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A Caspase-Independent Pathway Mediates Macrophage Cell Death in Response to Mycobacterium tuberculosis Infection

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Macrophages can undergo apoptosis after infection with Mycobacterium tuberculosis. This macrophage response deprives the bacillus of its niche cell and supports the host response through better antigen presentation. The intracellular pathways of apoptosis that elaborate this macrophage response are not well understood. To address this issue, we investigated the contribution of various apoptosis pathways to M. tuberculosis-induced macrophage cell death. We found that macrophages die in a caspase-independent manner after infection with M. tuberculosis (at multiplicities of infection ranging from 1 to 20). There was evidence for the involvement of both the mitochondria (cleavage of Bid) and the lysosomes (cathepsin-mediated DNA fragmentation) in this cell death pathway. Dying macrophages displayed several features typical of apoptosis, including DNA fragmentation, nuclear condensation, and exposure of phosphatidylserine on the plasma membrane. However, nuclear fragmentation was not observed, which suggests that M. tuberculosis-induced cell death differs in some respects from classical apoptosis. This novel mechanism of cell death was blocked by serine protease inhibitors. A better understanding of this protective macrophage response may direct new vaccine and treatment options.

Macrophage apoptosis can occur after infection with Mycobacterium tuberculosis (20). This response is thought to deprive the mycobacteria of a niche (29), cause bacillary killing (21, 26, 36, 39), and lead to cross-priming of M. tuberculosis-specific CD8+ T cells (43). Thus, apoptosis of M. tuberculosis-infected macrophages may be an important part of the host response to this pathogen.

Caspases are critical mediators of classical apoptosis. However, their activation does not appear to be obligatory for all forms of apoptosis. Classical apoptosis can be initiated via two distinct biochemical mechanisms, both of which result in the activation of caspases: (i) an extrinsic pathway that involves ligation of cell surface death receptors by their respective ligands and (ii) an intrinsic mitochondrial pathway largely controlled by the Bcl-2 family of pro- and antiapoptotic proteins. Suppression of caspase activity may be cytoprotective when cells are stimulated to undergo apoptosis via ligation of death receptors. However, caspases are terminal effectors, rather than initiators, of the mitochondrial pathway, and this type of cell death is often caspase independent (24, 49). Recent evidence indicates that apoptotic cell death can also occur in the absence of caspases or with mixed caspase and noncaspase protease activity (19, 24). For example, tumor necrosis factor alpha (TNF-α), as well as activating caspases, can trigger several cell death pathways, involving lysosomal permeabilization, that are mediated by release of cathepsin B, including cleavage of the Bid to its active form which, in turn, triggers the mitochondrial apoptosis pathway (11, 17). Calpains and serine proteases have also been implicated as mediators of cell death, although these caspase-independent pathways have not been fully characterized (24, 33, 37, 46).

The mechanism of macrophage death appears to be of critical importance in the host response to mycobacterial infection (21, 26, 36, 38, 40). Previous studies have shown that human macrophages undergo TNF-α-dependent apoptosis when infected with avirulent M. tuberculosis at a low multiplicity of infection (MOI) (20) which involves activation of initiator caspases, whereas virulent strains avoid causing macrophage apoptosis at a low MOI (2, 21, 41, 44). In contrast, murine macrophages infected at a high MOI (>25) with the virulent Erdman strain of M. tuberculosis undergo TNF-α- and caspase-independent apoptosis (28). Virulent M. tuberculosis can inhibit apoptosis in part by upregulation of the antiapoptotic Bcl-2 family member Mcl-1, suggesting that these bacilli can prevent macrophage apoptosis from occurring via the mitochondrial pathway (44). Several reports suggest that virulent strains of M. tuberculosis cause necrosis (4, 15, 50). Although the importance of cell death in the host response to M. tuberculosis infection is now being recognized, the underlying molecular and biochemical mechanisms have not been fully characterized.

To examine the mechanisms of cell death during M. tuberculosis infection, we infected phorbol myristate acetate (PMA)-differentiated THP-1 cells and monocye-derived macrophages with virulent and avirulent strains of M. tuberculosis at a range of MOIs in vitro. THP-1 cells differentiate to macrophages in the presence of PMA and have previously been shown to respond to M. tuberculosis infection in a manner similar to that of primary human alveolar macrophages (41). In the present study we investigated the mechanism of macrophage death in response to infection with avirulent H37Ra at low and high MOIs and the virulent strain H37Rv at a high MOI. We found that caspase and cathepsin activity was necessary for DNA fragmentation in response to M. tuberculosis...
infection. However, macrophage death was caspase and cathepsin independent. Cell death was prevented by the serine protease inhibitors AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride] and TPCK (N-p-tosyl-phenylalanine chloromethyl ketone). These results indicate that whereas caspases and cathepsins determine the mode of cell death that occurs in M. tuberculosis infection, they appear to be dispensable in terms of determining whether the cell lives or dies. In addition, our data suggest the existence of a serine protease-dependent signaling pathway that may play a role in the initiation of M. tuberculosis-mediated macrophage apoptosis.

MATERIALS AND METHODS

Myobacteria. M. tuberculosis strains H37Ra and H37Rv were obtained from the American Type Culture Collection (Manassas, VA). Stocks were propagated in Middlebrook 7H9 medium (Difco/Becton Dickinson, Sparks, MD) made up in low-endotoxin water (Sigma, St. Louis, MO) supplemented with albumin-dextrase-catalase supplement (Becton Dickinson) and 0.05% Tween 80 (Difco). Aliquots were frozen at −80°C, thawed, and propagated in Middlebrook 7H9 medium to log phase before use.

Cell culture. The THP-1 cell line was obtained from the American Type Culture Collection and maintained in RPMI 1640 (with Glutamax; Gibco) containing 10% fetal calf serum (FCS; Gibco). The medium was supplemented with 100 ng of PMA for 72 to 96 h. Adherent cells were harvested by scraping them into PARP lysis buffer (62.5 mM Tris-HCl [pH 6.8], 10 mM EDTA, 10% sodium deoxycholate, 0.001% bromophenol blue, 5% β-mercaptoethanol; Sigma). Liquids and detached cells were combined, vortexed, sonicated for 15 s, and incubated at 65°C for 15 min. Equal volumes were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12% Tris glycine minigels. Protein was electrophoretically transferred onto a 0.2-μm semipermeable nitrocellulose membrane (Immun-Blot PVDF membrane; Bio-Rad, Hercules, CA), which was then washed and blocked for 1 h with 5% nonfat milk in Tris-buffered saline–Tween (TBST) containing 0.01% sodium azide. The membrane was incubated with the appropriate primary antibody in TBST–5% milk, followed by incubation with horse radish peroxidase-conjugated secondary antibody (Chemicon). Signal was detected by using enhanced chemiluminescence and exposure to Hyperfilm MP (GE Healthcare). The cells were fixed with 1% paraformaldehyde for 5 min immediately before being analyzed.

Evaluation of nuclear morphology by fluorescence microscopy (Hoechst staining). Macrophages cultured in eight-well glass chamber slides (Nalge Nunc) were infected at various MOIs for 1 to 3 days. To evaluate nuclear morphology, the cells were stained with 2% paraformaldehyde, stained with 10 μg of Hoechst 33342/ml in phosphate-buffered saline (Molecular Probes/Invitrogen) for 20 min, and then observed by fluorescence microscopy using a Nikon TE inverted microscope equipped with a Leica DC-100 digital camera.

Western blotting. After experimental exposure the medium was removed from the wells and centrifuged at 100 × g for 10 min. The adherent detached cells were collected by scraping them into PARP lysis buffer (25 mM HEPES [pH 7.5], 0.1% CHAPS [3-[3-Cholamidopropyl]-dimethy lammonium]-1-propanesulfonate], 1 mM di thiothreitol). The cells were fixed with 1% paraformaldehyde for 5 min immediately before being analyzed.

Macrophages were cultured for infection of macrophages with M. tuberculosis. On the day of infection log-phase bacilli growing in Middlebrook 7H9 medium were centrifuged at 3,000 × g for 10 min and resuspended in RPMI 1640 containing 10% FCS (THP-1 cells) or RPMI containing 10% human serum (monocyte-derived macrophages). Clumps were broken up by passing the bacilli through a 25-gauge needle six to eight times, and the sample was centrifuged at 100 × g for 3 min to remove any remaining clumps. To determine the amount of M. tuberculosis necessary to activate the macrophages, a 10-fold serial dilution of the bacilli was plated onto two- and eight-well Labtek slides at a density of 0.5 × 10⁶ cells/ml and differentiated with 100 nM PMA for 72 to 96 h. Adherent cells were harvested by scraping them into PARP lysis buffer (25 mM HEPES [pH 7.5], 0.1% CHAPS [3-[3-Cholamidopropyl]-dimethylammonio]-1-propanesulfonate], 1 mM dithiothreitol), followed by three washes to remove extracellular bacilli, and fresh medium was added. The medium was replaced, with washing to remove any remaining nonadherent cells, every 2 to 3 days. Macrophages were cultured for 7 to 10 days before infection with M. tuberculosis.

Infection of macrophages with M. tuberculosis. On the day of infection log-phase bacilli growing in Middlebrook 7H9 medium were centrifuged at 3,000 × g for 10 min and resuspended in RPMI 1640 containing 10% FCS (THP-1 cells) or RPMI containing 10% human serum (monocyte-derived macrophages). Clumps were broken up by passing the bacilli through a 25-gauge needle six to eight times, and the sample was centrifuged at 100 × g for 3 min to remove any remaining clumps. To determine the amount of M. tuberculosis necessary to activate the macrophages, a 10-fold serial dilution of the bacilli was plated onto two- and eight-well Labtek slides (Nalge Nunc, Naperville, IL) were infected with various amounts of resuspended M. tuberculosis for 4 h. Extracellular bacteria were then washed off, and the cells were fixed for 5 min in 2% paraformaldehyde and stained with auramine-M (Becton Dickinson) for acid-fast bacteria. Macrophage nuclei were counterstained with 10 μg of Hoechst 33358 (Sigma)/ml. The percentage of infected cells and the number of bacilli per cell were determined by observing the slides under an inverted fluorescence microscope (Nikon TE 300). Based on this result macrophages were infected at various MOIs for 4 h, extracellular bacteria were removed by washing, and the cells were maintained in culture at 37°C for 2 h to 3 days before harvesting.

Protease inhibitors. The inhibitors used in the present study were obtained from Calbiochem (Merck Biosciences, Nottingham, United Kingdom) unless otherwise indicated. Stock solutions were prepared by dissolving each inhibitor in dimethyl sulfoxide (DMSO). To block enzyme activity in macrophages, the inhibitors were diluted 1,000-fold into cell culture medium to give a final concentration of 0.1% DMSO. The cells were preincubated with the inhibitors 5-benzyloxycarbonyl-Val-Ala-Asp-CH2F (z-VAD-fmk; a cathepsin B and L inhibitor), [3-2-(trans)-propanoyl]amino-2-carboxylyl-1-isocyanate-2-propenal methyl ester (CAO74Me; an inhibitor of cathepsin B), or benzyloxycarbonyl-Phe-Tyr-(Bu)-diazoalkylketone [z-FY(Bu)-dmk; an inhibitor of cathepsin L] or with vehicle (0.1% DMSO; Sigma) for 3 h before infection. AEBSF (a general inhibitor of serine proteases), TLCK (Nα-tosyl-l-lysine chloromethyl ketone; an inhibitor of trypsin-like serine proteases), TPCK (an inhibitor of chemotrypsin-like serine proteases), and diphenyleneiodonium (DPI; an NADPH oxidase inhibitor) were added to cells 30 min before infection. After 4 h of exposure to M. tuberculosis the cells were washed to remove extracellular bacilli, and fresh medium containing inhibitors or DMSO was added to the wells. Fresh z-VAD-fmk, z-FY(Bu)-dmk, CAO74Me, z-FY(Bu)-dmk, or DMSO was added to the cells only 24 h thereafter until the end of the experiment. Incubation of macrophages with AEBSF and DPI alone caused caspase-dependent apoptosis. For that reason these inhibitors were used in combination with 50 μM z-VAD-fmk to counteract their apoptotic effect unless otherwise stated.

DNA fragmentation (cell death ELISA). The cell death detection ELISA PLUS kit (Roche Applied Science, United Kingdom) was used to quantify M. tuberculosis-induced DNA fragmentation as recommended by the manufacturers. Briefly, 1 to 3 days after infection 24-well plates were centrifuged at 200 × g to sediment detached cells, the medium was discarded, and the cells were lysed. The lysate was subjected to antigen capture enzyme-linked immunosorbent assay (ELISA) to measure free nucleosomes, and the optical density at 405 nm (OD405) was read on a Wallac Victor plate reader. Triplicate wells were assayed for each condition.

Phosphatidylserine (PS) exposure. PMA-differentiated THP-1 cells were infected as described above and at the indicated times were detached from the plate by incubation with cold 10 mM EDTA in phosphate-buffered saline for 45 min. The cells were washed with Hanks balanced salt solution containing 0.1% bovine serum albumin, resuspended in 100 μl of calcium-binding buffer (100 mM HEPES-NaOH [pH 7.5] containing 1.4 M NaCl and 25 mM CaCl₂), incubated for 15 min at room temperature with 8 μl of annexin V-Alexa Fluor 488 (Molecular Probes/Invitrogen) and 5 μl of propidium iodide (PI; 1 μg/ml), washed, and analyzed within 30 min by using a FACSCalibur flow cytometer (Becton Dickinson). The cells were fixed with 1% paraformaldehyde for 5 min immediately before being analyzed.
counted by fluorescence microscopy. Each condition was assayed in triplicate, and at least 200 cells were counted in each well.

Detection of serine proteases with FSFCK. Detection of serine proteases with FSFCK [5(6) carboxyfluorescein L-phenylalanyl-chloromethyl ketone with spacer; Immunochemistry Technologies, Bloomington, MN] was carried out according to the supplier’s instructions. Briefly, 3 h after infection extracellular bacilli were removed by washing, and the cells were exposed to FSFCK for 50 min at 37°C and 5% CO₂. The cells were stained for a further 10 min with Hoechst (0.5% [vol/vol]), and the cells were then washed three times. FSFCK and Hoechst were detected by fluorescence microscopy.

Statistical analysis. Results are expressed as means ± the standard error of the mean. The data were analyzed by using Microsoft Excel statistical software using the Student t test. A P value of <0.05 was considered statistically significant.

RESULTS

Dose-dependent apoptosis of M. tuberculosis-infected macrophages. Infection of human macrophages with the H37Ra strain of M. tuberculosis at a low MOI is known to cause apoptosis. However, the effects of higher bacterial loads have not been characterized. To assess the relationship between bacillary load and macrophage apoptosis, cells infected with M. tuberculosis H37Ra over a range of MOIs (1 to 2 bacilli per cell to 10 to 20 bacilli per cell). Apoptosis was assessed by determining the amount of DNA fragmentation in infected and uninfected cells by nucleosome
ELISA 72 h after infection. Infection with H37Ra caused a dose-dependent increase in DNA fragmentation in THP-1 cells consistent with an apoptotic mechanism of cell death over the range of MOIs tested (Fig. 1A). Early exposure of PS, while the cell membrane remains intact, is a marker of apoptosis (10). To confirm that infected cells were undergoing apoptosis, staining for cell surface PS was carried out at both low (1 to 5) and high (10 to 20) MOIs with annexin V conjugated to AlexaFluor 488. PI was used to identify cells with a compromised plasma membrane. Adherent and floating cells were combined for each well and subjected to flow cytometry (Fig. 1B). Upon withdrawal of PMA a proportion of uninfected PMA-differentiated THP-1 cells detach from the substrate, undergo apoptosis, and contribute to background annexin V and PI staining. However, an increase above background levels in single positive annexin V-stained cells was detectable at both low and high MOIs, further supporting an apoptotic mechanism for cell death (Fig. 1C). The majority of dying cells stained with both PI and annexin V, suggesting that they were in the late stages of apoptosis (Fig. 1B, upper right quadrants).

Fluorescence microscopy of Hoechst-stained infected cells indicated that cell shrinkage and nuclear condensation occurred at all of the MOIs tested. While pronounced nuclear condensation occurred in cells dying due to \textit{M. tuberculosis} infection, there was no evidence of nuclear fragmentation (Fig. 1D). Treatment with other apoptotic stimuli (staurosporine and TNF-\textalpha-cycloheximide) caused extensive nuclear fragmentation, indicating that this can occur in THP-1 cells under certain conditions (Fig. 1D and data not shown).

Cell death can occur in the absence of DNA fragmentation, especially in the presence of caspase inhibitors. Since we sought to determine the mechanism of macrophage death in \textit{M. tuberculosis} infection, it was necessary to distinguish between nuclear apoptotic changes and cell death. For that reason DNA fragmentation was used in subsequent experiments as a marker for apoptosis, and exclusion of PI was used as an indicator of cell viability.

**Macrophage DNA fragmentation is caspase and cathepsin dependent.** Caspases are responsible for many of the nuclear hallmarks of apoptosis, including DNA fragmentation and nuclear condensation and fragmentation. To determine whether \textit{M. tuberculosis}-induced apoptosis requires caspase activation, macrophages were infected with \textit{M. tuberculosis} at a range of MOIs in the presence or absence (DMSO) of the pan-caspase inhibitor z-VAD-fmk (50 \mu M). z-VAD-fmk significantly inhibited DNA fragmentation at all MOIs tested but was most effective at low MOIs (Fig. 2A).

Activation of cathepsin B has been demonstrated in TNF-\textalpha-driven apoptosis of hepatocytes (17) and fibrosarcoma cells (11). We used the cathepsin B/L inhibitor z-FA-fmk (50 \mu M) to investigate a possible role for lysosomal proteases in \textit{M. tuberculosis}-mediated apoptosis. z-FA-fmk inhibited DNA fragmentation at low MOIs with an efficacy comparable to that of z-VAD-fmk. High concentrations of z-FA-fmk (>100 \mu M) can also inhibit the activity of the effector caspase-2, -3, -6, and -7 (31). To control for this possibility and to better define the role of cathepsins in macrophage apoptosis, macrophages were infected with one to five bacilli per cell in the presence or absence of the specific cathepsin B inhibitor CAO74Me (5 and 10 \mu M) and the specific cathepsin L inhibitor z-FY(t-Bu)-dmk.

The cathepsin B inhibitor CAO74Me did not inhibit the DNA fragmentation of \textit{M. tuberculosis}-infected macrophages (MOI of 1 to 5). However, the cathepsin L inhibitor (5 \mu M) significantly reduced DNA fragmentation of infected cells compared to infected cells treated with DMSO (Fig. 2B). These data suggest that z-FA-fmk is probably preventing DNA fragmentation in \textit{M. tuberculosis}-infected macrophages by inhibiting the proteolytic activity of cathepsin L.

**Loss of macrophage viability due to \textit{M. tuberculosis} infection is caspase and cathepsin independent.** Our results thus far indicated that active caspases and cathepsins are intrinsic to the execution of the apoptotic program triggered by \textit{M. tuberculosis} infection, particularly at low MOIs. However, inhibition of caspases does not always lead to protection from cell death (24). To analyze the effects of cysteine proteases on cell viability, we measured the capacity of macrophages infected with \textit{M. tuberculosis} at an MOI of one to five mycobacteria/cell to exclude PI in the presence of z-VAD-fmk or z-FA-fmk. The total number of cells per field and the number of PI-positive cells were counted by using a fluorescence microscope, and the percentage of PI-positive (dead) cells was calculated for each
confirmed that this caspase was not activated during infection with H37Ra. Consequently lysates of cells infected with H37Ra for 24 to 72 h were assayed for caspase-2-like activity using the fluorescent substrate VDVAD-AMC. We did not detect an increase in VDVAD-ase enzyme activity in infected macrophage lysates above that of uninfected cells, indicating that caspase-2 is not activated during M. tuberculosis infection (data not shown). Alternatively, the z-VAD-fmk could have degraded over time in culture, resulting in incomplete inhibition of caspase activity. However, z-VAD-fmk blocked DNA fragmentation and cleavage of caspase-3 and -8 in THP-1 cells treated for 3 days with TNF-α-cycloheximide (data not shown), indicating that the inhibitor was active throughout the course of our experiments. The lack of inhibition of M. tuberculosis-induced cell death by z-VAD-fmk and z-FA-fmk suggests that caspases and cathepsins are activated downstream of an event that determines the fate of the infected cells consistent with the mechanism of the intrinsic apoptosis pathway.

Evidence for activation of Bid and caspases. Macrophages undergo TNF-α-dependent apoptosis during H37Ra infection (20, 41). TNF-α can activate executioner caspases directly via the extrinsic pathway by activation of caspase-8, and it can also activate the intrinsic pathway by cleavage of the BH-3-only protein Bid to its active truncated form tBid by caspase-8, cathepsins, or calpain (23, 30, 32). Western blotting for Bid showed that the levels of full-length Bid decreased in lysates of infected cells undergoing apoptosis, suggesting that Bid may be cleaved to its active form (Fig. 3C). There was also a decrease in the levels of the 56- to 58-kDa pro-caspase-8 and 32-kDa caspase-3 indicative of activation. This is in agreement with a previous report that showed that caspase-8 activity is increased after infection with a low MOI of H37Ra in THP-1 cells (41). Taken together with the lack of inhibition of cell death by z-VAD-fmk, these data indicate that when infected with H37Ra the cells may undergo apoptosis via the mitochondrial pathway. Unexpectedly, neither z-VAD-fmk nor z-FA-fmk prevented the cleavage of Bid, caspase-8, or caspase-3, indicating that they are not activated by caspases or cathepsins (Fig. 3C).

The serine protease inhibitor AEBSF prevents cell death during M. tuberculosis infection. Since neither caspase nor cathepsin inhibitors prevented macrophage death or cleavage of caspases and Bid by M. tuberculosis, we investigated whether other proteases might be involved. Calpains have been identified as mediators of cell death in other models. However, we were unable to test their possible involvement in M. tuberculosis-induced apoptosis due to the toxic effect of calpain inhibitors on THP-1 cells (data not shown). Serine proteases have also been implicated as mediators of cell death in several in vitro models (7, 33, 37, 46). We found that, similar to previous findings in melanoma cell lines (7), the general serine protease inhibitor AEBSF (200 μM) alone, when incubated with THP-1 cells for more than 24 h, caused caspase-dependent apoptosis that was inhibited by z-VAD-fmk (data not shown). Therefore, although we could not use this concentration of AEBSF alone to test the involvement of serine protease activity in M. tuberculosis-induced cell death, the combination of z-VAD-fmk (50 μM) with 200 μM AEBSF prevented the loss of viability of THP-1 cells infected with H37Ra at low (1–5) and high (10–20) MOIs (Fig. 4A and B). Incubation times for low (72 h) and high (20 h)-MOI challenges were optimized to provide a sig-
significant increase in the percentage of PI-positive cells above background and to minimize the loss of dead cells due to detachment from the cell culture plate. As before (Fig. 3A), treatment with z-VAD-fmk alone did not significantly inhibit cell death, although it did inhibit DNA fragmentation (Fig. 4C and D). Cleavage of caspase-3 and -8 was also prevented by AEBSF/z-VAD-fmk but not by z-VAD-fmk alone (Fig. 4E).

In addition to modulating serine protease activity, AEBSF is also known to inhibit the activity of NADPH oxidase (8), which is responsible for the production of superoxide. Since reactive oxygen intermediates such as superoxide can be involved in promoting cell death, we compared the NADPH oxidase inhibitor DPI (10 μM) to AEBSF for its effect on H37Ra-mediated cell death at an MOI of 1 to 5. Due to its toxicity DPI was combined with z-VAD-fmk (50 μM) for these experiments. As expected, AEBSF/z-VAD-fmk treatment of infected cells significantly decreased the percentage of PI-positive cells to 30.56% ± 4.96% (P = 0.002, n = 3). However, there was no significant difference (P > 0.20, n = 3) in cell death of infected cells treated with DPI (73.14% ± 3.68%) compared to control infected cells treated with vehicle (80.72% ± 4.95%), indicating that the prevention of cell death by AEBSF was not due to inhibition of NADPH oxidase activity.

High-dose virulent M. tuberculosis causes serine protease-dependent macrophage cell death. Virulent strains of mycobacteria cause less macrophage apoptosis than avirulent strains at low MOIs (21, 41, 44). To determine the effect of higher infectious doses of H37Rv, we infected THP-1 cells and human peripheral blood monocyte-derived macrophages at MOIs of 10 to 20 and assessed nuclear morphology, DNA fragmentation, and cell viability as before. High-MOI infection with H37Rv led to loss of viability of monocyte-derived macrophages (81.14% ± 8.1% PI-positive cells compared to 2.22% ± 0.52% PI-positive cells in uninfected cultures [n = 4]). Macrophage cell death was accompanied by nuclear condensation (Fig. 5A). To test whether the mechanism of cell death due to high-dose virulent M. tuberculosis infection resembled that caused by H37Ra, THP-1 cells were infected with H37Rv (at an MOI of 10 to 20) in the presence of protease inhibitors.
Viability and DNA fragmentation were measured 24 h after infection. Inhibition of serine protease activity by AEBSF/z-VAD-fmk caused a small but significant decrease in cell death (64.02% ± 3.14% PI-positive cells, \( P = 0.04, n = 3 \)) compared to control cells (79.10% ± 5.24%) after \( M. \) *tuberculosis* infection. Treatment with z-VAD-fmk did not have a statistically significant effect on cell death (70.04% ± 0.82%, \( P = 0.07 \)).

The virulent strain H37Rv appeared to kill macrophages at a faster rate than H37Ra. Therefore, we wondered whether decreasing the incubation time of the cells with H37Rv would improve the ability of the inhibitors to prevent cell death. This also allowed us to test whether AEBSF (100 \( \mu \)M) alone could prevent cell death due to \( M. \) *tuberculosis* infection. When the infection period was reduced to 6 h both AEBSF alone and AEBSF/z-VAD-fmk significantly inhibited cell death compared to control infected cells (Fig. 5B). The caspase inhibitor z-VAD-fmk also decreased the percentage of PI-positive cells detected 6 h after infection, but this inhibition did not reach statistical significance, indicating that the abrogation of caspase activity may delay cell death in the very early stages of infection but is not able to prevent it in the long term. In contrast, DNA fragmentation was significantly inhibited in infected cells by z-VAD-fmk, similar to results obtained with H37Ra (Fig. 4C and D). Treatment with AEBSF alone prevented DNA fragmentation to a similar extent as z-VAD-fmk, and the combination of z-VAD-fmk and AEBSF reduced it to background levels (Fig. 5C). Therefore, similar to the situation with H37Ra, DNA fragmentation occurred in a caspase-dependent manner in macrophages infected with H37Rv. Serine proteases are well known to be involved in regulating internucleosomal DNA cleavage in other models of apoptosis (9, 25, 35, 37, 47). Whether serine proteases play a role in DNA degradation in the present study could not be determined because, unlike z-VAD-fmk, AEBSF is acting at a proximal stage of cell death in \( M. \) *tuberculosis*-infected macrophages.

**M. tuberculosis** activates chemotrypsin-like serine proteases.

Serine proteases can be classified on the basis of their broad substrate specificity: trypsin-like serine proteases cleave after basic amino acids, chemotrypsin-like serine proteases cleave after bulky hydrophobic residues and elastase-like proteases cleave after small aliphatic residues. The inhibitors TLCK and its phenylalanine analogue TPCK inhibit trypsin-like and chemotrypsin-like serine proteases, respectively, and were used to assess the involvement of these proteases in \( M. \) *tuberculosis*-induced apoptosis. Differentiated THP-1 cells were preincubated for 30 min with 25 \( \mu \)M TLCK or TPCK and then infected with H37Ra or H37Rv at an MOI of approximately 10 to 20. The viability of the cells was determined by PI staining 6 h after infection. TLCK did not significantly influence the viability of infected cells. However, TPCK almost completely abrogated cell death (Fig. 6A) caused by both strains of \( M. \) *tuberculosis*.

To confirm that chemotrypsin-like serine proteases were activated during \( M. \) *tuberculosis*-induced cell death we used FSFCK, a fluorescent analogue of TPCK. Binding of FSFCK to the enzyme active site leads to an increase in cellular fluorescence, which is considered to be an indicator of serine protease activity (16). THP-1 cells were infected as before with H37Ra and H37Rv at an MOI of 10 to 20. After 3 h extracellular mycobacteria were removed by washing the monolayers, and the cells were incubated with FSFCK for 1 h. FSFCK staining, detected by fluorescence microscopy, was increased in cells infected with H37Ra and H37Rv compared to uninfected cells. As expected, treatment of macrophages with the serine protease inhibitors AEBSF and TPCK during infection reduced FSFCK fluorescence to background levels (Fig. 6B).

**DISCUSSION**

In this report we demonstrate that \( M. \) *tuberculosis* causes caspase-independent death of macrophages that is characterized by DNA fragmentation, PS exposure, and nuclear condensation but lacks nuclear fragmentation. Inhibition of caspases or...
cathepsins, while effective at preventing DNA degradation, did not have a significant impact on cell viability or nuclear condensation. This suggests that caspases and cathepsins are activated but are dispensable for macrophage cell death after M. tuberculosis infection. Consistent with this we found that processing of Bid and caspases is caspase and cathepsin independent. In contrast, the general serine protease inhibitor AEBSF prevented DNA fragmentation, nuclear condensation, and cell death, placing caspase and cathepsin activity downstream of a serine protease-mediated event that determines the fate of the infected cell. Taken together, our data suggest that M. tuberculosis triggers a cell death pathway leading to an “apoptosis-like” phenotype that differs in some respects from classical apoptosis.

Macrophage apoptosis caused by M. tuberculosis infection at low MOIs has previously been shown to be dependent on TNF-α-mediated death signals (21, 22, 41). Signaling through the TNF-α receptor activates diverse cell death pathways leading to both caspase-dependent and caspase-independent apoptosis or to necrosis, depending on the type of cell and the stimulus involved (23). Our results indicate that cell death due to M. tuberculosis infection is unlikely to be initiated solely via the extrinsic TNFR1 pathway because, if this were the case, z-VAD-fmk would be expected to have a significant impact on the viability of infected cells. As well as triggering the extrinsic apoptosis pathway via activation of caspase-8 or caspase-10, death receptor ligation can also engage the intrinsic apoptotic pathway via an amplification loop involving the generation of tBid by caspase-8 and caspase-10 (30, 32). Bid can also be cleaved by cathepsins after lysosomal permeabilization, thus linking the lysosomes to the intrinsic mitochondrial pathway (5). Lee et al. have recently demonstrated a role for cathepsins in apoptosis of murine macrophages infected with M. tuberculosis Erdman at an MOI of >25 (28). In the present study we found evidence of cathepsin L activation in M. tuberculosis-infected cells. However, the lack of inhibition of caspase and Bid cleavage by z-VAD-fmk and z-FA-fmk implicated another class of protease in the processing of these apoptotic mediators. This further highlights the fact that M. tuberculosis-induced cell death differs in several key respects from classical apoptosis.

It has been suggested that macrophage apoptosis represents a successful host immune response to M. tuberculosis infection in vivo (40, 42). M. tuberculosis is an intracellular pathogen and, as such, this organism is dependent on the continuing survival of the host cell to provide it with a refuge in which to replicate. The recent discovery of the Ipr1 gene in mice reinforces the importance of this cell death mechanism in influencing the host response to infection. Mice lacking Ipr1 are extremely susceptible to M. tuberculosis, and infected macrophages from these mice undergo necrosis. Expression of Ipr1 limits replication of M. tuberculosis and drives infected macrophages toward apoptosis (40). Macrophage apoptosis was associated with a reduction in the viability of intracellular mycobacteria in this model. In contrast, macrophage necrosis leads to the survival and growth of mycobacteria (26, 36, 38, 40). Since alveolar macrophages are required to establish M. tuberculosis infection in the lung (29), their elimination by apoptosis in the early stages of infection would deprive the pathogen of a niche cell in which to replicate. Apoptosis in response to avirulent M. tuberculosis at low MOIs may reflect the host response of the majority of individuals who successfully contain M. tuberculosis infection.

Eventually, in order to disseminate, M. tuberculosis must escape from the macrophage into the extracellular environment. Macrophages infected with M. tuberculosis have been reported to undergo both apoptotic and necrotic cell death in vitro (4, 6, 15, 20, 50). It is thought that M. tuberculosis-laden macrophages “burst” open via necrosis. Uncontrolled cell lysis would allow the bacilli to avoid the microbicidal effect of apoptosis, allowing them access to the extracellular environment to infect other cells and spread to new hosts. We found...
that both low- and high-MOI challenge with avirulent *M. tuberculosis* and high-MOI challenge with virulent *M. tuberculosis* led to a similar caspase-independent, apoptosis-like phenotype that included early PS exposure, DNA fragmentation, and nuclear pycnosis; the main difference we observed between low and high MOIs was the more rapid progression of cells infected at a high MOI to apoptosis and the loss of plasma membrane integrity. The mechanism of necrotic cell death is poorly defined and cannot be easily distinguished from secondary necrosis in cultured cells because apoptotic cells eventually lyse and become necrotic if they are not phagocytosed. Therefore, due to a lack of reliable markers we cannot exclude the possibility that a proportion of *M. tuberculosis*-infected macrophages undergo death by necrosis. However, based on our results, it is possible that the mechanism of macrophage death is similar at both high and low MOIs but that at high MOIs the cells progress from apoptosis to secondary necrosis at an accelerated rate, allowing the bacilli to escape from the macrophage before they are killed. This would fit with the findings of Remold and coworkers, who showed that delaying secondary necrosis by treating infected cells with cyclosporine results in reduced survival of intracellular *M. tuberculosis* compared to that in untreated cells (13). Our observation that virulent *M. tuberculosis* can cause cell death at a high MOI and that it occurs at a faster rate than H37Ra-induced cell death at the same MOI concurs with recent findings in murine macrophages (28) and supports the notion that rapid cell death may provide an escape mechanism for replicating mycobacteria (28, 50).

Caspase activity is usually, directly or indirectly, responsible for most of the stereotypical morphological features characteristic of apoptosis. Since *M. tuberculosis*-induced cell death was accompanied by caspase activation but seemed to be caspase independent, the question then is whether caspase activity has any impact on the host response to infection. Activation of caspasess allows the cell to be dismantled while at the same time maintaining plasma membrane integrity, thus preventing the release of potentially harmful proteases and minimizing inflammation. It remains to be established whether the type of cell death we describe here has the same outcome as classical apoptosis in eliciting an anti-inflammatory response. Caspase activity also results in the release of chemotactic lipids from apoptotic cells, attracting uninfected phagocytes to dispose of dying cells (27). Indeed, phagocytosis of apoptotic macrophages by naïve macrophages has been shown to prevent the dissemination of mycobacteria (12). In addition, apoptotic macrophages ingested by dendritic cells lead to cross-priming of cytolytic T cells in a caspase-dependent manner in response to several pathogens, including *M. tuberculosis* (1, 43). Therefore, although caspases are not essential for macrophage death in *M. tuberculosis* infection, their activation may influence the host immune response to this pathogen.

Serine proteases have been implicated as mediators of cell death based on experiments with synthetic and naturally occurring inhibitors (33, 37, 46). In the present study inhibition of cell death and cleavage of caspases in infected macrophages was prevented by the serine protease inhibitors AEBSF and TPCK, placing serine protease activity upstream of caspase and cathepsin activation. The serpin PAI-2 inhibits macrophage cell death caused by infection with *M. avium*, presumably by inhibiting an as-yet-undefined serine protease (14). Serine proteases known to be involved in apoptosis include HtrA2/omi (18), AP24 (48), and cathepsin G (34). HtrA2/omi is a trypsin-like serine protease and thus is unlikely to be involved since cell death was not significantly inhibited by TPCK. However, further experiments with more specific inhibitors are needed to confirm these findings. Interestingly, the expression of cathepsin G, a highly cationic serine protease with antimicrobial activity, is upregulated in murine macrophages by *M. tuberculosis* infection (45). We are currently investigating the possibility that one or more of these serine proteases is involved in *M. tuberculosis*-mediated cell death.

The serine protease inhibitors were less effective at preventing cell death due to H37Rv compared to H37Ra at high MOIs at the 24-h time point. H37Rv appeared to be able to overcome the inhibition of serine proteases: the treatment of macrophages with AEBSF led to an almost complete inhibition of cell death caused by H37Ra at high MOIs, whereas cell death due to H37Rv was less effectively inhibited. This suggests that another cell death pathway is activated in the absence of serine protease activity. This may reflect a fundamental difference in the macrophage response to H37Rv, or it may simply be a consequence of the faster kinetics of cell death caused by H37Rv (Fig. 6A). It is possible that H37Ra-infected macrophages might also eventually succumb to this alternative cell death pathway at a time later than 24 h.

In conclusion, we show here that macrophages undergo a type of cell death similar to, but differing in some respects from, classical apoptosis when infected with H37Ra (at both high and low MOIs) or the virulent strain H37Rv (at high MOIs). In addition, our data suggest that caspases, cathepsins, and serine proteases all play a role in modulating cell death pathways of *M. tuberculosis*-infected macrophages. Further definition of this aspect of the host response to *M. tuberculosis* may contribute to the design of future therapeutics or vaccine options.

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