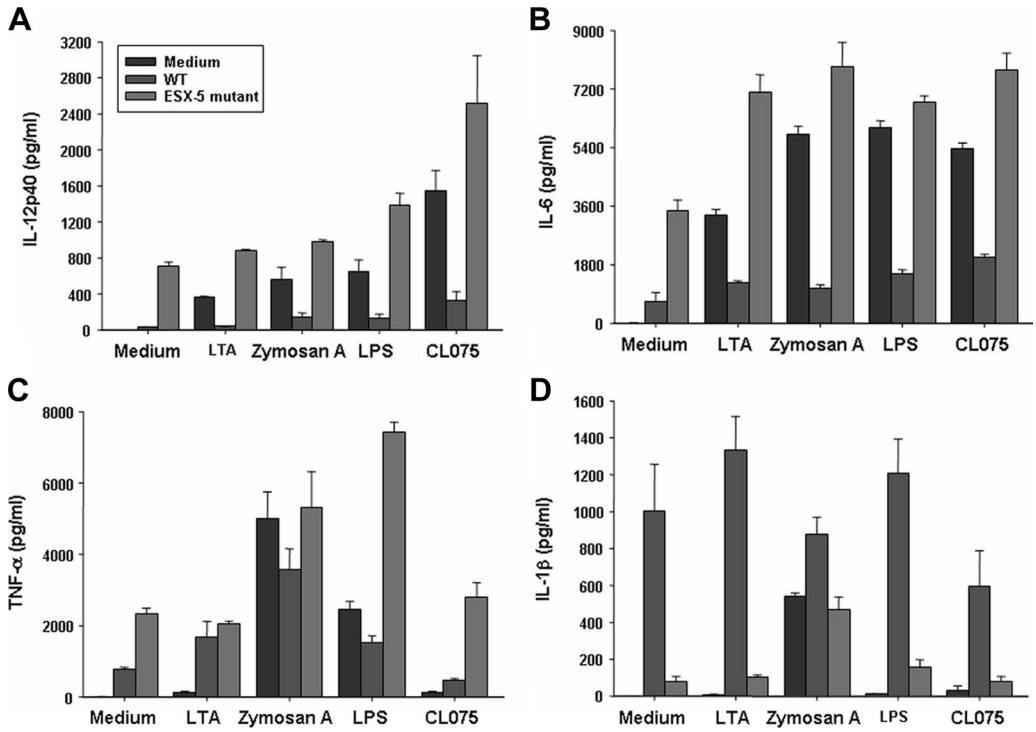

FIGURE 7. ESX-5 inhibits cytokine production elicited by TLR2, TLR4, TLR2/6, and TLR8/7. Mϕ-1 were infected and stimulated with various TLR ligands. After 24 h, culture supernatants were collected and the concentration of IL-12p40 (*A*), IL-6 (*B*), TNF-α (*C*) and IL-1β (*D*) was measured by ELISA and by Fluorescent Bead Immunoassay as previously mentioned. Results represent mean $\pm$ SE of two separate experiments.

ligands (Fig. 7*B*). The results for TNF-α were more variable, and wt *M marinum* only seemed to affect induction of this cytokine by LPS and CL075 (Fig. 7*C*). Conversely, whereas wt bacilli always showed induction of IL-1β, no considerable release of IL-1β was observed if the cells were stimulated through TLR2, TLR4, or TLR8/7 ligands in the presence of the ESX-5 mutant. Only the addition of the TLR2/6 ligand Zymosan A triggered significant IL-1β secretion (Fig. 7*D*). From these results we conclude that ESX-5 effector molecules attenuate signaling through TLR2, TLR4, TLR2/6, and TLR7/8.

Discussion

Pathogenic mycobacteria such as *M. tuberculosis* and *M. marinum* manipulate innate immune responses to achieve balanced inflammation, which allows long-term persistence. Although several studies have demonstrated that mycobacteria are able to dampen or subvert the innate immune response (13, 14, 17), the molecular mechanism underlying this phenomenon is still largely unknown. Our previous results have indicated that the ESX-5 secretion system might be responsible for a defect in the Mϕ infection cycle (26). However, a problem of these experiments is that they were performed in a strain that was later shown to be impaired in ESAT-6 secretion (A. M. Abdallah et al., submitted for publication). Therefore, we analyzed in this study the effect of a virulent wt strain and its isogenic ESX-5 mutant on cell infection, cytokine induction and immune regulation. ESX-5 is responsible for the secretion of a large number of proteins, including the PE-PGRS and PPE-MPTR proteins (A. M. Abdallah et al., submitted for publication). The proline-glutamic acid and proline-proline-glutamic acid protein family are both specific for mycobacteria, but their function is as yet unknown. However, their number is dramatically increased in several pathogenic mycobacteria, such as *M. marinum* and *M. tuberculosis*, suggesting a role in virulence.

First, the expression of Mϕ and DC cell surface markers was analyzed. Mϕ-2 and MoDC showed a significant down-modulation of HLA-DR, CD80, and CD86 following *M. marinum* wt infection as previously reported (41, 45). Conversely, ESX-5 mutant infected Mϕ-2 and MoDC showed induced expression of these surface markers. Conversely, the enhanced binding, uptake and endocytosis of mycobacteria by Mϕ-2 compared with Mϕ-1 (34), may account for the difference in the expression of costimulatory and molecules. Thus, our results suggest that the ESX-5 pathway impairs expression of class II and CD80, CD86 in Mϕ-2, and MoDC cells; this impairment in turn may affect subsequent T cell activation.

Next, the production of cytokines by Mϕ infected with the ESX-5 mutant was studied. It is well established that the production of proinflammatory cytokines, such as IL-12 and TNF-α, is crucial for optimal host defense against mycobacterial infection (6, 8). Furthermore, studies comparing pathogenic vs nonpathogenic mycobacteria have indicated that mycobacterial virulence has a tendency to be inversely correlated with the secretion of proinflammatory cytokines (46, 47). In the present study, we demonstrate that wt bacteria, but not the ESX-5 mutant, are able to suppress the production of IL-12p40, IL-6, and TNF-α. Previous studies showed that ESAT-6 modulates TLR2 signaling and thus cytokine secretion (23). However, this modulation is not reason for the observed changes in cytokine secretion in this study because ESX-5 mutant is not impaired in ESAT-6 secretion (A. M. Abdallah et al., submitted for publication). Perhaps an active ESX-5 secretion system could, in analogy to the ESX-1 system (25), be essential for the proper routing of the mycobacteria within the Mϕ to manipulate the host immune system. This possibility is, however, refuted by us because we showed that ESX-5 mutants, like wt bacteria, are able to escape from the phagolysosome into the cytosol. Interestingly, although the cellular localization between the ESX-1 and the ESX-5 mutant is different, the observed effects on Mϕ function of both mutants are comparable (21). A possible explanation for this observation is that the ESX-1 system is needed for the cytosolic localization of *M. marinum*, which allows ESX-5 effector molecules to be secreted in the cytosol and directly or indirectly manipulate the immune response from within the infected cells.

Verreck et al. (34) demonstrated that mycobacterial stimulation of Mϕ-1 initially results in the secretion of IL-23, but that the secretion of IL-12p70 is dependent on IFN-γ as an essential second signal. Therefore, the high IL-12p40 levels, observed in response to ESX-5 mutant infection after addition of exogenous IFN-γ suggest that the ESX-5 effector molecules suppress the capacity of Mϕ-1 to produce both IL-12p70 and IL-23.

Targeting TLR signaling is a strategy of immune evasion used by different intracellular bacteria and viruses. There are several negative regulators of IL-1R/TLR signaling, most of which seem to target the MyD88-dependent signaling cascade, including MyD88s (the short form of MyD88), IRAK-M, SOCS1, and Toll-interacting protein, depending on the cell type and the nature of the stimuli (48). In our present study, we examined the effect of ESX-5 on TLR signaling and show that many different TLRs are repressed by an ESX-5-dependent mechanism. Our data presented are intriguing, as they suggest ESX-5 could exert its inhibitory effect by attenuating MyD88-dependent TLR signaling. Further studies are required to assess the detailed mechanisms underlying this inhibitory effect of ESX-5 and to assess the effects of ESX-5 on MyD88-independent TLR signaling.

Although the ESX-5 mutant, compared with the wt bacilli, had enhanced induction of IL-12p40, IL-6, and TNF-α in infected Mϕ, the cells infected with the ESX-5 mutant triggered no IL-1β secretion. In contrast, Mϕ infected with *M. marinum* wt showed enhanced IL-1β secretion, suggesting that a different mechanism is involved in this process. A possible explanation for this observation is that ESX-5 effectors are, either directly or indirectly, involved in the induction of IL-1β activation, possibly through activation of caspase-1 (49, 50). Activation of caspase-1 via microbial components in the host cytosol through NLR proteins has been previously described. For instance, *Salmonella typhimurium* uses a type III secretion system to secrete SipB into the cytosol of Mϕ, which binds and activates caspase-1 in infected cells (51, 52). Similarly, *Helicobacter pylori* uses a type IV secretion system for the delivery in the cytosol of infected host cells of peptidoglycan-derived molecules, which activates Nod1, another NLR family member (53). Thus, ESX-5 effector molecules may interact, either directly or through another host factor in the cytosol, with inflammasome adaptors to promote the activation of caspase-1. This activation will lead to the concomitant release of proinflammatory cytokine IL-1β and the induction of osmotic death of the infected cells in vitro. Additional studies are required to unravel the mechanism that is responsible of the absence of IL-1β production from ESX-5 mutant infected Mϕ.

The wt mycobacteria were able to induce cell death, whereas the ESX-5 mutant was significantly impaired in this process. At present it is unclear, however, whether host or bacterial cell factors are driving *M. marinum*-specific apoptosis. Although *M. marinum*-infected wt Mϕ-1 did not secrete significant amounts of TNF-α, they did secrete IL-1β, which may contribute to cytotoxicity and cell death. Additional studies to explore the

precise mechanisms leading to apoptosis and cell death are required.

In conclusion, we have demonstrated that a functional ESX-5 pathway is a pivotal component required for the successful manipulation of the host Mϕ by mycobacteria. The effects were found at several levels, namely in induction of cell death, expression of cell surface Ags and cytokine production, all of which favor immune evasion by the bacilli. Future experiments are necessary to determine which effector molecule or, more likely, molecules are involved in these processes and what the host targets are of these secreted effector molecules.

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Disclosures

The authors have no financial conflict of interest.

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