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Fas Ligand-Induced Apoptosis of Infected Human Macrophages Reduces the Viability of Intracellular *Mycobacterium tuberculosis*¹

Mauro Oddo,* Toufic Renno,‡ Antoine Attinger,‡ Talitha Bakker,‡ H. Robson MacDonald,‡ and Pascal R. A. Meylan^{2*†}

Mycobacterium tuberculosis-specific cytolytic activity is mediated mostly by CD4⁺ CTL in humans. CD4⁺ CTL kill infected target cells by inducing Fas (APO-1/CD95)-mediated apoptosis. We have examined the effect of Fas ligand (FasL)-induced apoptosis of human macrophages infected in vitro with *M. tuberculosis* on the viability of the intracellular bacilli. Human macrophages expressed Fas and underwent apoptosis after incubation with soluble recombinant FasL. In macrophages infected either with an attenuated (H37Ra) or with a virulent (H37Rv) strain of *M. tuberculosis*, the apoptotic death of macrophages was associated with a substantial reduction in bacillary viability. TNF-induced apoptosis of infected macrophages was coupled with a similar reduction in mycobacterial viability, while the induction of nonapoptotic complement-induced cell death had no effect on bacterial viable counts. Infected macrophages also showed a reduced susceptibility to FasL-induced apoptosis correlating with a reduced level of Fas expression. These data suggest that apoptosis of infected macrophages induced through receptors of the TNF family could be an immune effector mechanism not only depriving mycobacteria from their growth environment but also reducing viable bacterial counts by an unknown mechanism. On the other hand, interference by *M. tuberculosis* with the FasL system might represent an escape mechanism of the bacteria attempting to evade the effect of apoptosis. *The Journal of Immunology*, 1998, 160: 5448–5454.

Apoptosis (or programmed cell death) is a distinct form of cell death that is essential for the regulation of the immune system. Fas (APO-1/CD 95) (1), a member of the TNF receptor family, and its ligand (FasL)³ (2) play an important role in various processes involving the induction of apoptosis. Binding of FasL to Fas results in the transduction of a signal into the cell leading to apoptosis (3). The FasL system is implicated in both the clonal deletion of autoreactive T cells and the elimination of activated T cells after they have responded to foreign antigens (4). In addition, FasL-induced apoptosis has recently emerged as a major cytolytic pathway that CTL, particularly of the CD4⁺ subset, use to kill susceptible target cells (5, 6) (reviewed in Ref. 3), including activated macrophages (7).

Protective immunity against the intracellular pathogen *Mycobacterium tuberculosis* is dependent on the activation of T cells (8). Initially, it was thought that Ag-specific helper T cells mediate their antimicrobial activity exclusively via the release of macrophage-activating cytokines (9). However, studies in mice and humans have demonstrated that mycobacteria, like other intracellular

pathogens, induce Ag-specific, MHC-restricted CD4⁺ (10–14) and CD8⁺ (15) CTL. In humans, the bulk of *M. tuberculosis*-specific cytolytic activity is mediated by CD4⁺ CTL (16, 17), and the killing by CD4⁺ CTL clones of *Mycobacterium*-infected human macrophages has been shown, in some instances, to inhibit the growth of the bacilli (18), probably by destroying the intracellular habitat of the microorganisms (19). Since CD4⁺ CTL kill target cells via FasL-induced apoptosis (20), we analyzed whether the incubation with sFasL could kill macrophages infected with intracellular mycobacteria and possibly affect bacillary viability.

We show here that this is indeed the case. Moreover, we demonstrate that another physiologic inducer of apoptosis, TNF- α , has the same effect on infected macrophages, suggesting that apoptosis-related reduction of bacterial viable counts in *M. tuberculosis*-infected macrophages is not restricted to one particular apoptosis induction pathway.

Materials and Methods

Bacteria

M. tuberculosis H37Ra (attenuated strain) and H37Rv (virulent strain) (American Type Culture Collection, Manassas, VA) were obtained from Thomas Bodmer (Institute of Microbiology, Bern, Switzerland). Mycobacterial strains were grown in 7H9 broth (Difco Laboratories, Detroit, MI) containing 10% ADC supplement (Difco) and 0.05% Tween 80 (Difco), up to an OD₆₀₀ of 1, corresponding to a bacterial suspension of $\sim 1 \times 10^8$ CFU/ml.

Reagents

Soluble recombinant Fas ligand (sFasL, 200 U/ml) was a gift from Jürg Tschopp (Institute of Biochemistry, University of Lausanne, Epalinges, Switzerland). sFasL was obtained by transfecting Neuro-2a cells (5×10^6) with cloned human FasL cDNA, as described (21). The activity of sFasL-containing supernatant was determined by measuring the viability of A20 cells (22) exposed to serial dilutions of sFasL. One unit of sFasL was defined as the amount causing 75% cell death in 12 h. Anti-APO-1 IgG3 hybridoma supernatant was a gift from Peter H. Krammer (Division of Immunogenetics, German Cancer Research Center, Heidelberg, Germany).

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³ Abbreviations used in this paper: FasL, Fas ligand; CHX, cycloheximide; sFasL, soluble recombinant Fas ligand; PI, propidium iodide; rhu, recombinant human.

TNF- α (recombinant human (rhu) TNF- α , Boehringer Ingelheim, Basel, Switzerland), purified mouse IgG3 isotype control monoclonal Ab and FITC-conjugated goat anti-mouse Ig antiserum (PharMingen, San Diego, CA), cycloheximide (CHX, Sigma Chemical, St. Louis, MO), Annexin V-FITC (Nexins Research BV, Hoeven, The Netherlands), Propidium iodide (PI, Sigma), 4',6-Diamidino-2-phenylindole (Boehringer-Mannheim, Mannheim, Germany) and Lympho-kwik reagent (One Lambda, Canoga Park, CA) were obtained commercially.

Isolation of monocyte-derived macrophages

Human macrophages were derived from blood monocytes obtained from healthy volunteers and prepared by centrifugation over a Ficoll-Hypaque (Seromed, Biochrom, Berlin, Germany) gradient followed by a fibronectin adherence step (23). Monocytes were resuspended in RPMI 1640 (Life Technologies, Gaithersburg, MD) with 2 mM L-alanyl-L-glutamine (Life Technologies) and 5% heat-inactivated human AB⁺ serum (Sigma), plated at 4×10^5 cells/cm² on 24-well Falcon *Primaria* plates (Becton Dickinson, Lincoln Park, NJ), and allowed to differentiate for 5 to 7 days at 37°C and 5% CO₂, before infection. Cells obtained with this protocol gave rise to an even monolayer and were >95% esterase positive.

Infection of macrophages with *M. tuberculosis*

After four washings by centrifugation in RPMI 1640 to avoid any LPS contamination from the bacterial culture medium, the bacterial pellet was resuspended in RPMI 1640 containing 10% fresh human AB⁺ serum and sonicated for 20 s in a 50-ml conical polystyrene tube (Falcon) in a cup-horn transducer at maximum output (400 W, Branson 450, Branson Ultrasonics, Danbury, CT). This amount of sonication has been previously determined to be optimal for dispersing bacteria without causing significant loss of viability. Finally, the inoculum was filtered through a 5- μ m pore size polyvinylidene difluoride Milllex filter membrane (Millipore, Bedford, MA) to produce a strictly single-cell suspension. The bacterial density of the final suspension was estimated by phase contrast microscopy and adjusted to $\sim 10^7$ bacilli/ml. The viable counts of the inoculum were then checked by serial dilution and plating on 7H10 agar with 10% Middlebrook OADC supplement (Difco, Detroit, MI). Monolayers of macrophages were infected by replacing the culture medium with the *M. tuberculosis* inoculum for 2 h. The cells were then washed three times with RPMI 1640 and cultured in RPMI 1640 with 10% FCS (PAA, Linz, Austria). To obtain a morphologic assessment of the infection, macrophages differentiated and infected in parallel on slide chambers (Nunc, Naperville, IL) were fixed and stained by the Kinyoun method.

Induction of apoptosis in human macrophages

Uninfected and infected macrophages (5×10^5 cells) were incubated for 6 to 18 h in RPMI 1640 with 10% FCS containing recombinant sFasL (final concentration, 40 U/ml) or rhuTNF α (10 ng/ml). All samples, including controls, were treated with 1 μ g/ml CHX. At the end of the incubation time, cells were incubated with PBS containing 5 mM EDTA and detached by gentle scraping. After detachment, the viability of control macrophages was >90% as assessed by trypan blue exclusion.

Quantification of macrophages undergoing apoptosis by annexin V-FITC assay

The binding of annexin V-FITC was used as a sensitive measurement of macrophage apoptosis and performed by modification of a previously described method (24). Cells were incubated in 100 μ l of binding buffer (HEPES-buffered saline solution with 2 mM CaCl₂, pH 7.5) containing 1/500 dilution of annexin V-FITC for 30 min at 4°C and then with propidium iodide (PI) (2 μ g/ml). Macrophages were immediately analyzed, without fixation, on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). After gating on the basis of forward vs side scatter to exclude cell debris, macrophage apoptosis was quantitatively evaluated by measuring the proportion of annexin V-FITC, excluding or not PI-positive cells. Macrophages infected with H37Rv strain were stained with annexin V-FITC only and fixed before analysis with 1% paraformaldehyde (Sigma) dissolved in HEPES buffer with 2 mM Ca²⁺, pH 7.5.

Assessment of macrophage apoptosis by TUNEL

Cells undergoing DNA fragmentation were detected in situ with the TUNEL method (terminal deoxyribonucleotidyl transferase labeling of DNA strand breaks with dUTP, Boehringer-Mannheim), according to the manufacturer's instructions. Stained cells were analyzed under light microscopy.

Assessment of bacillary viability in human macrophages

To evaluate bacillary viability, monolayers of 5×10^5 *M. tuberculosis*-infected macrophages differentiated in 24-well plates were used in triplicates. Culture medium was removed and replaced with 200 μ l of 0.1% saponin (Sigma) in RPMI 1640, and the adherent cells were incubated at 37°C for 20 min. The supernatant of each well was also lysed separately by adding saponin to the supernatant to a final concentration of 0.1%, so that no colony forming unit could escape detection due to cell detachment or lysis. After gentle mixing, the supernatant and cell lysates were collected and sonicated for 20 s to disperse the residual clumps of bacilli. Serial 10-fold dilutions were made in 7H9 broth and plated on 7H10 agar plates. Plates were sealed in plastic, kept at 37°C, and counted after 11 to 13 days, with the aid of a dissecting microscope. The bacterial counts were calculated based on the counting of colonies from a dilution containing at least 20 colonies and expressed as the number of CFU per well of both adherent cell and supernatant lysate. In every experiment, >95% of the CFU counts were cell associated.

Induction of cell lysis in infected macrophages

Cell lysis was induced by incubating macrophages for 18 h with mAbs against nonlymphoid leukocytes and complement (Lympho-kwik reagent) and was assessed by phase contrast microscopy.

Detection of Fas expression on human macrophages

Cell surface Fas expression was quantitated on human macrophages by flow cytometry. Uninfected and infected adherent human macrophages were detached with 5 mM PBS-EDTA and gentle scraping. Viability was >90% as determined by trypan blue exclusion. Cells (5×10^5) were incubated with 100 μ l of anti-APO-1 IgG3 hybridoma supernatant (5 μ g/ml) in PBS-5% FCS for 45 min at 4°C and then with a 1:100 dilution of FITC-conjugated goat anti-mouse Ig (PharMingen, San Diego, CA). Macrophages were also stained with goat anti-mouse FITC alone (negative control) or purified mouse IgG3 plus goat anti-mouse FITC (isotype control). Cells were then analyzed on a FACScan flow cytometer.

Results

FasL-induced apoptosis in human macrophages

Apoptosis of human macrophages was analyzed by cytofluorometric detection of annexin V and PI staining. In living cells, phosphatidylserine is stringently located in the membrane leaflet that faces the cytosol. The surface expression of phosphatidylserine is an early feature of apoptosis and occurs before the loss of membrane integrity (25). Early apoptotic cells bind annexin V, a Ca²⁺-dependent phospholipid-binding protein with high affinity for phosphatidylserine, but exclude PI and can therefore be specifically detected and quantified by FACS analysis (26). Recent studies have shown that CHX blocks the synthesis of proteins that prevent cell death and thus enhances the killing mediated by death factors of the TNF family, including FasL (27) and TNF- α (28). Incubation of macrophages with sFasL (40 U/ml), in combination with CHX (1 μ g/ml), led to a substantial increase in the percentage of cells undergoing apoptosis (Fig. 1), showing that human macrophages are indeed susceptible to FasL-induced killing. The induction of apoptosis by sFasL was dose dependent from 20 to 200 U/ml and time dependent from 6 to 24 h (data not shown). When the analysis was restricted to PI-negative cells, representing cells that have maintained their membrane integrity, the percentage of early apoptotic annexin V-positive cells significantly increased in FasL-treated cells. A similar increase in the percentage of annexin V-positive cells was observed among the total cell population. In all additional experiments, the analysis of apoptotic cells was specifically restricted to PI-negative macrophages, which represented >70% of the total cell population. Macrophage apoptosis was shown to be specifically FasL dependent in all our experiments, as the incubation of cells with supernatant of mock-transfected Neuro-2a cells, CHX, or both did not induce any significant increase in apoptosis (data not shown). The occurrence of apoptosis in sFasL-treated macrophages was further confirmed by the observation of chromatin condensation, a hallmark of apoptosis, using

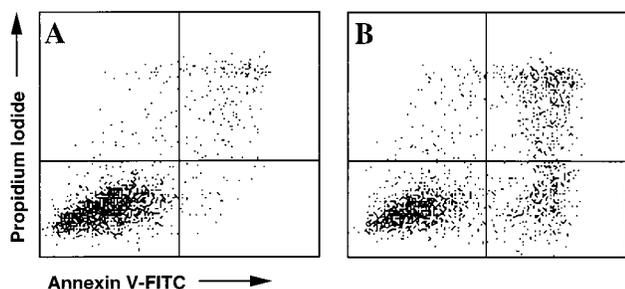


FIGURE 1. FasL-induced apoptosis in human macrophages. Human macrophages were incubated for 12 h with sFasL as described in *Materials and Methods*, stained with annexin V-FITC and PI and analyzed by flow cytometry. Two-dimensional dot plot analyses of control (A) and sFasL-treated (B) macrophages are depicted. Lower left quadrant, viable cells (annexin V-FITC and PI negative); lower right quadrant, early apoptotic cells (annexin V-FITC positive and PI negative); upper right quadrant, cells that have already died (annexin V-FITC and PI positive). Data are representative of six separate experiments.

4',6-diamidino-2-phenylindole nuclear staining (data not shown), as well as by the in situ observation of DNA fragmentation using the TUNEL assay (Fig. 2, B–E).

Apoptosis of infected macrophages is associated with a reduction in viability of intracellular M. tuberculosis

There are potential caveats when evaluating the intracellular survival and growth of *M. tuberculosis* in macrophages; high density inocula, and in particular bacterial clumps, lead to an overload of the macrophages with concomitant cytotoxicity and early macrophage lysis (29) (our unpublished results). It is therefore crucial to prepare a strictly single-cell suspension, such that the vast majority of macrophages are infected with 5 to 10 isolated bacilli each. Monolayers of adherent human macrophages were infected 5 to 7 days after isolation with a single-cell suspension of *M. tuberculosis*. With this procedure, the infection of >95% of the macrophages was routinely achieved (see, e.g., H37Ra-infected macrophages in Fig. 2A). Similar results were observed using H37Rv). After 2 h of infection, cells were washed three times to eliminate bacteria that were not cell associated and incubated with sFasL. To investigate whether apoptosis affected the viability of *M. tuberculosis*, measurements of annexin V binding and bacterial colony counts were performed after 18 h in macrophages infected with the attenuated H37Ra strain. *M. tuberculosis*-infected macrophages were susceptible to FasL-induced apoptosis (Fig. 3A, sFasL). Bacillary viability was determined in parallel by CFU counting. FasL-induced apoptosis of macrophages infected with the attenuated H37Ra strain was associated with a $63 \pm 7\%$ reduction in bacterial colony counts after 18 h (sFasL), compared with the colony counts immediately after infection (T0). During the same time interval, no change in mycobacterial viability was observed in control cells treated with supernatant of mock-transfected Neuro-2a cells and CHX (Fig. 3B, CTRL). The separate culture of cell and supernatant lysates confirmed that the decreased bacterial counts in macrophages undergoing FasL-induced apoptosis were not accompanied by increased bacterial counts in the supernatant (data not shown). This excludes cell detachment or cell lysis with release of bacteria as the cause for the reduced bacterial viable counts in adherent macrophages. Neither the addition of sFasL nor that of CHX alone to the bacterial inoculum suspension prepared in RPMI medium affected mycobacterial viability (data not shown). Furthermore, when infected macrophages were completely resistant to FasL-induced apoptosis, as observed in one of the donors tested

(see below), no reduction of mycobacterial viability was observed (data not shown).

We next asked whether another apoptosis inducer could also increase bacterial killing. TNF- α induces apoptosis by activating the same cascade of cellular proteases as FasL (3). Indeed, like sFasL, rhuTNF- α (10 ng/ml) induced apoptosis in infected macrophages (Fig. 3A, TNF) when associated with cycloheximide. TNF-induced apoptosis of *M. tuberculosis*-infected human macrophages was associated with a comparable reduction in mycobacterial viability as sFasL-induced apoptosis. (Fig. 3B, TNF). Macrophages treated with rhuTNF- α alone did not undergo noticeable apoptosis, and there was no reduction in bacterial counts (data not shown).

Finally, we tested in parallel the effect of apoptosis on the viability of the attenuated (Ra) and the virulent (Rv) strains. FasL treatment induced a similar increase of the percentage of annexin V-binding cells (data not shown) and a comparable reduction of viable counts with either strain (Fig. 4).

Nonapoptotic cell death of infected human macrophages does not affect the viability of intracellular M. tuberculosis

To test whether the reduction in mycobacterial counts was specifically related to FasL-induced killing of infected macrophages, cells were killed by complement-mediated cell lysis, using a mix of Abs directed against nonlymphoid leukocytes and complement. Cell lysis, as assessed by phase contrast microscopy, was complete after 30 min, with only macrophage ghosts remaining. *M. tuberculosis* viability was determined after 18 h by CFU assay in cultures of infected macrophages treated with complement and compared with parallel cultures of untreated and sFasL-treated macrophages. As shown in Figure 5, complement-mediated lysis of infected macrophages had no effect on bacillary viability, whereas FasL-induced apoptosis was coupled with a significant reduction in cell-associated bacillary counts. In contrast to apoptotic death, cell lysis resulted in threefold increased bacterial numbers in the culture supernatant (data not shown).

M. tuberculosis-infected macrophages showed a reduced susceptibility to FasL-induced apoptosis and reduced levels of Fas expression

With sFasL treatment, the proportion of apoptotic cells in infected macrophages was diminished, to a variable extent, compared with that of uninfected cells from the same donor (Fig. 6). The decreased proportion of apoptotic cell death in *M. tuberculosis*-infected macrophages was observed whether the analysis was restricted to PI-negative cells or extended to the total cell population, including live and dead cells. This suggests that intracellular *M. tuberculosis* was able to prevent its host macrophage from FasL-induced apoptosis. These observations led us to ask whether the intracellular *M. tuberculosis* infection might modulate the expression of Fas on human macrophages. Using FACS analysis, we could demonstrate that Fas expression was substantially down-regulated on *M. tuberculosis*-infected cells (Fig. 7), potentially explaining the reduced susceptibility of infected macrophages to FasL-induced apoptosis.

Discussion

In the present work, we demonstrate that FasL-induced apoptosis of human macrophages, infected in vitro with an attenuated strain of *M. tuberculosis*, was associated with a substantial reduction of the viability of the intracellular mycobacteria. A similar effect on *M. tuberculosis* viability was observed when apoptosis was induced by TNF- α .

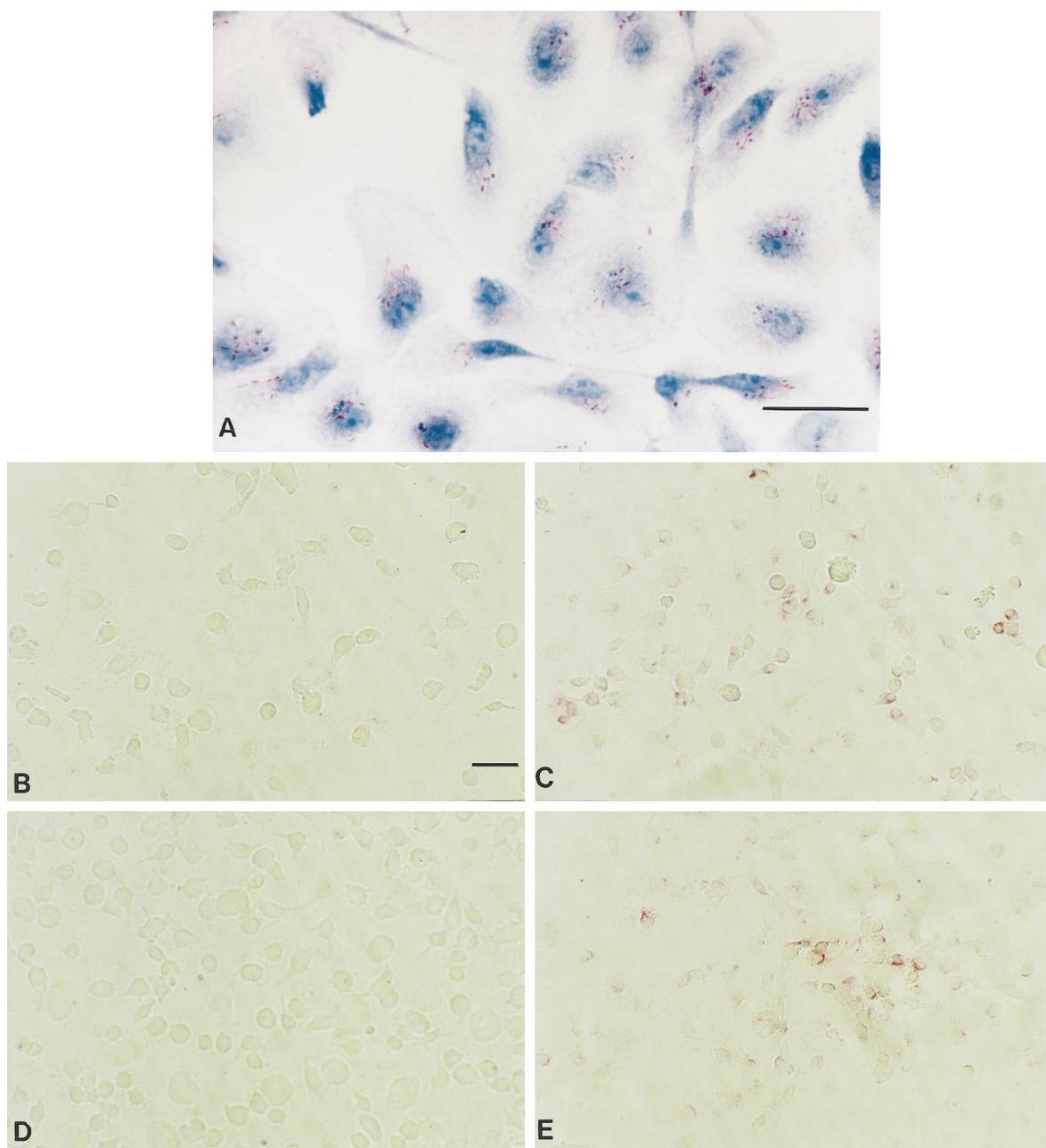


FIGURE 2. Morphology of *M. tuberculosis*-infected human macrophages and detection of apoptosis in situ by TUNEL. Monocyte-derived macrophages were cultivated in slide chambers for 5 to 7 days and exposed to a single-cell suspension of *M. tuberculosis* H37Ra for 2 h. Cells were then washed three times to remove the bacteria that were not cell associated. After the infection, the medium was gently removed, and the slide was fixed and stained with the Kinyoun method (A). Apoptosis of macrophages was detected in situ using the TUNEL assay. Cells were incubated in RPMI-10% FCS medium. After 12 h of incubation with sFasL (40 U/ml), uninfected (C) and H37Ra-infected (E) macrophages, underwent apoptotic cell death, as demonstrated by the labeling of DNA strand breaks with fluorescein-dUTP using terminal deoxynucleotidyl transferase and subsequent detection by an anti-fluorescein-alkaline phosphatase conjugate. Controls, uninfected (B) and infected (D), were incubated with medium alone. All the samples were also incubated with CHX, 1 μ g/ml. Bars represent 40 μ m.

A specific relationship between apoptosis and diminished viable counts is supported by the following observations. Bacterial counts were not reduced when FasL-induced apoptosis was completely inhibited by *M. tuberculosis* infection as observed in one experiment. TNF- α has been claimed to act as a macrophage-arming factor for *M. tuberculosis* (30, 31). In our experiments, however, a reduction in bacterial counts was observed only when TNF- α was associated with cycloheximide to induce apoptosis in infected macrophages, while TNF- α alone did not affect bacterial viability. Finally, nonapoptotic cell death induced by complement-mediated lysis had no effect on

bacillary viability, suggesting that only apoptotic cell death may reduce bacterial viability inside human macrophages.

Molloy et al. (32) recently showed that the induction of apoptosis by chemical agents such as ATP in *Mycobacterium bovis* bacillus Calmette-Guérin-infected macrophages was accompanied by a reduction of intracellular bacterial viable counts. Others have observed that the apoptosis of *Mycobacterium avium*-infected macrophages favored the adherence of freshly added uninfected macrophages and that this process was accompanied by a reduction of *M. avium* viable counts without promoting the release of

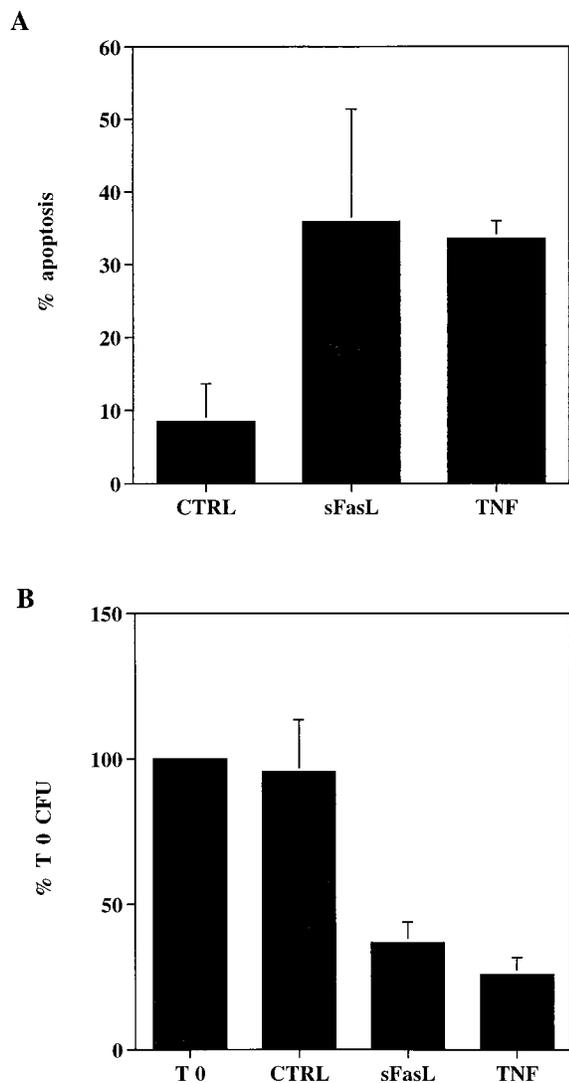


FIGURE 3. Apoptosis of infected macrophages is associated with a reduction of the viability of intracellular *M. tuberculosis*. *A*, Percentage of apoptosis was determined in macrophages infected with the H37Ra strain by the flow cytometric analysis of annexin V-FITC-positive cells only. Cells were incubated for 18 h with medium (RPMI-10% FCS) alone (CTRL), sFasL (40 U/ml), or TNF- α (10 ng/ml). All the samples were incubated with CHX, 1 μ g/ml. Data represent means \pm SD of six separate experiments conducted with sFasL, of which two were performed in parallel with TNF- α . *B*, FasL- and TNF-induced apoptosis was associated with a concomitant reduction of mycobacterial viability in macrophages infected with the attenuated H37Ra strain. Viability of the bacteria was measured by serial dilutions, plating, and colony counting of triplicate cultures of each condition. Bacillary viability was calculated for each experiment as the ratio of mean CFU counts after 18 h for the various conditions compared with the mean CFU counts at the beginning of the infection (T0). The data shown are the mean \pm SD of six experiments with sFasL, of which two were performed in parallel with TNF- α .

extracellular bacteria (33). While this reduction in the bacterial counts was specifically associated with the apoptosis of infected macrophages, the exact relationship between these observations in a complex system and our data awaits further understanding of the relationship between macrophage apoptosis and bacterial loss of viability.

In the present study, we now demonstrate a similar effect using physiologic apoptosis-inducing agents that are relevant to the immune system. In addition, the observation that FasL-induced ap-

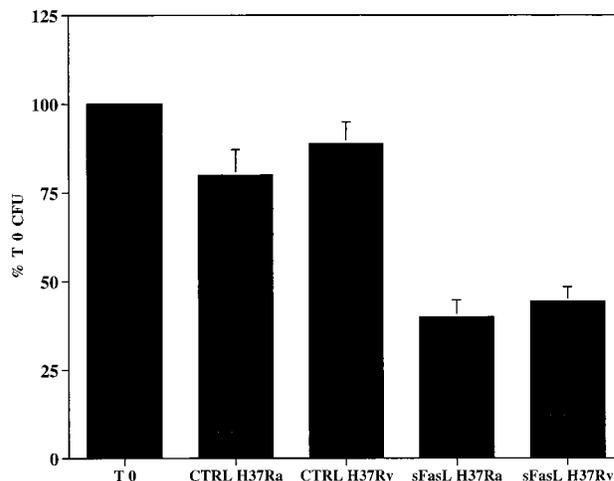


FIGURE 4. Reduction of the viability of the virulent H37Rv *M. tuberculosis* strain by FasL-induced apoptosis of infected macrophages. Induction of apoptosis of macrophages infected in parallel with the attenuated H37Ra or the virulent H37Rv strain of *M. tuberculosis* was associated with a comparable reduction of bacillary viability of both strains. Bacterial viability was calculated as described in Figure 3. Data represent the means \pm SD of two independent experiments. CTRL, control.

optosis of macrophages infected with the virulent H37Rv *M. tuberculosis* strain was also coupled with a reduction in bacterial viability extend these findings to a pathogenic strain of greater clinical relevance.

M. tuberculosis has been shown to inhibit phagosome-lysosome fusion in human macrophages (34) and to reside in a compartment with endosomal characteristics (35), thereby avoiding the hostile environment of the phagolysosome. Conceivably, the induction of apoptosis via the FasL system might reverse the block imposed by *M. tuberculosis* to phagosome-lysosome fusion. In addition, *M.*

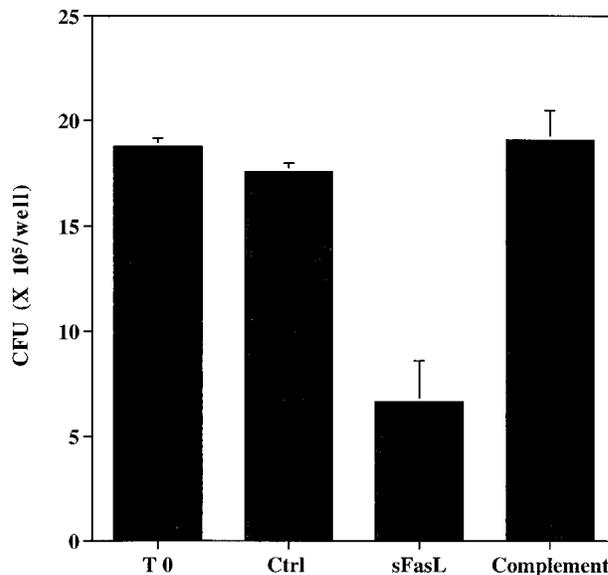


FIGURE 5. Complement-mediated cell death of infected human macrophages does not affect intracellular *M. tuberculosis* viability. Control CFU counts at the beginning of the experiment (T0) were compared with parallel cultures treated with medium alone (CTRL), sFasL-containing medium (sFasL) or complement-containing medium (Complement) for 18 h. Data are expressed as the mean \pm SD of triplicate well cultures and are representative of two separate experiments.

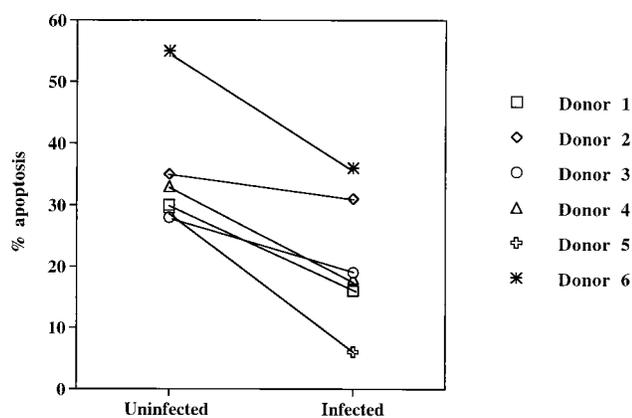


FIGURE 6. *M. tuberculosis*-infected macrophages showed a reduced susceptibility to FasL-induced apoptosis. Uninfected macrophages and macrophages infected with H37Ra strain were incubated in parallel with sFasL-containing medium, and the proportion of annexin V-binding cells was determined by flow cytometry among live (PI-negative) cells. The reduced susceptibility to FasL-induced apoptosis of infected macrophages was significant compared with uninfected macrophages in six separate donors ($p = 0.03$, two-tailed paired Wilcoxon signed rank test).

tuberculosis-containing phagosomes fail to acidify by exclusion of the vesicular proton-ATPase (36). Recent observations showed that FasL-induced apoptosis of Jurkat cells is accompanied by cytoplasmic acidification at a relatively early stage (37). Whether this would result in a reduced pH in *Mycobacterium*-containing phagosomes remains to be determined.

FasL can induce apoptosis in vitro as a membrane-bound molecule or in a soluble form (21), and it has been shown that the supernatant of cultured activated T cells contains FasL and triggers apoptosis in vitro (38). In our experiments, we induced apoptosis using a soluble form of FasL. Other investigators have examined the ability of CD4⁺ CTL to induce lysis of *Mycobacterium*-infected macrophages. In some experiments, a concomitant decrease of bacterial viable counts was observed (18), while no such effect was detected in other studies (39). It is possible that differences in the mechanism of cell death involved in individual experimental systems, the different *M. tuberculosis* strains used, and the differential susceptibility of infected cells to apoptosis may explain these discordant results. Recently, Stenger et al. (40) derived CD1-restricted human T cell lines of two distinct phenotypes that were able to lyse efficiently *M. tuberculosis*-infected macrophages. Interestingly, CD4⁻CD8⁻ T cells lysed macrophages in a Fas-FasL-dependent manner without effect on bacterial viability. In contrast, CD8⁺ T cells lysed macrophages in a granule-dependent mechanism that resulted in killing of bacteria (40). While these data are difficult to reconcile with ours, it should be noted that macrophages presenting mycobacterial antigens in a CD1-restricted manner were obtained through a differentiation procedure involving GM-CSF and IL-4 quite different from our preparation of adherent macrophages (40).

Few data are available concerning the role of CTL-induced apoptosis on the outcome of intracellular pathogen infections in vivo. In humans, the lack of CD4⁺-restricted cytolytic activity against mycobacteria correlate with high bacillary load and a disseminated disease in tuberculous and leprosy patients (17, 41) suggesting that CD4⁺CTL could have a protective role against mycobacterial infections in vivo. This seems to be further confirmed by the experiments conducted in vivo with spontaneous mutants *gld* mice. These mice lack functional FasL on mature T cells and have a strikingly reduced CD4⁺-restricted cytolytic activity (42). Recent

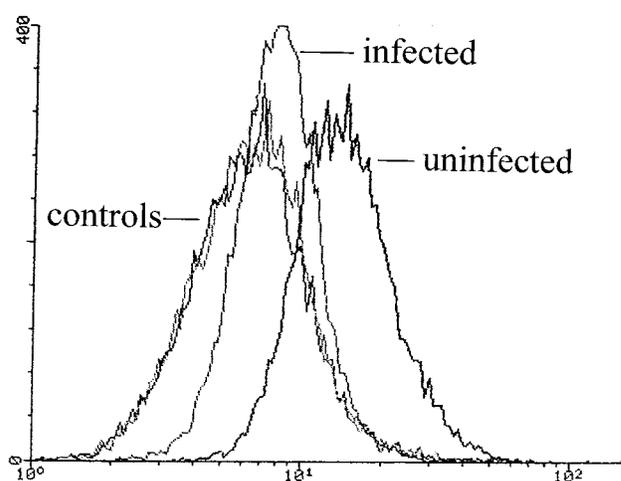


FIGURE 7. Down-regulation of surface Fas expression on *M. tuberculosis*-infected human macrophages. Fas expression of uninfected and H37Ra-infected macrophages was analyzed by flow cytometry. Cells (5×10^5) were stained with anti-APO-1 IgG3 hybridoma supernatant and 1:100 diluted goat anti-mouse-FITC. For negative and isotype match controls, cells were stained with goat anti-mouse-FITC alone or with purified mouse IgG3 isotype and goat anti-mouse-FITC, respectively. Data show histograms of fluorescence intensity (representative of two independent experiments).

results have shown that *gld* mice, which fail to eliminate *Leishmania major*, are indeed able to clear cutaneous lesions after the in situ injection of soluble FasL (47), suggesting a direct implication of the FasL-induced apoptotic cell death pathway in the protection against intracellular pathogens. Laochumroonvorapong et al. (43) recently reported that in Fas receptor-defective *lpr* mice the course of primary *M. bovis* bacillus Calmette-Guérin infection was not altered. However, Kägi et al. (44) have demonstrated that while CTL have no effect on the course of *Listeria monocytogenes* primary infection in mice, they do affect the bacterial load in the organs once these animals have mounted a specific immune response. Therefore, additional studies are needed to test whether the FasL system is implicated in the host defenses against *M. tuberculosis*, beyond the time of the primary infection.

We also show that *M. tuberculosis*-infected macrophages display a reduced susceptibility to FasL-induced apoptosis, together with reduced levels of surface Fas expression. The modulation in macrophages of cell surface molecules expression by intracellular mycobacteria is not unprecedented. *M. tuberculosis* infection has been shown to reduce the expression of the costimulatory molecule B7 on murine macrophages (45) and of HLA-DR on human macrophages (46), allowing the bacteria to dampen the immune response by interfering with antigen presentation.

In summary, *M. tuberculosis* has developed the capacity to escape killing mechanisms in human macrophages. Our results suggest that *M. tuberculosis*-specific CD4⁺ CTL might induce in vivo FasL-dependent macrophage apoptosis, not only depriving mycobacteria from their growth environment but also reducing viable bacterial counts during the apoptotic process. In so doing, CTL may complement the effector functions of IFN- γ -secreting effector cells by the specific killing of infected cells. An advantage of such a mechanism would be that mycobacteria inside apoptotic macrophages would remain intracellular and prone to phagocytosis in apoptotic debris by freshly attracted monocytes, thus preventing bacterial dissemination. In turn, the capacity of *M. tuberculosis* to modulate the Fas expression and the susceptibility of infected macrophages to FasL-induced killing might appear as another escape mechanism of *M. tuberculosis* to evade immune control.

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