Mycobacterium tuberculosis Subverts Innate Immunity to Evade Specific Effectors[1]

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The macrophage is the niche of the intracellular pathogen *Mycobacterium tuberculosis*. Induction of macrophage apoptosis by CD4$^+$ or CD8$^+$ T cells is accompanied by reduced bacterial counts, potentially defining a host defense mechanism. We have already established that *M. tuberculosis*-infected primary human macrophages have a reduced susceptibility to Fas ligand (FasL)-induced apoptosis. To study the mechanisms by which *M. tuberculosis* prevents apoptotic signaling, we have generated a cell culture system based on PMA- and IFN-γ-differentiated THP-1 cells recapitulating the properties of primary macrophages. In these cells, nucleotide-binding oligomerization domain 2 or TLR2 agonists and mycobacterial infection protected macrophages from apoptosis and resulted in NF-κB nuclear translocation associated with up-regulation of the antiapoptotic cellular FLIP. Transduction of a receptor-interacting protein-2 dominant-negative construct showed that nucleotide-binding oligomerization domain 2 is not involved in protection in the mycobacterial infection system. In contrast, both a dominant-negative construct of the MyD88 adaptor and an NF-κB inhibitor abrogated the protection against FasL-mediated apoptosis, showing the implication of TLR2-mediated activation of NF-κB in apoptosis protection in infected macrophages. The apoptosis resistance of infected macrophages might be considered as an immune escape mechanism, whereby *M. tuberculosis* subverts innate immunity signaling to protect its host cell against FasL$^+$-specific cytotoxic lymphocytes. ***The Journal of Immunology,*** **2006, 177: 6245–6255.**

Infection by *Mycobacterium tuberculosis* is efficiently controlled by the immune system, as the vast majority of the 2 billion infected humans contain infection unless their immune system is compromised (1). The preferred niche of *M. tuberculosis* is the macrophage, which fails to control the growth of *M. tuberculosis* in the absence of T cells (1). Several cytokines may act as macrophage-arming factors in the control of *M. tuberculosis* (1). Mice deficient for IFN-γ or its receptor fail to generate reactive nitrogen intermediates and are unable to restrict mycobacterial growth (2, 3). Humans with similar genetic defects also show an increased susceptibility to mycobacterial infections (4, 5). However, the ability of human macrophages to generate reactive nitrogen derivatives in response to IFN-γ is controversial (6), and in vitro, treatment of human macrophages with IFN-γ enhances rather than restricts the growth of *M. tuberculosis* (7–9). Thus, the exact role of IFN-γ in antimycobacterial immunity in humans remains unclear.

Other cytokines have been implicated in host defenses against *M. tuberculosis*. In particular, TNF-α (10) and lymphotoxin (11) have been demonstrated crucial for granuloma formation and for limiting pathology, and therapies aimed at blocking TNF-α do increase the risk of reactivation tuberculosis in humans (12). However, in vitro experiments have failed to demonstrate that TNF-α can arm macrophages to kill intracellular bacilli (13). These observations have led investigators to look for alternate mechanisms for the killing of mycobacteria.

Several observations have suggested ways for T cells to kill mycobacteria independent of macrophage activation, "dealing a direct blow to the pathogens" (14). Indeed, different T lymphocyte populations have the potential to restrict the intracellular growth of mycobacteria when cocultured with infected macrophages, in part through soluble mediators and in part by a cell contact-mediated mechanism (15, 16). How could T cells kill mycobacteria? Stenger et al. (17) have demonstrated that CD8$^+$ T cell killing infected macrophages kill intracellular mycobacteria in a granule-dependent mechanism involving granulysin (18). Others have, however, demonstrated that the induction of apoptosis in infected macrophages by stimuli such as Fas ligand (FasL)[3] (19) and extracellular ATP (20, 21) is accompanied by a reduction of viable intracellular bacilli independently of granulysin. FasL signaling thus provides CD4$^+$ cells with a potential to participate directly to host defense mechanisms against *M. tuberculosis*. Indeed, CD4$^+$ T cell responses, including responses with cytolytic activity against *M. tuberculosis* Ags, are found both in humans, especially in clinical settings characterized by an effective immunity such as tuberculous pleurisy (22–28) and in mice (29, 30). Thus, induction of macrophage apoptosis by *M. tuberculosis*-specific CD4$^+$ T cells may operate in vivo to control *M. tuberculosis* survival. Granuloma do contain CD4$^+$ T cells and apoptotic figures involving macrophages (31, 32) as well as cells expressing FasL (33).

If the induction of apoptosis in infected macrophages is a host defense mechanism, then preventing apoptosis may represent for

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[3] Abbreviations used in this paper: FasL, Fas ligand; LAM, lipoarabinomannan; AraLAM, noncapped LAM; c-FLIP, cellular FLIP; CARD, caspase recruitment domain; ManLAM, mannose-capped LAM; MDP, muramyl dipeptide; Nod, nucleotide-binding oligomerization domain; PDTC, ammonium pyrrolidine dithiocarbamate; RIP2, receptor-interacting protein-2; TRAF, TNFR-associated factor.

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mycobacteria a way to preserve their habitat and escape host immune responses. Indeed, we have observed that *M. tuberculosis*-infected macrophages are relatively resistant to apoptosis induced by FasL (19).

Two major pathways are described for mammalian apoptosis: the death receptor (type I) and mitochondrial (type II) pathways (34). The type I pathway is initiated by extrinsic signals, such as the FasL-dependent triggering of the death receptor Fas, which leads to the formation of the death-inducing signaling complex and the activation of the initiator caspase-8. The type II pathway is triggered by intrinsic signals with the formation of an apoptosome complex containing active caspase-9. Both pathways converge on the activation of executioner caspases and cell death.

We first hypothesized that intracellular *M. tuberculosis* may secrete a factor that may gain access to the phagocyte cytoplasm (35) and interfere with apoptotic pathways. We therefore did a profile search of the *M. tuberculosis* genome for proteins containing motifs such as a death domain, death effector domain, and caspase recruitment domain (CARD), using the Prosite database, and did not find any significant signal (L. Falquet and P. Meylan, unpublished observations). We then hypothesized that cell wall components, such as muramyl dipeptide (MDP), lipoarabinomannan (LAM), and 19-kDa lipoprotein of *M. tuberculosis*, i.e., agonists of nucleotide-binding oligomerization domain (Nod)-2 or TLR-2 (36–38), might act to prevent apoptosis through the induction of antiapoptotic genes by the NF-κB or other signaling pathways.

In this study, we describe a model using the THP-1 cell line that recapitulates the properties of human primary macrophages with respect to the effect of *M. tuberculosis* infection on their susceptibility to FasL-induced apoptosis. We have found that following infection, the activation of NF-κB through the TLR2 signaling pathway and the subsequent induction of cellular FLIP (c-FLIP) expression are able to protect infected cells from FasL-induced apoptosis.

Materials and Methods

Cell culture and infection with M. tuberculosis *or* Escherichia coli

THP-1 cells (TIB 202; American Type Culture Collection) were cultured in RPMI 1640/Glutamax-I medium (Invitrogen Life Technologies) supplemented with 10% FCS (Inotech) and 0.05 mg/ml gentamicin (Invitrogen Life Technologies), subsequently named R10. For their differentiation, cells were plated in R10 plus PMA (20 nM; Fluka) for 3 days, at 37°C, 5% CO_2. Differentiated cells were then treated with human rIFN-γ (PBL Biomedical Laboratories), 500 U/ml, for 1 day. These cells were named THP-1/PMA/IFN-γ.

The virulent (H37Rv) and attenuated (H37Ra) *M. tuberculosis* strains were used and cultured, as previously described (19). FITC labeling of mycobacteria was done, as previously described (39). Single-cell suspensions of *M. tuberculosis* were prepared, as previously described (19), with a slight modification. Before infection, bacteria were opsonized in RPMI 1640 plus 50% AB^+ fresh human serum (Inotech) by rocking at 200 rpm, at 37°C, for 30 min. Heat-killed inocula were generated by exposing bacteria to 70°C for 30 mn. THP-1/PMA/IFN-γ were infected by replacing culture medium with the mycobacteria inoculum at a density of 10–20 bacteria per cell for 2 h, at 37°C, 5% CO_2. After three washes with RPMI 1640, cells were placed in R10 without antibiotics.

E. coli strain was cultured in LB medium and heat killed as *M. tuberculosis*.

Primary human macrophages

Blood was collected in CPT tubes (BD Biosciences). After centrifugation (20 min, 1600 × *g*), PBMC were collected, washed three times in RPMI 1640, resuspended in RPMI 1640 supplemented with 5% AB^+ human serum, and seeded in 24-well Primaria plates (Costar). After 2 h of adhesion, at 37°C, 5% CO_2, cells were washed with PBS, and 1 ml of RPMI 1640/5% AB serum was added. After 6 days, human macrophages were infected with *M. tuberculosis*, as described above.

Induction of apoptosis

THP-1/PMA/IFN-γ cells were incubated for 18 h in R10 containing human rFasL (1 μg/ml) or human rTRAIL (200 ng/ml) in the presence of an enhancer for ligands (1 μg/ml), by human rTNF-α (10 ng/ml) (all obtained from Apotech), or by staurosporine (1 μM; Sigma-Aldrich). Samples and controls were treated with 1 μg/ml cycloheximide (Sigma-Aldrich).

Quantification of apoptosis

Annexin V binding. Apoptosis was assessed by using the annexin V-FITC apoptosis detection kit (BD Pharmingen) with a slight modification. Cells undergoing apoptosis were detached using Accutase (Inotech), washed twice with cold PBS, and resuspended in 1× binding buffer. A total of 5 μl of annexin V-FITC was added to the cell suspension and incubated for 15 min at room temperature. Labeled cells were then washed with binding buffer, and the pellet was resuspended in 100 μl of 1× Cellfix (BD Biosciences) solution. Fixed cells were analyzed by flow cytometry using a FACSCalibur system (BD Biosciences). Data acquisition and analysis were performed using the CellQuest Pro software, measuring 20,000 events. Apoptosis was quantified by setting a fluorescence threshold above the fluorescence of cycloheximide-treated cells and expressed as the percentage of cells above the threshold.

Caspase activity. Apoptosis was also detected using a fluorogenic substrate for caspase-3, as previously described (40). Data were acquired on a Spectra Man Gemini reader (Molecular Devices), and analysis was performed with the SOFT Max PRO3.1 software. Results were expressed as relative fluorescence units per milligram of protein.

Flow cytometry

Staining of THP-1/PMA ± IFN-γ cells was done as follows: cells were washed, resuspended in PBS/0.5% BSA (Sigma-Aldrich), and incubated with primary mAb or isotypes for 30 min at 4°C. After washing, cells were incubated, if necessary, with a secondary Ab for 30 min at 4°C. Cells were washed, fixed with CellFix, and analyzed using a FACSCalibur system, as described above. Primary mAb were: anti-CD95 FITC (1/20, APO1-1); anti-FasL (1/100, Mike1), anti-TRAIL-R1 (1/200, HS101), anti-TRAIL-R2 (1/200, HS201), anti-TRAIL (1/100, III6F), and anti-TLR2 (1/100, TL2.1), all from obtained Apotech. Secondary Abs were as follows: anti-mouse or anti-rat IgG FITC (1/50; Sigma-Aldrich). Cells were analyzed using a FACSCalibur system, as described above. Positive events were defined as a fluorescence level superior to that of isotypic control.

Treatment with mycobacterial cell wall components or with chemical compounds

THP-1/PMA/IFN-γ cells were treated with 2 μg/ml noncapped LAM (AraLAM), mannose-capped LAM (ManLAM), 19-kDa lipoprotein (all obtained from National Institutes of Health, National Institute of Allergy and Infectious Diseases Contract AI-75320 and Colorado State University's contribution of Research Material), or LPS (Sigma-Aldrich) for 2 h, at 37°C, 5% CO_2, with 1 μg/ml Pam_3Cys (2 h; EMC Microcollections), with 1 μg/ml Ac-muramyl-Ala-D-Glu-NH2 (MDP-D, 6 h; Bachem) or the inactive enantiomer Ac-muramyl-D-Ala-D-Glu-NH2 (MDP-DD), or with ammonium pyrrolidine dithiocarbamate (PDTC; 250 mM, 10 h; Fluka).

NF-κB nuclear detection

Immunofluorescence. THP-1/PMA/IFN-γ cells were cultured in Labtek chambers (Nunc). The day after infection, the cells were washed, fixed with 4% paraformaldehyde (Fluka) for 30 min, at 4°C, and permeabilized with acetone (Fluka) for 5 min, at −20°C. After three washes, cells were stained with tetramethylrhodamine isothiocyanate-conjugated mAb anti-NF-κB p65 (Santa Cruz Biotechnology) for 30 min, at room temperature, washed once, and counterstained with Hoechst 33528 (0.05 μg/ml; Sigma-Aldrich) for 10 min, at room temperature. Slides were examined with an Axioplan 2 microscope (Zeiss), and images were acquired with a κ camera using the κ image base control (κ).

Nuclear translocation of NF-κB p65. NF-κB p65 was measured in nuclear lysates using a kit (NF-κB p65 transcription factor assay; Chemicon International). THP-1/PMA/IFN-γ cells were cultured in a 48-well plate. The day after infection, nuclear cell lysates were collected and the assay was performed. Measure of absorbance of the samples at 450 nm was conducted using a microplate reader with the reference length set to 650 nm (Bio-Rad).

Lentiviral constructions, production, and transduction

The two lentiviral constructions used were pRDI292-CMV-MyD88DN (expressing the dominant analog of TLR domain-containing adaptor,

MyD88) or pRDI292-CMV-RIP2DN (expressing the dominant analog of protein kinase receptor-interacting protein-2 (RIP2)). The two genes of interest are under the control of the CMV promoter in these constructs.

Lentiviral vector (MyD88DN) expressing the dominant analog of MyD88. The plasmid pL502 containing the MyD88DN gene (41) was digested by *Bam*HI and *Apa*I, and the resulting fragment was cloned into pSL1180 (Pharmacia Diagnostics). The CMV promoter was subcloned from the vector SIN.cPPT.CMV.eGFP-WHV (D. Trono, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland), using *Cla*I and *Bam*HI, into pSL1180/MyD88DN to obtain PSL1180/CMV/MyD88DN. This vector was digested by *Apa*I and *Bam*HI and blunted to obtain the CMV-MyD88DN fragment, which was cloned into the lentiviral vector pRDI292 (R. Iggo, Institut Suisse de Recherches Experimentales surle Cancer, Lausanne, Switzerland) digested by *Bam*HI and blunted. The vector obtained was designed as pRDI292-CMV-MyD88DN.

Lentiviral vector expressing the dominant analog of RIP2 (RIP2DN). RIP2DN (42) was cloned *Apa*I/*Cla*I into PSL1180-CMV-MyD88DN digested by the same enzymes to remove the MyD88DN insert, to yield the vector pSL1180-CMV-RIP2DN. The CMV-RIP2DN fragment was then cloned into the lentiviral vector pRDI292, as described above, to obtain the pRDI292-CMV-RIP2DN construct.

Lentivirus production. The lentiviruses were produced by cotransfecting the Gag-Pol construct (pCMV._R8.92), the Rev expression plasmid (pRSVRev), the vesicular stomatitis virus G protein envelope construct (pMD.G), and the packaged construct of interest (expressing MyD88DN, RIP2DN, or GFP as a control of transduction efficiency) (see ⟨www.tronolab.epfl.ch⟩ for vector details).

Cells transduction. Transduction of THP-1 cell was performed by spinoculation of 0.5×10^6 cells with 400 μl of lentivirus-containing supernatant in the presence of 4 μg/ml polybrene (Sigma-Aldrich) for 3 h, at $1500 \times g$ and 22°C. After 48 h, cells were selected overnight in R10 with 2 μg/ml puromycin (Fluka), and maintained with 1 μg/ml puromycin.

Western blot

THP-1/PMA/IFN-γ cells were washed with cold PBS, and lysed in 1× radioimmunoprecipitation buffer containing a protease inhibitor mixture (Complete TM; Roche Diagnostics). The samples were mixed with sample buffer, separated on 12% SDS-PAGE, transferred to polyvinylidene difluoride membrane (NEN Life Science Products), and immunoblotted with the appropriate dilution of anti-c-FLIP (1/800, Dave2; Apotech), anti-tubulin (1/10000, B512; Sigma-Aldrich), anti-FLAG M2 (10 μg/ml; Sigma-Aldrich), or anti-vesicular stomatitis virus (1/1000, P5D4; Sigma-Aldrich). Blots were developed using an ECL detection system (Pierce).

Statistical analysis

Values are expressed as the mean ± SEM. Statistically significant differences between series were assessed by Student's *t* test using GraphPad Prism software.

Results

Development of THP-1 cells as a model system for the study of apoptosis susceptibility

We have reported that infection of primary macrophages by *M. tuberculosis* induced a relative resistance of these cells to apoptotic stimuli (19). To facilitate the study of the mechanisms of this resistance, we aimed at developing a system using a continuous cell line to recapitulate the behavior of primary macrophages regarding infection and apoptosis susceptibility. THP-1 cells treated by PMA became phagocytic, but did not express Fas (Fig. 1*A*). We examined whether IFN-γ treatment might affect the differentiation and apoptosis susceptibility of THP-1 cells. IFN-γ treatment of PMA-differentiated THP-1 cells induced surface expression of Fas receptor from 0 to 50 ± 12% of the cell population (Fig. 1*B*). FasL expression was also up-regulated (41 ± 21 vs 25 ± 8%). The IFN-γ treatment led to the diminution of TRAIL receptors 1 and 2 (9 and 12 vs 19 and 18%, respectively) and TRAIL expression (15 vs 11%), whereas TLR2 was equally expressed in THP-1/PMA cells, irrespective of IFN-γ treatment (25–30%).

THP-1/PMA/IFN-γ cells were susceptible to FasL-induced apoptosis, as demonstrated by annexin V staining (Figs. 1*C* and 2*A*) with ~45 ± 7% of cells undergoing apoptosis. These cells were also susceptible to TRAIL-induced apoptosis (up to 22 ± 5%), but not to TNF-α-induced apoptosis. Because the highest level of apoptosis induction was achieved using FasL, this ligand was used in further experiments.

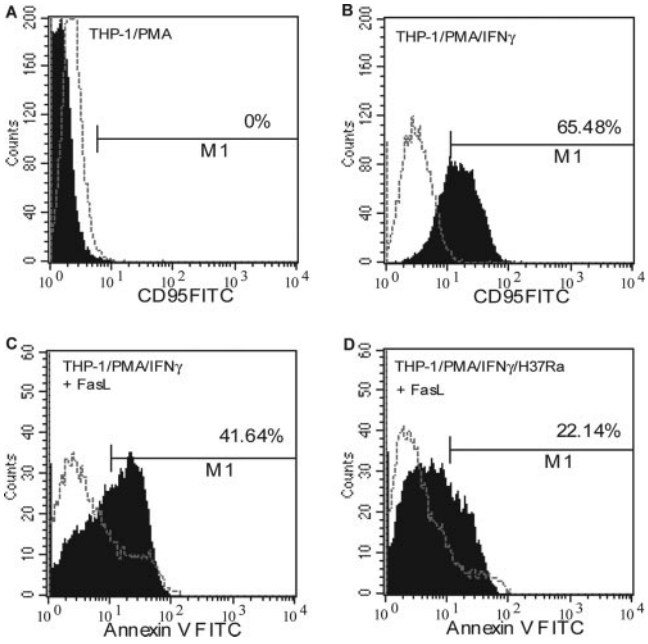

FIGURE 1. Fas expression following IFN-γ treatment and analysis of FasL-induced apoptosis in *M. tuberculosis*-infected THP-1 cells. *A* and *B*, Expression of Fas receptor was measured by flow cytometry in THP-1/PMA cells (filled black curve) before and after IFN-γ treatment. Overlay of the corresponding isotype staining control is shown (dotted line). *C* and *D*, Apoptosis was detected by annexin V staining in noninfected or in *M. tuberculosis*-infected cells treated with FasL. Overlay of the annexin V staining in cells without apoptosis induction is shown (dotted line). Percentages of positive cells (M1) are indicated.

Effect of M. tuberculosis *infection on the susceptibility of THP-1 cells to FasL*

To test whether infection induces apoptosis resistance in THP-1/PMA/IFN-γ cells as in primary macrophages, the cells were infected at a bacterium/cell ratio of 10–20 using *M. tuberculosis* H37Ra or H37Rv. The remaining nonphagocytosed mycobacteria were removed by three washes. This led to the infection of a majority of the cells, with ~2–6 bacteria per cell (see Fig. 2 in Ref. 19). *M. tuberculosis* infection did not by itself induce apoptosis in our system, as witnessed by annexin V staining (data not shown). Two hours after infection, the cells were incubated with FasL or staurosporin to induce apoptosis. Similar to primary human macrophages (19), infection of differentiated THP-1 cells with H37Rv or H37Ra strains protected them from FasL-induced apoptosis, reducing apoptosis by close to 50% (Figs. 1*D* and 2*B*). When using FITC-labeled mycobacteria and annexin V-allophycocyanin staining, we were unable to observe a difference in apoptosis in infected vs noninfected cells. However, we have also tested whether apoptosis resistance can be induced in noninfected cells by cytokines or soluble factors potentially secreted by infected ones (data not shown). This was performed using a permeable membrane system for cell culture (Transwells; Costar). Cells were infected in the lower well, and noninfected cells were seeded in the upper well. FasL apoptosis was induced immediately after infection or 4 h postinfection. A similar resistance to apoptosis was observed in

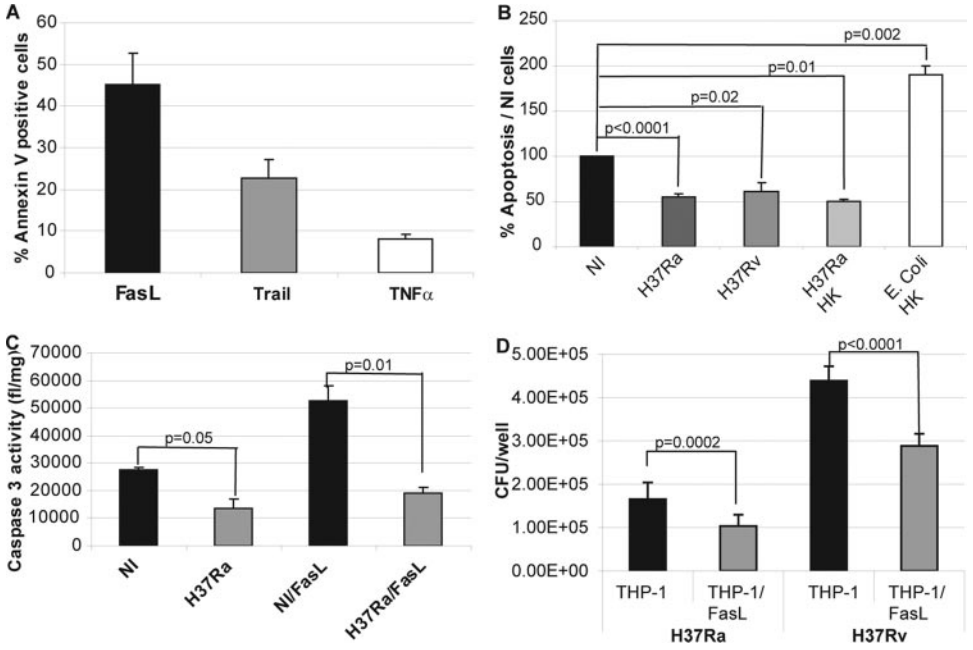

FIGURE 2. THP-1 cells as a model to study *M. tuberculosis*-induced resistance to apoptosis. *A*, THP-1 cells were treated by the indicated proapoptotic agents and apoptosis quantified by FACS using annexin V-FITC staining. Shown is the mean ± SEM of at least four independent experiments. *B*, The relative level of FasL-induced apoptosis was measured by annexin V staining of THP-1 cells after *M. tuberculosis* infection (seven and four independent experiments were performed for H37Ra and H37Rv infection, respectively) or with heat-killed H37Ra or *E. coli* (three independent experiments). Apoptosis was normalized to 100% in uninfected cells. *C*, Infected and noninfected cells were submitted to FasL-induced apoptosis, and caspase activity was determined (shown is the mean ± SEM of three independent experiments). *D*, Infected THP-1 cells were treated with FasL, and bacterial viability was determined at day 1 after infection (mean ± SEM of three independent experiments performed in duplicate).

both wells. However, apoptosis resistance of noninfected cells cultured in the presence of the supernatant of infected cell was observed only when apoptosis was induced 4 h postinfection. It is likely that this 4-h delay is necessary for the synthesis and the diffusion of a soluble mediator to the noninfected cells present in the upper well. We hypothesize that soluble factors produced by the infected cells are sufficient to induce apoptosis resistance.

This reduced apoptosis was not due to a diminution of Fas expression at the cell surface (data not shown). *M. tuberculosis* infection did not protect cells from staurosporin-induced apoptosis (data not shown). Thus, *M. tuberculosis* was able to protect cells from apoptosis mediated by death receptor binding, but not via the mitochondrial pathway.

A similarly diminished susceptibility to FasL-induced apoptosis was observed in THP-1/PMA/IFN-γ cells infected with either viable or heat-killed H37Ra bacteria (Fig. 2*B*), suggesting that binding and phagocytosis of mycobacteria are sufficient to induce apoptosis resistance. This phenomenon was specific to *M. tuberculosis* because infection with heat-killed *E. coli* was unable to protect cells from FasL-induced apoptosis (Fig. 2*B*), but rather induces apoptosis by itself (data not shown).

Apoptosis protection was also demonstrated by the diminution of caspase-3 activity in *M. tuberculosis*-infected cells (Fig. 2*C*). *M. tuberculosis* infection per se reduced caspase activity, suggesting a basal level of caspase activation in untreated cells that was inhibited by infection. Treatment of uninfected cells using FasL increased caspase activity by ~2-fold. This increase of caspase activity upon FasL treatment was almost completely prevented in infected cells.

Apoptosis and bacterial viability in THP-1 cells

FasL-induced apoptosis was associated with a diminution of bacterial viability (Fig. 2*D*) by ~38% in H37Ra-infected cells and 35% in H37Rv-infected cells. These observations are comparable to results obtained with primary macrophages (19).

MDP provides cell protection against FasL-induced apoptosis

Because the apoptosis protection was also conferred by heat-killed bacteria, we tested the effect of bacterial cell wall components on FasL-induced apoptosis susceptibility.

MDPs are the smallest peptidoglycan degradation products. They bind to intracellular Nod2, leading to the activation of RIP2 and subsequently of NF-κB and antiapoptotic genes (43). To test whether MDP-induced NF-κB activation was implicated in *M. tuberculosis*-mediated apoptosis resistance of macrophages, THP-1/PMA/IFN-γ cells were treated with MDP-D or with MDP-DD, an inactive enantiomer as a negative control. Apoptosis was then induced by FasL and assessed by annexin V staining or by caspase assay. MDP-D treatment resulted in apoptosis protection against FasL-induced apoptosis (40% reduction), whereas MDP-DD did not (Fig. 3*A*). Caspase activity was also reduced in MDP-D-treated cells, although not significantly (Fig. 3*B*). Thus, the activation of the Nod2 pathway is a potential pathway for mycobacteria to induce apoptosis resistance.

Role of the Nod2 pathway in apoptosis resistance of infected cells

To test whether blocking the Nod2 pathway could abrogate apoptosis protection of cells infected by *M. tuberculosis*, we used a transdominant-negative approach. RIP2 is an NF-κB-activating protein interacting with Nod2 through a homophilic CARD-CARD interaction (44). A dominant-negative RIP2 construct (RIP2DN), corresponding to RIP2 truncated of its CARD domain (42), was transduced into THP-1 cells using a lentiviral vector, and after several days of puromycin selection, the presence of the RIP2DN protein was detected by Western blot (Fig. 3, *inset*).

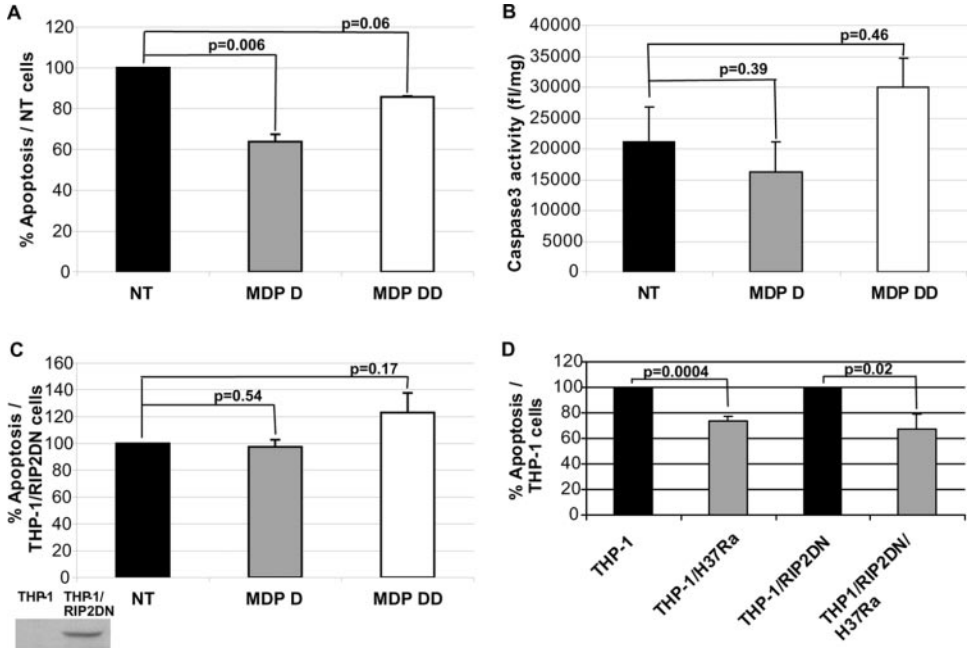

FIGURE 3. The Nod2 pathway is not involved in apoptosis protection following *M. tuberculosis* infection. *A*, THP-1 cells were treated with MDP for 6 h, and apoptosis was induced by FasL (mean ± SEM of five independent experiments). Results are expressed as annexin V-positive cells, normalized to 100% for untreated cells. *B*, Determination of caspase activity in THP-1 cells treated with MDP (mean ± SEM of three independent experiments). *C*, Effect of MDP-D and MDP-DD on FasL-induced apoptosis in THP-1 cells transduced with a lentiviral vector encoding a dominant-negative form of RIP2 (mean ± SEM of two independent experiments performed in triplicate). Expression of RIP2DN was checked by Western blot (*inset*). *D*, Effect of *M. tuberculosis* infection on FasL-induced apoptosis in THP-1 cells transduced with a mock or RIP2DN construct (mean ± SEM of two independent experiments performed in triplicate).

The functionality of RIP2DN was first tested using MDP-D as an agonist of the Nod2 pathway. MDP-D protected THP-1/PMA/IFN-γ cells against apoptosis, and the expression of RIP2DN abrogated this protection (Fig. 3*C*).

In contrast to the effect of RIP2DN on MDP-D-induced resistance, the expression of RIP2DN did not abrogate the apoptosis protection in infected cells (Fig. 3*D*). Together, these findings suggest that the Nod2 pathway does not play a significant role in the apoptosis resistance of *M. tuberculosis*-infected THP-1/PMA/IFN-γ cells.

M. tuberculosis *cell wall TLR2 agonists induce apoptosis resistance*

M. tuberculosis cell wall components such as the 19-kDa lipoprotein and AraLAM are known to signal through TLR2, with a potential for activating proinflammatory and antiapoptotic pathways (36). We therefore tested the capability of such cell wall components of *M. tuberculosis* to protect THP-1/PMA/IFN-γ cells from apoptosis (Fig. 4*A*). FasL-induced apoptosis was significantly reduced when these cells were pretreated with the TLR2 agonists (19-kDa lipoprotein and AraLAM), but not with ManLAM. In contrast, a TLR4 agonist (LPS) increased cell susceptibility to apoptosis by 110% when compared with untreated cells (Fig. 4*A*). Similar results were observed when apoptosis was assessed measuring caspase activity (Fig. 4*B*).

To confirm that this observation was not due to a contamination of the mycobacterial preparation, cells were treated with the selective purified chemical Pam_3Cys TLR2 ligand, and level of FasL-induced apoptosis was assessed. We observed again that activation of the TLR2 pathway allows cell protection from FasL-induced apoptosis (Fig. 4*A*).

We also checked the level of Fas expression by FACS analysis after 19-kDa lipoprotein treatment. No change was found, suggesting that the decrease in apoptosis susceptibility was not related to a concomitant decrease in Fas expression (data not shown).

Thus, TLR2 agonists purified from the cell wall of *M. tuberculosis* recapitulated the ability of *M. tuberculosis* to induce a relative resistance to FasL-induced apoptosis in phagocytes.

Role of the TLR2 signaling pathway in apoptosis resistance of infected cells

To test whether TLR2 signaling was involved in apoptosis resistance in infected cells, THP-1 cells were transduced with a transdominant-negative mutant of the adaptor molecule MyD88 truncated of its death domain (MyD88DN (41)), using a lentiviral vector. After puromycin selection, expression of MyD88DN was detected by Western blotting (Fig. 4*C*, *inset*). No change in basal apoptosis was detected in MyD88DN-expressing cells (data not shown).

To verify the functionality of the MyD88DN protein, control and MyD88DN-expressing THP-1/PMA/IFN-γ cells were treated with the TLR2 agonist 19-kDa lipoprotein (Fig. 4*C*). MyD88DN partly abrogated the apoptosis protection conferred by the 19-kDa lipoprotein, and completely abrogated the apoptosis protection conferred by *M. tuberculosis* infection (Fig. 4*D*). This result confirms that the TLR2 signaling pathway is involved in inducing apoptosis protection, accounting for most of the apoptosis resistance induced by *M. tuberculosis* infection in THP-1 cells.

NF-κB activation protects cells from apoptosis

The activation of the TLR2 signaling pathway culminates in the nuclear translocation of the transcription factor NF-κB. To assess whether NF-κB activation was involved in apoptosis protection in our model, we first blocked NF-κB activation by PDTC, a pyrrolidine derivative of dithiocarbamate that suppresses the release of IκB from the latent cytoplasmic form of NF-κB (45). Treatment

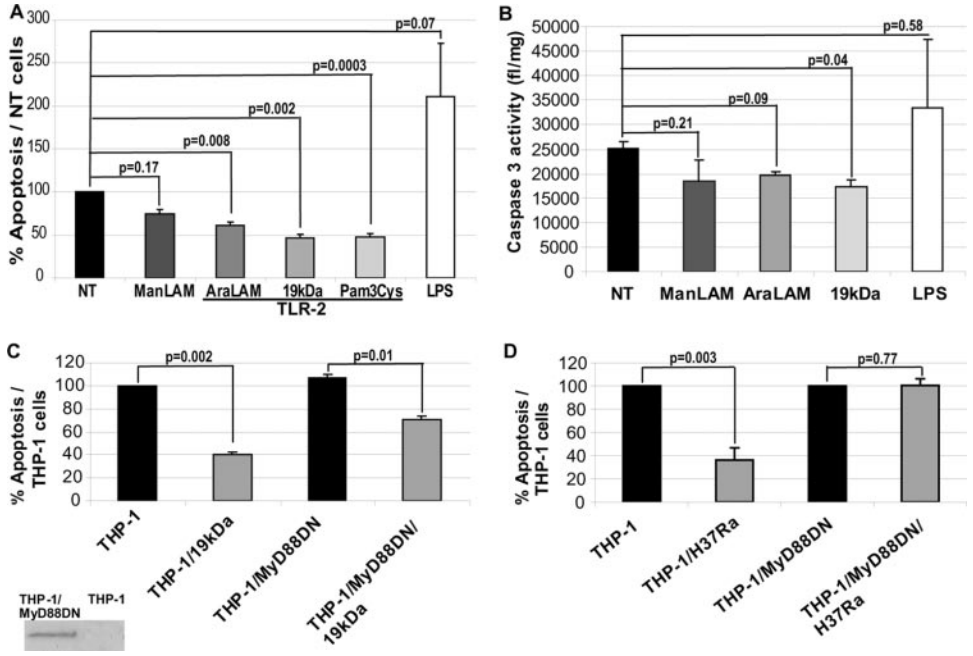

FIGURE 4. TLR2 signaling is involved in apoptosis protection following *M. tuberculosis* infection. *A*, FasL-induced apoptosis in THP-1 cells was measured after treatment with TLR2 or TLR4 agonists. Results are expressed as annexin V-positive cells, normalized to 100% for untreated cells (mean ± SEM of at least three to four independent experiments). *B*, Determination of caspase activity in cells treated with TLR2 or TLR4 agonists (mean ± SEM of three independent experiments performed in triplicate). *C*, Effect of 19-kDa lipoprotein on FasL-induced apoptosis in THP-1 cells transduced with a mock or a dominant-negative construct of MyD88 (mean ± SEM of two independent experiments performed in triplicate). Expression of MyD88DN was checked by Western blot (*inset*). *D*, Effect of *M. tuberculosis* infection on FasL-induced apoptosis in THP-1 cells transduced with a mock or MyD88DN construct (mean ± SEM of three independent experiments performed in duplicate).

of THP-1/PMA/IFN-γ cells with PDTC increased the basal rate of apoptosis by ~35% (Fig. 5*A*), suggesting that NF-κB protects these cells from apoptosis in the absence of FasL-mediated signaling. In infected cells, not only did PDTC abrogate the protection induced against apoptosis, but it increased the level of apoptosis by 250% compared with infected untreated cells, thus to a degree that is beyond the apoptosis level of control cells. In noninfected primary human macrophages, no difference was observed between treated and nontreated cells, suggesting that primary cells are less dependent on NF-κB protection following FasL-apoptosis induction (Fig. 5*B*). However, in infected primary human macrophages, the same concentration of PDTC was sufficient to reverse the protection conferred by mycobacterial infection against FasL-induced apoptosis, with an increase of 340% of apoptosis. This confirmed the role of NF-κB activation in apoptosis protection, in particular in infected cells.

To corroborate these data, nuclear translocation of NF-κB was quantified using the transcription factor assay on nuclear cell fractions. THP-1/PMA/IFN-γ cells were infected by *M. tuberculosis*. After 24 h of infection, cells were processed and NF-κB was assayed in nuclear extracts. Upon infection, there was a 3-fold increase in NF-κB detectable in nuclear fractions (data not shown).

We also looked for cellular localization of NF-κB by immunofluorescence. THP-1/PMA/IFN-γ cells were infected using FITC-labeled H37Ra *M. tuberculosis* and, after 24 h, fixed and stained for NF-κB p65 transcription factor and for DNA by Hoechst 33528 (Fig. 6). In uninfected cells, NF-κB localized to the cell cytoplasm (Fig. 6*A*). When the cells were infected with H37Ra, NF-κB translocated to the nucleus (Fig. 6*B*), colocalizing with the Hoechst DNA staining. This translocation was especially obvious in cells containing the largest number of bacteria.

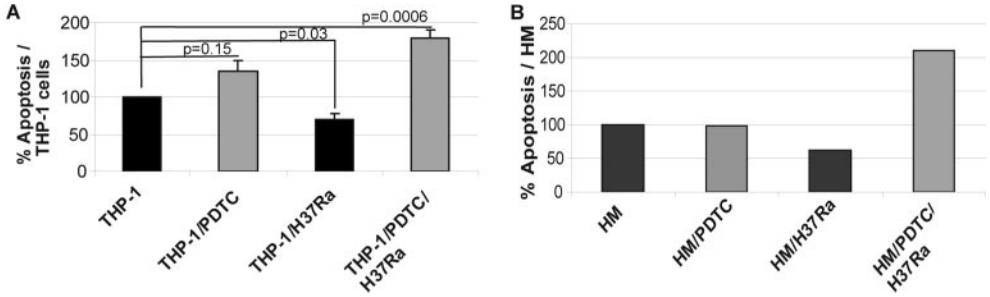

FIGURE 5. *M. tuberculosis* infection mediates NF-κB-dependent protection from FasL-induced apoptosis. *A*, THP-1 cells were treated with PDTC, infected or not with *M. tuberculosis*, and apoptosis was induced by FasL. Results are expressed as annexin V-positive cells, normalized to 100% for untreated cells (mean ± SEM of five experiments). *B*, Primary human macrophages were treated with PDTC, infected or not with *M. tuberculosis*, and apoptosis was induced by FasL. Results are expressed as annexin V-positive cells, normalized to 100% for untreated cells (one experiment).

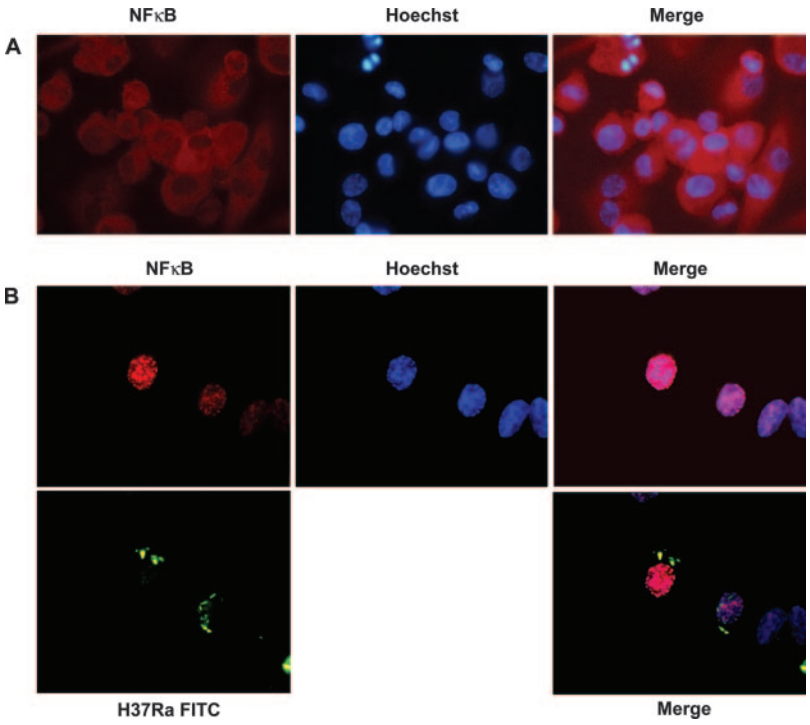

FIGURE 6. Nuclear localization of NF-κB is induced by *M. tuberculosis* infection. *A*, NF-κB is present in the cytoplasm of noninfected cells. *B*, THP-1 cells infected with FITC-labeled *M. tuberculosis* and stained with anti-NF-κB Ab and Hoechst. Infected cells show NF-κB nuclear localization whose intensity is proportional to the number of ingested bacteria.

Thus, these three independent approaches suggest that TLR2-mediated NF-κB nuclear translocation is crucial in mediating *M. tuberculosis*-induced protection of phagocytes from apoptosis.

Effect of TLR signaling on the expression of proteins involved in apoptosis regulation

In an attempt to understand how TLR2 signaling may protect from apoptosis, we tested whether TLR2 agonists or infection would affect cellular levels of proteins known to regulate apoptosis. Among these, the levels of Fas, TNFR-associated factor (TRAF)-1, caspase-3, caspase-8, and caspase-9 were not affected by TLR2 agonist treatment or by *M. tuberculosis* infection (data not shown). We also looked for c-FLIP, a major inhibitor of death receptor-induced apoptosis (46), which is known to be regulated by NF-κB (47), and for TRAF6, an adaptor molecule in the TLR2 signaling cascade (37), which, if regulated by NF-κB signaling, would have a potential for feedback amplification of this pathway.

TLR2 agonists increased the levels of c-FLIP (Fig. 7*A*) and, to a weaker extent, of TRAF6 (data not shown), whereas the TLR4 agonist LPS had no effect either on c-FLIP or on TRAF6. In comparison, both H37Rv and H37Ra *M. tuberculosis* increased the level of c-FLIP 18 h after infection (Fig. 7*B*), but had no effect on TRAF6 levels (data not shown).

These data suggest that an increase of c-FLIP expression upon TLR2 activation may account, at least in part, for the relative resistance of infected cells to apoptosis.

Together, these findings suggest that *M. tuberculosis*-induced NF-κB activation by the TLR2, but not the Nod2 pathway, protects infected THP-1 cells from FasL-induced apoptosis.

Discussion

Depending on the experimental system, *M. tuberculosis* phagocytosis has been shown to promote or protect phagocytes from spontaneous apoptosis (48–51). In contrast, the effect of *M. tuberculosis* phagocytosis on apoptosis induced by various apoptotic stimuli is to reduce phagocyte susceptibility to undergo apoptosis (52). In addition, virulent *M. tuberculosis* strains, but not the avirulent ones, have been shown to evade apoptosis not only in human alveolar macrophages (53), but also in differentiated THP-1 cells (54). In some of these systems, this effect has been ascribed to the induction of an antiapoptotic Bcl-2 family member (52, 55) or soluble TNF-R2 (52).

We also described a reduced susceptibility of *M. tuberculosis*-infected primary human macrophages to FasL-induced apoptosis (19). Because FasL-induced apoptosis was associated with a reduction in viability of intracellular bacteria, this reduced susceptibility may appear as a potential immune escape mechanism. To facilitate the study of the cellular mechanisms that underlie this altered apoptosis susceptibility, we aimed at developing a phagocyte infection system based on a continuous cell line. Upon PMA differentiation, THP-1 cells recapitulate the properties of human monocyte-derived macrophages regarding receptor-mediated uptake, survival, replication, and drug sensitivity of *M. tuberculosis* (56), as well as for ranking the intracellular growth characteristics

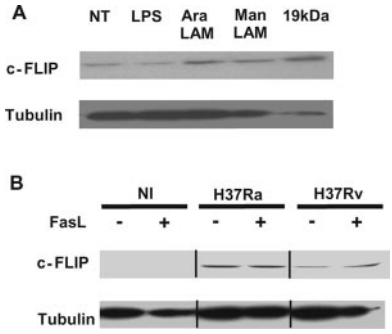

FIGURE 7. Increased c-FLIP levels following cell treatment with *M. tuberculosis* ligands or *M. tuberculosis* infection. *A*, Cells were treated with TLR2 or TLR4 ligands for 2 h, and c-FLIP expression was determined by Western blot. *B*, THP-1 cells were infected with virulent or attenuated *M. tuberculosis* strains, apoptosis was induced by FasL for 18 h, and c-FLIP expression was monitored by Western blot. Noninformative slots have been cut out.

of various strains (56). Recently, activated THP-1 cells have been used in a range of studies of the intracellular life style of *M. tuberculosis*, including studies that address the host cell susceptibility to apoptosis (54).

In this study, we demonstrate that upon PMA-induced differentiation and IFN-γ stimulation, THP-1 cells acquire characteristics reminiscent of human primary macrophages (19) with respect to the following: 1) susceptibility to FasL; 2) effect of apoptosis on intracellular *M. tuberculosis* viability; and 3) effect of infection on susceptibility to apoptotic stimuli, suggesting that these cells are an appropriate model to study the determinants of this potential immune escape mechanism.

Because apoptosis protection also occurred when THP-1 cells were exposed to heat-killed *M. tuberculosis*, we tested the effect of various cell wall components on THP-1 apoptosis susceptibility. MDP is the smallest peptidoglycan degradation product of the bacterial cell wall. These peptidoglycan subunits are recognized by proteins of the NOD family, in particular by the Nod2 protein. Recognition of MDP by Nod2 is followed by NF-κB activation (43) and NF-κB-dependent activation of genes, including anti-apoptotic genes. We first looked for the effect of MDP-D on FasL-induced apoptosis of THP-1 cells. Indeed, treating differentiated THP-1 cells with MDP-D induced apoptosis protection. The Nod2 protein is an activator of NF-κB via its interaction with RIP2/RICK/CARDIAK through its CARD domain (57). A dominant-negative form of RIP2 (RIP2DN) (42) abrogated protection against apoptosis induced by MDP-D, but not by *M. tuberculosis* infection. These results indicate that whereas MDP-D can protect cells from FasL-induced apoptosis, this signaling pathway is not involved in protection of infected cells in our system. It is unclear whether this observation reflects truly the fact that NOD signaling is not involved in apoptosis protection, or whether in our infection system, the time of apoptosis induction after infection is not long enough for *M. tuberculosis* to release peptidoglycan fragments and thus for MDP-D or similar Nod2 ligands to reach the infected cell cytoplasm, as has been described for other microorganisms (38). We cannot therefore exclude that after a prolonged time of infection, as in granulomas in vivo, Nod2 ligands may arise from bacterial metabolism or degradation after killing, and thus, could also protect infected cells from FasL-induced apoptosis.

Because the role of pattern recognition receptors (in particular, TLRs) in immune defense mechanisms against microbial pathogens is increasingly recognized (37), we then turned toward the possible activation of TLRs, and subsequently of NF-κB, by *M. tuberculosis*. These TLRs, especially TLR2, are expressed at the site of *M. tuberculosis* infection, i.e., macrophages in granulomas (58), and they are known to mediate responses triggered by cell wall-associated mycobacterial LAM and lipoproteins (37). The LAM exists in various forms with mannosylated LAM found in slow growing mycobacteria such as *M. tuberculosis* and nonmannosylated or phosphoinositide LAM found in avirulent mycobacteria such as *Mycobacterium smegmatis* (37). These latter are known to signal in a TLR-dependent manner. Another important TLR2 agonist is the major 19-kDa lipoprotein of *M. tuberculosis* (37).

These compounds have been described as potentially participating to the immune escape of *M. tuberculosis* by interrupting the cross talk between $CD4^+$ T lymphocytes and macrophages. Indeed, they inhibit IFN-γ-mediated cell surface expression of MHC class II and FcγR1, potentially interfering with Ag presentation (59, 60) and interfere with the IFN-γ-mediated activation of mouse macrophages, preventing their cidal effect against various targets (61). Furthermore, ManLAM has also been shown to antagonize *M. tuberculosis*-induced macrophage apoptosis, protecting the *M. tuberculosis* host cell niche (62).

In this study, we present several lines of evidence that the relative refractoriness to FasL-induced apoptosis of *M. tuberculosis*-infected THP-1 cells is due to TLR2-mediated NF-κB signaling and the resulting overexpression of c-FLIP.

First, the apoptosis resistance induced by infection could be reproduced by mycobacterial products known as TLR2 agonists (37). In our model, AraLAM and the 19-kDa lipoprotein were able to protect cells from FasL-induced apoptosis with the most potent effect observed with the 19-kDa lipoprotein. Others have observed that a nonacylated recombinant 19-kDa lipoprotein induces human macrophage apoptosis in a TLR2- and dose-dependent manner up to 200 ng/ml (63). In contrast, using purified mycobacterial 19-kDa lipoprotein, we did not observe spontaneous apoptosis of our THP-1 cells even when treated with a dose as high as 2 μg/ml, whereas A. Ciaramella et al. (64) observed increasing apoptosis at 19-kDa lipoprotein levels ranging from 0.5 to 10 μg/ml. These discordant results may reflect differences in the purity of 19-kDa lipoprotein preparations or differences in the macrophages used (for example, different relative expression of MyD88, Toll/IL-1

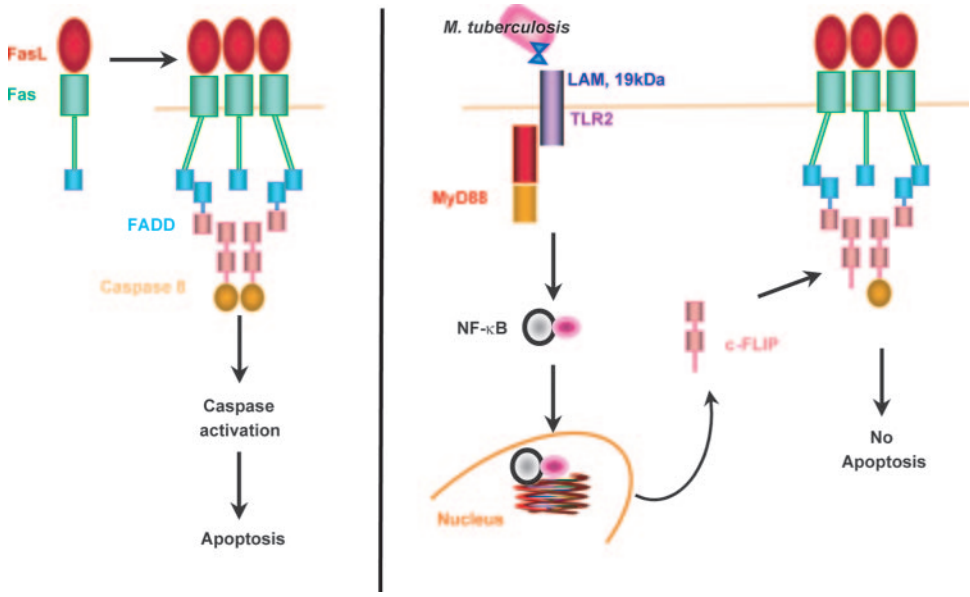

FIGURE 8. Model of *M. tuberculosis*-induced apoptosis evasion. *Right*, In classical apoptosis, ligation of FasL to its receptor leads to caspase activation and apoptosis. *Left*, Infection of macrophages by *M. tuberculosis* leads through TLR2 to the nuclear translocation of NF-κB. This leads to the expression of anti-apoptotic target genes such as c-FLIP, which inhibits death receptor-induced apoptosis.

receptor domain-containing adaptor-inducing IFN-β, or other signaling components) that affect the balance between NF-κB-activating and apoptosis-activating TLR pathways (65, 66). However, when using a specific TLR2 ligand (Pam_3Cys), we observed a similar apoptosis protection of our cells. This suggests that our 19-kDa preparation is not contaminated and that our results reflect a specific TLR2 activation.

Second, to confirm the involvement of TLR2 in FasL-induced apoptosis protection of *M. tuberculosis*-infected cells, we disrupted the TLR2 signaling pathway using a dominant-negative form of the MyD88 adapter molecule that disconnects the receptor and the first protein of the TLR signaling pathway, i.e., IL-1R-associated kinase, Ser/Thr kinase-1 (37). MyD88 has been described as essential in the control of *M. tuberculosis* infection, and the $MyD88^{-/-}$ mice rapidly succumb to infection (67). In mice, disrupting the TLR pathway probably prevents the induction of the production of the antimycobacterial agent NO, which has been shown to be induced by the 19-kDa lipoprotein (36). However, this mechanism is unlikely to play an important role in humans, as human macrophages produce only minimal amounts, if any, of NO (6, 36). Our results support the idea that the MyD88DN construct abrogated the *M. tuberculosis*-induced apoptosis protection by specific inhibition of the TLR2-induced NF-κB pathway.

Indeed, in the present model, NF-κB was shown to translocate into the nucleus following *M. tuberculosis* infection by immunofluorescence and subcellular fractionation. To verify the involvement of NF-κB in apoptosis protection induced by *M. tuberculosis*, THP-1 cells were also treated by a chemical compound PDTC known to antagonize the release of NF-κB from IκB by inhibiting IκB degradation (68). Indeed, PDTC abrogated *M. tuberculosis*-mediated apoptosis protection in FasL-treated THP-1 cells and human primary macrophages. Thus, NF-κB activation is necessary to protect *M. tuberculosis*-infected cells from FasL-induced apoptosis.

As a pleiotropic factor affecting potentially the expression of many genes, NF-κB may favor cell survival by up-regulating gene products with antiapoptotic properties, or down-regulating pro-apoptotic factors. We therefore tested the effect of TLR2 agonists and infection on cellular levels of a number of proteins participating in apoptotic pathways. Although a number of proteins involved in apoptosis signaling were unaffected, c-FLIP protein expression was induced in cells treated with 19-kDa lipoprotein and in *M. tuberculosis*-infected cells. The up-regulation of c-FLIP expression following NF-κB activation and its involvement in preventing apoptosis has also been observed after stimulation by CD40L, LPS, or TNF, and may be especially effective against death ligand-induced apoptosis (47). c-FLIP protein is known to inhibit death receptor-induced apoptosis by interacting with the receptor-associated initiator caspase-8 (46, 69). Because the caspase-8 levels were quite stable in our cells even after infection (data not shown), the increase of c-FLIP expression is likely to account for the increased FasL resistance of the infected cells.

In summary, we present in this study evidence for a mechanism to explain the resistance to death receptor-mediated apoptosis observed in *M. tuberculosis*-infected human macrophages, based on c-FLIP induction secondary to TLR2-dependent NF-κB activation (Fig. 8). Increased expression of the TRAF6 adapter may further amplify this response. In so doing, *M. tuberculosis* may protect macrophages from FasL-induced apoptosis, and thus allow their escape from $CD4^+$ T cell killing. The Nod2-dependent NF-κB activation did not play a role in our culture model, but could perhaps operate in vivo.

Subversion of signaling pathways by microorganisms to control host cell apoptosis is not unprecedented. Thus, HSV type I (70) or *Rickettsia rickettsii* (71) has been shown to protect against apoptosis by mobilizing NF-κB.

In conclusion, whereas there is no in vitro evidence that the old paradigm of enhanced mycobacterial activity of macrophages treated with IFN-γ holds true in humans, several alternate mechanisms for the human immune system have been identified: granulysin and perforin release by $CD8^+$ cells (18), induction by TLR2 ligands of an antimicrobial activity independent of NO and TNF-α (58), induction by IFN-γ of LRG-47, a member of a newly emerging 47-kDa guanosine triphosphatase family (72), and most recently autophagy (73). The respective role of these different mechanisms in the immune defenses against *M. tuberculosis* in vivo remains to be delineated. Although there is no evidence at the present time that death receptor-induced macrophage apoptosis is a host defense mechanism operating in vivo, the resistance to apoptosis induced by infection in macrophages might be considered as an immune escape mechanism, whereby *M. tuberculosis* subverts innate immunity signaling to escape acquired immunity effectors. As such, our observations might be an indirect testimony that death receptor-induced macrophage apoptosis is indeed a host defense mechanism in vivo.

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Disclosures

The authors have no financial conflict of interest.

References

1. Kaufmann, S. H. 2001. How can immunology contribute to the control of tuberculosis? *Nat. Rev. Immunol.* 1: 20–30.
2. Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom. 1993. An essential role for interferon-γ in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med.* 178: 2249–2254.
3. Kamijo, R., J. Le, D. Shapiro, E. A. Havell, S. Huang, M. Aguet, M. Bosland, and J. Vilcek. 1993. Mice that lack the interferon-γ receptor have profoundly altered responses to infection with bacillus Calmette-Guérin and subsequent challenge with lipopolysaccharide. *J. Exp. Med.* 178: 1435–1440.
4. Newport, M. J., C. M. Huxley, S. Huston, C. M. Hawrylowicz, B. A. Oostra, R. Williamson, and M. Levin. 1996. A mutation in the interferon-γ-receptor gene and susceptibility to mycobacterial infection. *N. Engl. J. Med.* 335: 1941–1949.
5. Jouanguy, E., F. Altare, S. Lamhamedi, P. Revy, J.-F. Emile, M. J. Newport, M. Levin, S. Blanche, E. Seboun, A. Fischer, and J.-L. Casanova. 1996. Interferon-γ-receptor deficiency in an infant with fatal bacille Calmette-Guérin infection. *N. Engl. J. Med.* 335: 1956–1961.
6. Chan, E. D., J. Chan, and N. W. Schluger. 2001. What is the role of nitric oxide in murine and human host defense against tuberculosis? Current knowledge. *Am. J. Respir. Cell Mol. Biol.* 25: 606–612.
7. Meylan, P. R. A., D. D. Richman, and R. S. Kornbluth. 1992. Reduced intracellular growth of mycobacteria in human macrophages cultivated at physiologic oxygen pressure. *Am. Rev. Respir. Dis.* 145: 947–953.
8. Douvas, G. S., D. L. Looker, A. E. Vatter, and A. J. Crowle. 1985. γ Interferon activates human macrophages to become tumoricidal and leishmanicidal but enhances replication of macrophage-associated mycobacteria. *Infect. Immun.* 50: 1–8.
9. Rook, G. A. W., J. Steele, M. Ainsworth, and B. R. Champion. 1986. Activation of macrophages to inhibit proliferation of *Mycobacterium tuberculosis*: comparison of the effects of recombinant γ-interferon on human monocytes and murine peritoneal macrophages. *Immunology* 59: 333–338.
10. Kindler, V., A.-P. Sappino, G. E. Grau, P.-F. Pituet, and P. Vassalli. 1989. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell* 56: 731–740.
11. Bopst, M., I. Garcia, R. Guler, M. L. Olleros, T. Rulicke, M. Muller, S. Wyss, K. Frei, M. Le Hir, and H. P. Eugster. 2001. Differential effects of TNF and LTα in the host defense against *M. bovis* BCG. *Eur. J. Immunol.* 31: 1935–1943.
12. Keane, J. 2004. Tumor necrosis factor blockers and reactivation of latent tuberculosis. *Clin. Infect. Dis.* 39: 300–302.
13. Warwick-Davies, J., J. Dhillon, L. O'Brien, P. W. Andrew, and D. B. Lowrie. 1994. Apparent killing of *Mycobacterium tuberculosis* by cytokine activated human monocytes can be an artifact of a cytotoxic effect on the monocytes. *Clin. Exp. Immunol.* 96: 214–217.
14. Kaufmann, S. H. 1999. Cell-mediated immunity: dealing a direct blow to pathogens. *Curr. Biol.* 9: R97–R99.

15. Silver, R. F., Q. Li, W. H. Boom, and J. J. Ellner. 1998. Lymphocyte-dependent inhibition of growth of virulent *Mycobacterium tuberculosis* H37Rv within human monocytes: requirement for $CD4^+$ T cells in purified protein derivative-positive, but not in purified protein derivative-negative subjects. *J. Immunol.* 160: 2408–2417.
16. Canaday, D. H., R. J. Wilkinson, Q. Li, C. V. Harding, R. F. Silver, and W. H. Boom. 2001. $CD4^+$ and $CD8^+$ T cells kill intracellular *Mycobacterium tuberculosis* by a perforin and Fas/Fas ligand-independent mechanism. *J. Immunol.* 167: 2734–2742.
17. Stenger, S., R. J. Mazzaccaro, K. Uyemura, S. Cho, P. F. Barnes, J.-P. Rosat, A. Sette, M. B. Brenner, M. A. Porcelli, B. R. Bloom, and R. L. Modlin. 1997. Differential effects of cytolytic T cell subsets on intracellular infection. *Science* 276: 1684–1687.
18. Stenger, S., D. A. Hanson, R. Teitelbaum, P. Dewan, K. R. Niazi, C. J. Froelich, T. Ganz, S. Thoma-Uszynski, A. Melian, C. Bogdan, et al. 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* 282: 121–125.
19. Oddo, M., T. Renno, A. Attinger, T. Bakker, H. R. MacDonald, and P. R. Meylan. 1998. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*. *J. Immunol.* 160: 5448–5454.
20. Molloy, A., P. Laochumroonvorapong, and G. Kaplan. 1994. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guérin. *J. Exp. Med.* 180: 1499–1509.
21. Fairbairn, I. P., C. B. Stober, D. S. Kumararatne, and D. A. Lammas. 2001. ATP-mediated killing of intracellular mycobacteria by macrophages is a $P2X_7$-dependent process inducing bacterial death by phagosome-lysosome fusion. *J. Immunol.* 167: 3300–3307.
22. Hancock, G. E., Z. A. Cohn, and G. Kaplan. 1989. The generation of antigen-specific, major histocompatibility complex-restricted cytotoxic T lymphocytes of the $CD4^+$ penotype: enhancement by the cutaneous administration of interleukin 2. *J. Exp. Med.* 169: 909–919.
23. Ottenhoff, T. H. M., and T. Mutis. 1990. Specific killing of cytotoxic T cells and antigen-presenting cells by $CD4^+$ cytotoxic T cell clones. *J. Exp. Med.* 171: 2011–2024.
24. Boom, W. H., R. S. Wallis, and K. A. Chervenak. 1991. Human *Mycobacterium tuberculosis*-reactive $CD4^+$ T-cell clones: heterogeneity in antigen-recognition, cytokine production, and cytotoxicity for mononuclear phagocytes. *Infect. Immun.* 59: 2737–2743.
25. Mutis, T., Y. E. Cornelisse, and T. H. M. Ottenhoff. 1993. Mycobacteria induce $CD4^+$ T cells that are cytotoxic and display Th1-like cytokine secretion profile: heterogeneity in cytotoxic activity and cytokine secretion levels. *Eur. J. Immunol.* 23: 2189–2195.
26. Lorgat, F., M. M. Keraan, P. T. Lukey, and S. R. Ress. 1992. Evidence for in vivo generation of cytotoxic T cells: PPD-stimulated lymphocytes from tuberculous pleural effusions demonstrate enhanced cytotoxicity with accelerated kinetics of induction. *Am. Rev. Respir. Dis.* 145: 418–423.
27. Kale Ab, B., R. Kiessling, J. D. A. Van Embden, J. E. R. Thole, D. Kumararatne, P. Pisa, A. Wondimu, and T. H. M. Ottenhoff. 1990. Induction of antigen-specific $CD4^+$ HLA-DR-restricted cytotoxic T lymphocytes as well as nonspecific non-restricted killer cells by the recombinant mycobacterial 65-kDa heat-shock protein. *Eur. J. Immunol.* 20: 369–377.
28. Tan, J. S., D. H. Canaday, W. H. Boom, K. N. Balaji, S. K. Schwander, and E. A. Rich. 1997. Human alveolar T lymphocyte response to *Mycobacterium tuberculosis* antigens: role for $CD4^+$ and $CD8^+$ cytotoxic T cells and relative resistance of alveolar macrophages to lysis. *J. Immunol.* 159: 290–297.
29. Chackerian, A. A., T. V. Perera, and S. M. Behar. 2001. γ Interferon-producing $CD4^+$ T lymphocytes in the lung correlate with resistance to infection with *Mycobacterium tuberculosis*. *Infect. Immun.* 69: 2666–2674.
30. Scanga, C. A., V. P. Mohan, K. Yu, H. Joseph, K. Tanaka, J. Chan, and J. L. Flynn. 2000. Depletion of $CD4^+$ T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon γ and nitric oxide synthase 2. *J. Exp. Med.* 192: 347–358.
31. Fayyazi, A., B. Eichmeyer, A. Soruri, S. Schweyer, J. Herms, P. Schwarz, and H. J. Radzun. 2000. Apoptosis of macrophages and T cells in tuberculosis associated caseous necrosis. *J. Pathol.* 191: 417–425.
32. Cree, I. A., S. Nurbhai, G. Milne, and J. S. Beck. 1987. Cell death in granulomata: the role of apoptosis. *J. Clin. Pathol.* 40: 1314–1319.
33. Mustafa, T., S. Phyu, R. Nilsen, G. Bjune, and R. Jonsson. 1999. Increased expression of Fas ligand on *Mycobacterium tuberculosis* infected macrophages: a potential novel mechanism of immune evasion by *Mycobacterium tuberculosis*? *Inflammation* 23: 507–521.
34. Green, D. R. 2000. Apoptotic pathways: paper wraps stone blunts scissors. *Cell* 102: 1–4.
35. Mazzaccaro, R. J., M. Gedde, E. R. Jensen, H. M. van Santen, H. L. Ploegh, K. L. Rock, and B. R. Bloom. 1996. Major histocompatibility class I presentation of soluble antigen facilitated by *Mycobacterium tuberculosis* infection. *Proc. Natl. Acad. Sci. USA* 93: 11786–11791.
36. Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, et al. 1999. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. *Science* 285: 732–736.
37. Quesniaux, V., C. Fremond, M. Jacobs, S. Parida, D. Nicolle, V. Yeremeev, F. Bihl, F. Erard, T. Botha, M. Drennan, et al. 2004. Toll-like receptor pathways in the immune responses to mycobacteria. *Microb. Infect.* 6: 946–959.
38. Boneca, I. G. 2005. The role of peptidoglycan in pathogenesis. *Curr. Opin. Microbiol.* 8: 46–53.
39. Hmama, Z., R. Gabathuler, W. A. Jefferies, G. de Jong, and N. E. Reiner. 1998. Attenuation of HLA-DR expression by mononuclear phagocytes infected with *Mycobacterium tuberculosis* is related to intracellular sequestration of immature class II heterodimers. *J. Immunol.* 161: 4882–4893.
40. Garcia-Calvo, M., E. P. Peterson, D. M. Rasper, J. P. Vaillancourt, R. Zamboni, D. W. Nicholson, and N. A. Thornberry. 1999. Purification and catalytic properties of human caspase family members. *Cell Death Differ.* 6: 362–369.
41. Burns, K., F. Martinon, C. Esslinger, H. Pahl, P. Schneider, J. L. Bodmer, M. F. Di, L. French, and J. Tschopp. 1998. MyD88, an adapter protein involved in interleukin-1 signaling. *J. Biol. Chem.* 273: 12203–12209.
42. Thome, M., K. Hofmann, K. Burns, F. Martinon, J. L. Bodmer, C. Mattmann, and J. Tschopp. 1998. Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. *Curr. Biol.* 8: 885–888.
43. Girardin, S. E., I. G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D. J. Philpott, and P. J. Sansonetti. 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278: 8869–8872.
44. Kobayashi, K., N. Inohara, L. D. Hernandez, J. E. Galan, G. Nunez, C. A. Janeway, R. Medzhitov, and R. A. Flavell. 2002. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 416: 194–199.
45. Schreck, R., B. Meier, D. N. Mannel, W. Droge, and P. A. Baeuerle. 1992. Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J. Exp. Med.* 175: 1181–1194.
46. Irmler, M., M. Thome, M. Hahne, P. Schneider, K. Hofmann, V. Steiner, J. L. Bodmer, M. Schroter, K. Burns, C. Mattmann, et al. 1997. Inhibition of death receptor signals by cellular FLIP. *Nature* 388: 190–195.
47. Micheau, O., S. Lens, O. Gaide, K. Alevizopoulos, and J. Tschopp. 2001. NF-κB signals induce the expression of c-FLIP. *Mol. Cell. Biol.* 21: 5299–5305.
48. Dürrbaum-Landmann, I., J. Gercken, H. D. Flad, and M. Ernst. 1996. Effect of in vitro infection of human monocytes with low numbers of *Mycobacterium tuberculosis* bacteria on monocyte apoptosis. *Infect. Immun.* 64: 5384–5389.
49. Klingler, K., K. M. Tchou-Wong, O. Brandli, C. Aston, R. Kim, C. Chi, and W. N. Rom. 1997. Effects of mycobacteria on regulation of apoptosis in mononuclear phagocytes. *Infect. Immun.* 65: 5272–5278.
50. Placido, R., G. Mancino, A. Amendola, F. Mariani, S. Vendetti, M. Piacentini, A. Sanduzzi, M. L. Bocchino, M. Zembala, and V. Colizzi. 1997. Apoptosis of human monocytes/macrophages in *Mycobacterium tuberculosis* infection. *J. Pathol.* 181: 31–38.
51. Rojas, M., L. F. Barrera, G. Puzo, and L. F. Garcia. 1997. Differential induction of apoptosis by virulent *Macobacterium tuberculosis* in resistant and susceptible murine macrophages: role of nitric oxide and mycobacterial products. *J. Immunol.* 159: 1352–1361.
52. Balcewicz-Sablinska, M. K., J. Keane, H. Kornfeld, and H. G. Remold. 1998. Pathogenic *Mycobacterium tuberculosis* evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF-α. *J. Immunol.* 161: 2636–2641.
53. Keane, J., H. G. Remold, and H. Kornfeld. 2000. Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. *J. Immunol.* 164: 2016–2020.
54. Riendeau, C. J., and H. Kornfeld. 2003. THP-1 cell apoptosis in response to mycobacterial infection. *Infect. Immun.* 71: 254–259.
55. Sly, L. M., S. M. Hingley-Wilson, N. E. Reiner, and W. R. McMaster. 2003. Survival of *Mycobacterium tuberculosis* in host macrophages involves resistance to apoptosis dependent upon induction of antiapoptotic Bcl-2 family member Mcl-1. *J. Immunol.* 170: 430–437.
56. Stokes, R. W., and D. Doxsee. 1999. The receptor-mediated uptake, survival, replication, and drug sensitivity of *Mycobacterium tuberculosis* within the macrophage-like cell line THP-1: a comparison with human monocyte-derived macrophages. *Cell. Immunol.* 197: 1–9.
57. Ogura, Y., N. Inohara, A. Benito, F. F. Chen, S. Yamaoka, and G. Nunez. 2001. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-κB. *J. Biol. Chem.* 276: 4812–4818.
58. Thoma-Uszynski, S., S. Stenger, O. Takeuchi, M. T. Ochoa, M. Engele, P. A. Sieling, P. F. Barnes, M. Rollinghoff, P. L. Bolcskei, M. Wagner, et al. 2001. Induction of direct antimicrobial activity through mammalian Toll-like receptors. *Science* 291: 1544–1547.
59. Noss, E. H., R. K. Pai, T. J. Sellati, J. D. Radolf, J. Belisle, D. T. Golenbock, W. H. Boom, and C. V. Harding. 2001. Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. *J. Immunol.* 167: 910–918.
60. Tobian, A. A., N. S. Potter, L. Ramachandra, R. K. Pai, M. Convery, W. H. Boom, and C. V. Harding. 2003. Alternate class I MHC antigen processing is inhibited by Toll-like receptor signaling pathogen-associated molecular patterns: *Mycobacterium tuberculosis* 19-kDa lipoprotein, CpG DNA, and lipopolysaccharide. *J. Immunol.* 171: 1413–1422.
61. Sibley, L. D., S. W. Hunter, P. J. Brennan, and J. L. Krahenbuhl. 1988. Mycobacterial lipoarabinomannan inhibits γ interferon-mediated activation of macrophages. *Infect. Immun.* 56: 1232–1236.
62. Maiti, D., A. Bhattacharyya, and J. Basu. 2001. Lipoarabinomannan from *Mycobacterium tuberculosis* promotes macrophage survival by phosphorylating Bad through a phosphatidylinositol 3-kinase/Akt pathway. *J. Biol. Chem.* 276: 329–333.
63. Lopez, M., L. M. Sly, Y. Luu, D. Young, H. Cooper, and N. E. Reiner. 2003. The 19-kDa *Mycobacterium tuberculosis* protein induces macrophage apoptosis through Toll-like receptor-2. *J. Immunol.* 170: 2409–2416.
64. Ciaramella, A., A. Cavone, M. B. Santucci, S. K. Garg, N. Sanarico,M. Bocchino,

D. Galati, A. Martino, G. Auricchio, M. D'Orazio, et al. 2004. Induction of apoptosis and release of interleukin-1β by cell wall-associated 19-kDa lipoprotein during the course of mycobacterial infection. *J. Infect. Dis.* 190: 1167–1176.

65. Dao, D. N., L. Kremer, Y. Guerardel, A. Molano, W. R. Jacobs, Jr., S. A. Porcelli, and V. Briken. 2004. *Mycobacterium tuberculosis* lipomannan induces apoptosis and interleukin-12 production in macrophages. *Infect. Immun.* 72: 2067–2074.
66. Ruckdeschel, K., G. Pfaffinger, R. Haase, A. Sing, H. Weighardt, G. Hacker, B. Holzmann, and J. Heesemann. 2004. Signaling of apoptosis through TLRs critically involves Toll/IL-1 receptor domain-containing adapter inducing IFN-β, but not MyD88, in bacteria-infected murine macrophages. *J. Immunol.* 173: 3320–3328.
67. Fremond, C. M., V. Yeremeev, D. M. Nicolle, M. Jacobs, V. F. Quesniaux, and B. Ryffel. 2004. Fatal *Mycobacterium tuberculosis* infection despite adaptive immune response in the absence of MyD88. *J. Clin. Invest.* 114: 1790–1799.
68. Lee, R., P. Beauparlant, H. Elford, P. Ponka, and J. Hiscott. 1997. Selective inhibition of lκBα phosphorylation and HIV-1 LTR-directed gene expression by novel antioxidant compounds. *Virology* 234: 277–290.
69. Thome, M., and J. Tschopp. 2001. Regulation of lymphocyte proliferation and death by FLIP. *Nat. Rev. Immunol.* 1: 50–58.
70. Goodkin, M. L., A. T. Ting, and J. A. Blaho. 2003. NF-κB is required for apoptosis prevention during herpes simplex virus type 1 infection. *J. Virol.* 77: 7261–7280.
71. Joshi, S. G., C. W. Francis, D. J. Silverman, and S. K. Sahni. 2003. Nuclear factor κB protects against host cell apoptosis during *Rickettsia rickettsii* infection by inhibiting activation of apical and effector caspases and maintaining mitochondrial integrity. *Infect. Immun.* 71: 4127–4136.
72. MacMicking, J. D., G. A. Taylor, and J. D. McKinney. 2003. Immune control of tuberculosis by IFN-γ-inducible LRG-47. *Science* 302: 654–659.
73. Gutierrez, M. G., S. S. Master, S. B. Singh, G. A. Taylor, M. I. Colombo, and V. Deretic. 2004. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* 119: 753–766.