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Mycobacterium tuberculosis Promotes Apoptosis in Human Neutrophils by Activating Caspase-3 and Altering Expression of Bax/Bcl-x_L Via an Oxygen-Dependent Pathway[1]

Nasrin Perskvist, Min Long, Olle Stendahl, and Limin Zheng[2]

In addition to direct bactericidal activities, such as phagocytosis and generation of reactive oxygen species (ROS), neutrophils can regulate the inflammatory response by undergoing apoptosis. We found that infection of human neutrophils with *Mycobacterium tuberculosis* (Mtb) induced rapid cell death displaying the characteristic features of apoptosis such as morphologic changes, phosphatidylserine exposure, and DNA fragmentation. Both a virulent (H37Rv) and an attenuated (H37Ra) strain of Mtb were equally effective in inducing apoptosis. Pretreatment of neutrophils with antioxidants or an inhibitor of NADPH oxidase markedly blocked Mtb-induced apoptosis but did not affect spontaneous apoptosis. Activation of caspase-3 was evident in neutrophils undergoing spontaneous apoptosis, but it was markedly augmented and accelerated during Mtb-induced apoptosis. The Mtb-induced apoptosis was associated with a speedy and transient increase in expression of Bax protein, a proapoptotic member of the Bcl-2 family, and a more prominent reduction in expression of the antiapoptotic protein Bcl-x_L. Pretreatment with an inhibitor of NADPH oxidase distinctly suppressed the Mtb-stimulated activation of caspase-3 and alteration of Bax/Bcl-x_L expression in neutrophils. These results indicate that infection with Mtb causes ROS-dependent alteration of Bax/Bcl-x_L expression and activation of caspase-3, and thereby induces apoptosis in human neutrophils. Moreover, we found that phagocytosis of Mtb-induced apoptotic neutrophils markedly increased the production of proinflammatory cytokine TNF-α by human macrophages. Therefore, the ROS-dependent apoptosis in Mtb-stimulated neutrophils may represent an important host defense mechanism aimed at selective removal of infected cells at the inflamed site, which in turn aids the functional activities of local macrophages. ***The Journal of Immunology*****, 2002, 168: 6358–6365.**

Human neutrophils play a significant protective role in the acute phase of mycobacterial infection (1, 2). This is clearly illustrated by the fact that tuberculosis is characterized by the predominant migration of monocytes/macrophages to the site of infection; the earliest response to the invasion of tissue by mycobacteria is primarily an influx of neutrophils (3, 4). Neutrophils encountering mycobacteria initially react by performing phagocytosis and generating reactive oxygen species (ROS)[3] to kill part of the microbes and thereby prevent them from spreading until the macrophages accumulate (5–7). Neutrophils containing ingested *Mycobacterium tuberculosis* (Mtb) have been shown to release an array of cytokines and chemokines that attract other cells of the immune system to the site of infection (2, 8). Furthermore, Kasahara et al. (8) observed that neutrophils stimulated with both heat-killed Mtb and TNF-α underwent rapid apoptosis, a process that is vital for the rapid resolution of inflammation.

Increasing numbers of bacterial pathogens have been identified as mediators of host cell apoptosis, and, by this action, the microbes may eliminate key immune cells or evade the host defense and thus modulate the pathogenesis of a variety of infectious diseases (9–11). However, recent studies indicated that induction of apoptosis represents not only the pathogenic strategies of pathogens, but also the protective mechanism developed by the host (9–12). For example, in human macrophages, it has been shown that apoptosis limits the intracellular growth of mycobacteria (11) and attenuated strains of Mtb have been found to be much more effective than virulent strains at inducing apoptosis (12). It is not yet known whether apoptosis in neutrophils is regulated by live Mtb or whether virulent and attenuated Mtb strains have different effects on this process.

Neutrophils undergo rapid spontaneous apoptosis both in vivo and in vitro. Specific changes in the plasma membrane of these apoptotic cells are recognized by macrophages, which can then ingest the neutrophils and thereby prevent them from releasing their histotoxic contents (13). Various inflammatory mediators, such as cytokines (14) and bacteria products (15), and local conditions, for example, hypoxia (16) and expression of Fas/Fas ligand (17), are known to promote or suppress neutrophil apoptosis at the site of infection and thus regulate the progression of inflammatory responses. Though apoptosis in neutrophils may occur through oxygen-independent mechanisms (16), the following findings in neutrophils suggest the potential role of ROS, produced via NADPH oxidase, in this process. Apoptosis is promoted by neutrophil-derived ROS during phagocytosis (18); apoptosis is inhibited by hypoxia and by the addition of antioxidants (16). Both spontaneous and Fas-induced apoptosis are decreased in neutrophils isolated from patients with chronic granulomatous disease, which have an inherited defect in their NADPH oxidase (19).

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[3] Abbreviations used in this paper: ROS, reactive oxygen species; Mtb, *Mycobacterium tuberculosis*; DPI, diphenylene iodonium; NAC, *N*-acetylsysteine; PI, propidium iodide; PS, phosphatidylserine; AMC, 7-amino-4-methylcoumarin.

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Less is known about the intracellular mechanisms by which these factors regulate neutrophil apoptosis. The execution of apoptotic pathway is mediated by a family of cysteine aspartases (caspases) (20–22), and caspase-3 and -8 are the main forms activated during neutrophil apoptosis (23–25). Neutrophils also express a number of proteins of the Bcl-2 families, which regulate apoptosis by, among other things, controlling the activity of caspases (15, 26). The Bcl-2 family includes proapoptotic proteins (e.g., Bax, Bad, and Bak) and antiapoptotic proteins (e.g., Bcl-2, Bcl-x_L, and Mcl-1), and the balance between these two groups determines the fate of cells in many systems (26). Recent studies have shown that human neutrophils express both the proapoptotic Bax and antiapoptotic Bcl-x_L and Mcl-1, and the levels of those proteins are correlated with the apoptotic processes that are either promoted or suppressed by a variety of inflammatory mediators (27–29).

In the present study, we establish an in vitro infection model of human neutrophils with both a virulent (H37Rv) and an attenuated (H37Ra) strain of Mtb to investigate if and how these bacteria regulate neutrophil apoptosis and the effect of uptake of these apoptotic neutrophils on the activation of human macrophages.

Materials and Methods

Reagents

The Ab and chemicals and their sources are as follows: anti-Bcl-x_L (H-62), anti-Bax (N-20), and anti-actin (C-2) Ab from Santa Cruz Biotechnology (Santa Cruz, CA); caspase-3 (CPP32) fluorogenic substrate DEVD-AMC and its competitive inhibitor DEVD-CHO from BD PharMingen (San Diego, CA); cell membrane permeable inhibitors of caspase-3 (zDEVD-fmk) and caspase-8 (zIETD-fmk) from Calbiochem (Darmstadt, Germany); annexin V and DNA laddering apoptosis detection kit from R&D Systems (Abingdon, U.K.); cell isolation and tissue culture reagents from Invitrogen (Lidingö, Sweden); electrophoresis and ECL reagents from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated in the text.

Preparation of bacteria

The virulent *M. tuberculosis* strain H37Rv was purchased from the Swedish Institute of Infection Laboratory (Stockholm, Sweden), and attenuated strain H37Ra (ATCC 25177) was obtained from the American Type Culture Collection (Manassas, VA). Initial colonies of Mtb were expanded and frozen in aliquot (7). Continuous subculturing of Mtb in liquid medium is associated with loss of virulence; therefore, freshly thawed aliquots of bacteria were cultured for each experiment. Single mycobacteria suspensions were prepared and then opsonized with human serum as previously described (5). All Mtb used along with this study were C3b/biopsonized but are simply referred to as H37Rv, H37Ra or Mtb. The integrity of the bacterial cell wall was confirmed by electron microscopy. The viability of Mtb was assessed in each step by comparing the results of bacterial counts determined by microscopy and assay of CFU.

Cell isolation, phagocytosis, and culture conditions

Human neutrophils were isolated from peripheral blood of healthy donors as previously described (5). Neutrophils of ~96% purity were resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml streptomycin (RPMI-medium). Human macrophages were prepared from PBMC as described (30, 31). The cells were plated at 4×10^6/well in 24-well plates for 1 h in DMEM alone, washed, and then cultured in DMEM containing 10% human serum. The medium was changed at day 3 and macrophages were used 6–8 days after culture (30–32). This yielded ~10^6 macrophages/well at the time of the assay.

Neutrophils were allowed to phagocytose Mtb as previously described (5). In short, incubations were performed for 30 min at 37°C under occasional agitation, using H37Rv or H37Ra (final volume 1 ml of RPMI-medium) at the indicated ratio of neutrophil to mycobacteria. The samples were then centrifuged ($110 \times g$, 10 min) to remove noningested Mtb, and the neutrophils were resuspended in 1 ml of RPMI-medium and cultured in a 24-well plate at 37°C in a humidified CO_2 incubator (5% CO_2, 95% air). At indicated time points, the cells were washed with PBS and used for further analysis. To measure the efficiency of infection, we used FITC-conjugated mycobacteria. The bacteria were conjugated with FITC as previously described (7), opsonized, and then incubated with neutrophils (1:20, cell-Mtb) at 37°C for 30 min. Under these conditions, the phagocytic index was 80 ± 5 bacteria/100 cells with 56% of neutrophil-ingested mycobacteria as determined by trypan blue exclusion (5).

Treatment of neutrophils with inhibitors

The NADPH-oxidase activity of neutrophils was inhibited by preincubating the cells with 5 μM diphenylene iodonium (DPI) at 37°C for 5 min (33). To examine the effect of antioxidants, cells were pretreated with 5 mM of glutathione or *N*-acetylsysteine (NAC) for 10 min and then infected with mycobacteria (18). The activity of caspase was prevented by preincubation with an inhibitor of caspase-3 or -8 (25 μM) at 37°C for 10 min (23–25). These inhibitors (at the indicated concentrations) were present throughout phagocytosis and the subsequent culture period. To ascertain whether the reduced ROS production in the inhibitor-treated neutrophils was due to impairment of phagocytosis, we performed experiments using FITC-conjugated and opsonized mycobacteria as described previously (5). To determine the source of ROS involved in Mtb-induced apoptosis, in some experiments we added 50 U superoxide dismutase and 2000 U catalase (cell-impermeable scavenger of O^-_2 and H_2O_2, respectively) to the cell cultures.

Morphological assessment of apoptosis

Giemsa-stained cytocentrifuged neutrophils were examined for morphological changes characteristic of apoptosis, as previously described (18). A minimum of 200 cells was scored in each sample to determine the percentage of apoptotic neutrophils.

Flow cytometric measurement of neutrophil apoptosis

Early apoptotic changes were identified by staining neutrophils with FITC-conjugated annexin V according to the protocol from the manufacturer (R&D Systems). Specific binding of annexin V was achieved by incubating 10^6 neutrophils in 60 μl of binding buffer with a saturated concentration of annexin V for 15 min at 4°C in the dark. To discriminate between early apoptosis and necrosis, the cells were simultaneously stained with annexin V and propidium iodide (PI) before analysis. The binding of FITC-annexin V (FL1) and PI (FL2) to the cells was measured by flow cytometry (FACS-Calibur; BD Biosciences) using CellQuest software. In some experiments, after incubation with annexin V, the neutrophils were washed once, fixed in 4% paraformaldehyde in binding buffer, and analyzed by flow cytometry. At least 10,000 cells were counted in each sample and a gate based on forward and side scatters was set to exclude cell debris.

DNA fragmentation assay

Neutrophils (2×10^6 cells/sample) were lysed, and the genomic DNA was extracted according to the protocol for apoptosis DNA laddering kit. The samples (2 μg of DNA/lane) were analyzed by gel electrophoresis (1.8% agarose) and ethidium bromide staining. The gel was visually examined under 305 nm UV illumination and scanned with a Bio-Rad FluorsMulti Imager (Hercules, CA).

Fluorometric assay for caspase-3 activity

DEVD-7-amino-4-methylcoumarin (AMC) cleavage was measured using a fluorometric assay modified from Parvathenani et al. (34). Neutrophils (5×10^6) were placed in 100 μl of lysis buffer (34) for 15 min on ice and then centrifuged (15,000 $\times$ *g*, 10 min). The protein concentrations of the lysates were determined by colorimetric analysis using DC protein assay reagents (Pierce, Rockford, IL). Cell lysate (100 μg) and the substrate DEVD-AMC (50 μM) were combined in a standard reaction buffer (10% sucrose, 10 mM DTT, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 50 mM HEPES, pH 7.5). After 1 h at 37°C, 0.5 ml of the mixture was diluted with 1.5 ml of reaction buffer and the cleavage of fluorogenic peptide substrate DEVD-AMC was monitored as liberation of AMC, using excitation and emission wavelengths of 355 and 460 nm, respectively. The fluorescence units were converted to picomoles of AMC using a standard curve generated with free AMC. The competitive inhibitor DEVD-CHO completely blocked the activity of caspase-3, which demonstrated the specificity of the assay. Blanks were measured in the absence of cell lysate to determine background fluorescence.

Western blotting

After incubation for various time periods, the neutrophils (2×10^6/sample) were pelleted, dissolved in Laemmli sample buffer (35), and heated for 5 min at 100°C. Total cellular proteins were separated by 12% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with BSA, and the presence of Bcl-x_L or Bax proteins on the blots was detected with specific Abs and a commercial ECL

kit. The intensity of the proteins was determined by densitometric assay, using a Howtek scanner and Quantity One software (Advanced American Biotechnology, Fullerton, CA).

Cytokine production in macrophages after phagocytosis of apoptotic neutrophils

Mtb-infected neutrophils were collected at 4 h postinfection. Uninfected apoptotic neutrophils were obtained by exposure to UV irradiation for 10 min followed by culture for 3 h (31). This routinely yielded populations containing 40–60% cells positively stained with annexin V and <4% cells positive for PI. The apoptotic neutrophils were washed twice and resuspended in DMEM without serum. Four million neutrophils or 5×10^6 Mtb were added per well of macrophages for 1 h, and washed out (31, 32). Under these conditions, the percentage of infected macrophages after stimulation with Mtb alone is slightly higher than that after exposure to Mtb-induced apoptotic neutrophils (46 vs 42%, respectively), as determined with FITC-conjugated Mtb. Fresh DMEM without serum was added and supernatants were collected 18 h later. As a control, UV- or Mtb-induced apoptotic neutrophils were cultured for 18 h in DMEM without macrophages. Supernatants were centrifuged at $2000 \times g$ to remove particular debris and then stored in aliquots at −70°C. To ensure that uptake was equivalent for the two different apoptotic neutrophils, the monolayers were stained for myeloperoxidase as previously described (30, 31). Phagocytosis of apoptotic neutrophils was quantified by phase-contrast microscope, and the macrophages that had engulfed neutrophils were scored as positive for staining (31).

Cytokine concentrations in the culture supernatants were determined by ELISA, using Quantikine immunoassays manufactured by R&D Systems. The cytokines analyzed were the proinflammatory TNF-α, which is required for control of acute Mtb infection (36), and the anti-inflammatory cytokine TGF-β1. For TGF-β1, the supernatants were activated with HCl before analysis. Assays were performed according to the instructions provided with each kit.

Statistical analysis

Differences between experimental groups comprised normally distributed data, which were analyzed for statistical significance using the Student *t* test or ANOVA. Values of $p < 0.05$ and $p < 0.01$ were considered to be statistically significant.

Results

M. tuberculosis-*induced apoptosis in neutrophils via an oxygen-dependent pathway*

To determine whether Mtb regulates apoptosis in human neutrophils, the cells were infected with Mtb for 30 min, then cultured for 18 h, and thereafter subjected to morphological examination, detection of phosphatidylserine (PS) exposure, and assaying of DNA fragmentation. We found that ~28% of the uninfected neutrophils had undergone spontaneous apoptosis, seen as typical apoptotic changes in morphology, including decreased cell volume as well as chromatin condensation with loss of multilobular nuclear structure (Fig. 1*A2* and Table I). Stimulation of neutrophils with Mtb markedly accelerates this process (Fig. 1*A3* and Table I). Another important feature of neutrophil apoptosis is the cell surface exposure of PS, which can be detected by FITC-conjugated annexin V. Flow cytometric analysis showed that the level of PS exposure increased from 26% in uninfected cells to 68% in Mtb-stimulated cells (Fig. 1*B*, *1* and *3*, respectively, and Table I). Most of the apoptotic cells were in the early stage of apoptosis and bound to annexin V only (Fig. 1*B*, *1* and *3*, *lower right quadrant*); a small portion of the cells were bound to both annexin V and PI, indicating necrosis or later apoptosis (Fig. 1*B*, *1* and *3*, *upper right quadrant*). Similar results were obtained in complementary experiments performed to assay DNA fragmentation, another well-known marker of apoptosis (Fig. 1*C*).

In a previous study, we observed that ingestion of Mtb was associated with increased production of ROS in neutrophils (5). Although controversial, ROS have been implicated as common mediators of apoptosis in a variety of cells, including neutrophils (15, 37, 38). In the present study, we used DPI, a flavoprotein inhibitor of NADPH oxidase (33), to address the possibility that ROS are involved in Mtb-induced apoptosis in neutrophils. We found that DPI had no effect on spontaneous apoptosis, whereas it nearly abolished the Mtb-induced apoptosis, as indicated by morphological changes, PS exposure, and DNA fragmentation in the neutrophils (Fig. 1 and Table I).

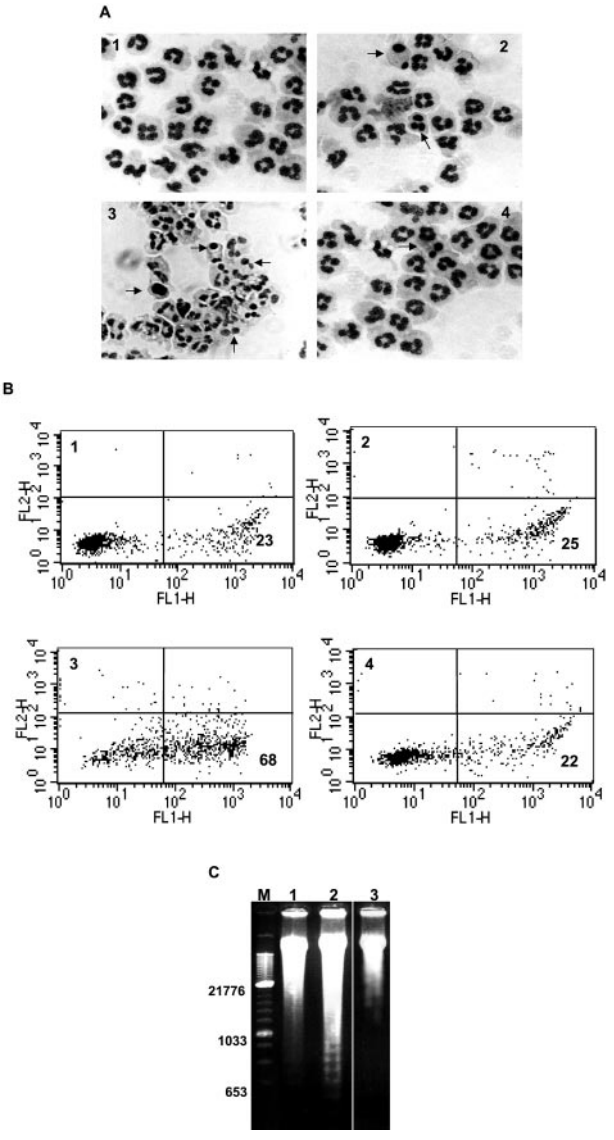

FIGURE 1. Mtb induce apoptosis in human neutrophils. *A*, Cytospine preparations of neutrophils, after 18-h culturing time, were stained with Giemsa and apoptotic cells (arrows) were quantified morphologically. The micrographs show neutrophils treated as follows: 1) freshly isolated; 2) incubated in medium alone for 18 h; exposed to the Mtb strain H37Rv in the (3) absence or (4) presence of 5 μM DPI. *B*, Flow cytometric analysis of binding of annexin V (FL1) and PI (FL2) in neutrophils incubated in medium alone for 18 h to undergo spontaneous apoptosis in the (1) absence or (2) presence of DPI, or with H37Rv for mycobacteria-induced apoptosis in the (3) absence or (4) presence of DPI. The percentage of PS-positive cells in each sample is indicated. For clarity, the fluorescence profiles of 10% of the analyzed events are shown. *C*, Agarose gel electrophoresis of internucleosomal DNA fragmentation during spontaneous apoptosis (*lane 1*) or H37Rv-induced apoptosis in the absence (*lane 2*) or presence of DPI (*lane 3*) for the indicated culture times. The molecular markers are indicated to the left (*lane M*). The results shown are representative of four independent experiments. Percentages of apoptotic cells (mean ± SD) are given in Table I.

Table I. *Effect of the NADPH oxidase inhibitor DPI on spontaneous and Mtb-induced apoptosis in human neutrophils*[a]

Treatment	Apoptotic Cells (%)	
	Morphology	Annexin V
Medium alone	28 ± 6.2	26 ± 4.1
+DPI	24 ± 5.0	23 ± 2.8
H37Rv	70 ± 6.0	72 ± 5.3
+DPI	32 ± 9.0	24 ± 3.2

[a] DPI-treated or untreated neutrophils were cultured for 18 h in medium alone or stimulated with H37Rv. The percentage of cells displaying apoptotic morphology and exposing PS were evaluated as outlined in the legend to Fig. 1. Each value represents the mean ± SD of four independent experiments.

To further confirm the role of ROS on Mtb-induced apoptosis, we assessed the effects of two antioxidants, glutathione and NAC, on the apoptotic process. Neutrophils were pretreated with 5 mM of these antioxidants, and then exposed to Mtb and subsequently cultured for 6 and 18 h. The results showed that these antioxidants inhibited ~60% of the Mtb-induced apoptosis in neutrophils cultured for 6 or 18 h, but had little effect on spontaneous apoptosis, as shown in Fig. 2 for 18 h. Moreover, DPI, glutathione, and NAC at the concentrations used efficiently blocked the Mtb-stimulated ROS production in neutrophils, as measured by luminol-ECL, but had no effects on phagocytosis of Mtb (data not shown). Addition of extracellular scavengers (superoxide dismutase and catalase) did not alter either spontaneous or Mtb-induced apoptosis (data not shown). These results clearly show that infection with Mtb induces apoptosis in neutrophils, a process that depends on intracellular generation of ROS.

Virulent and attenuated Mtb exerted the same effect on neutrophil apoptosis

A recent study in human macrophages has shown that apoptosis was induced more potently by attenuated than by virulent mycobacteria (12). To find out whether the same is true in neutrophils, we compared the influence of virulent H37Rv and attenuated H37Ra strain of Mtb on neutrophil apoptosis. Neutrophils were infected with Mtb at a ratio of 1:20 or 1:10 (cell to bacteria) for 30 min and then cultured for 3–24 h. We found that both virulent and attenuated Mtb at the indicated ratios were equally effective in inducing apoptosis in neutrophils with similar kinetics as shown for the 1:20 infection rate in Fig. 3. Under the same conditions, treatment of neutrophils with DPI efficiently reduced apoptosis elicited by either H37Rv or H37Ra (data not shown).

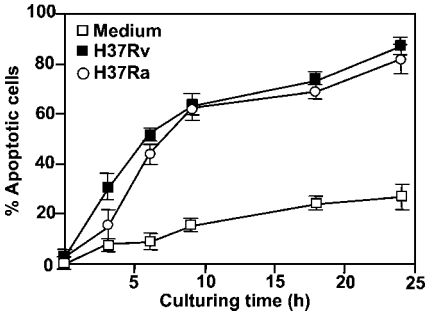

FIGURE 3. Virulent and attenuated strains of Mtb have similar effects on induction of neutrophil apoptosis. Neutrophils (2×10^6/ml) were exposed to virulent H37Rv or attenuated H37Ra strains of Mtb at a ratio of one cell-20 Mtb or incubated in medium alone, then cultured for 3–24 h. The percentage of apoptotic cells was determined by flow cytometry and each value represents the mean ± SD of four independent experiments.

Activation of caspase-3 in Mtb-induced apoptosis

A common feature of cells undergoing apoptosis is activation of caspases, in neutrophils primarily caspase-3 and -8 (23–25). We began our study by performing an experiment to identify the caspases that are involved in Mtb-induced apoptosis in neutrophils. The cells were pretreated with 25 μM zDEVD-fmk or zIETD-fmk which respectively inhibit caspase-3 and -8, and were then exposed to Mtb and subsequently cultured for 12 h. As shown in Fig. 4, inhibition of caspase-3 activity effectively reduced the Mtb-induced apoptosis from 70 ± 8% (Mtb alone) to 30 ± 12% (Mtb and zDEVD-fmk). Spontaneous apoptosis was also partially inhibited by zDEVD-fmk, which agrees with previously reported data (23). In contrast, Mtb-induced apoptosis was only slightly reduced by caspase-8 inhibitor zIETD-fmk (Fig. 4). In light of these results, we proceeded to determine the caspase-3 activity in neutrophils that had been treated as indicated.

A continuous fluorometric assay was used to measure cleavages of the caspase-3-specific fluorogenic substrate (DEVD-AMC), and the results were considered to represent caspase-3 activity. Extracts from neutrophils that had or had not been exposed to Mtb and were then cultured for various time periods were mixed with

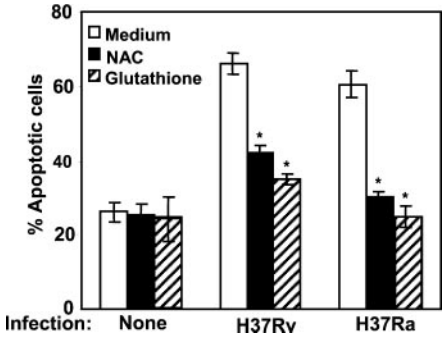

FIGURE 2. Effects of antioxidants on spontaneous and Mtb-induced neutrophil apoptosis. Neutrophils (2×10^6/ml) were pretreated for 10 min at 37°C with 5 mM of NAC or glutathione or medium alone. The cells were subsequently incubated for 30 min with or without Mtb (1:20, cell-Mtb), and then cultured for 18 h in the presence of antioxidants. The percentage of apoptotic cells was determined by flow cytometry and each value represents the mean ± SD of four independent experiments. *, Significantly different from neutrophils not treated with antioxidants; $p < 0.05$.

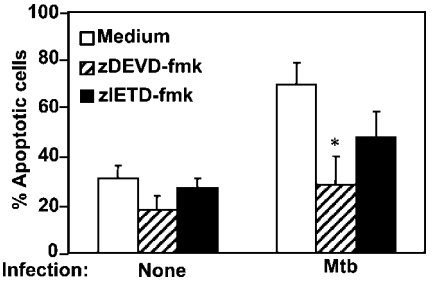

FIGURE 4. Effect of inhibitors of caspase-3 and -8 on spontaneous and Mtb-induced neutrophil apoptosis. Neutrophils (2×10^6/ml) were pretreated for 10 min at 37°C with 25 μM zDEVD-fmk or zIETD-fmk, to inhibit caspase-3 and -8 respectively. The cells were subsequently incubated with Mtb (1:20, cell-Mtb) or in medium alone and then cultured for 18 h. Untreated cells that had or had not been exposed to Mtb were cultured for the same period of time. The percentage of apoptotic cells was obtained from flow cytometry, using annexin V. The data represent means ± SD of four separate experiments. *, Significantly different from cells not exposed to inhibitors; $p < 0.05$.

DEVD-AMC, and the maximum linear rate of AMC release was measured. The results show substantial caspase-3 activity with a peak value of 86.4 ± 6 at 18 h during spontaneous apoptosis and a markedly higher peak value of 160 ± 10 that occurred much sooner (at 12 h) during Mtb-induced apoptosis (Fig. 5*A*). Subsequent measurements revealed that the caspase-3 activity declined more rapidly in Mtb-stimulated cells than in the uninfected neutrophils, which is not surprising since most of the former had become apoptotic. The specific caspase-3 inhibitor DEVD-CHO (50 nM) prevented the DEVD-AMC cleavages, which confirms the specificity of the assay. These findings suggest that activation of caspase-3 is a prerequisite of Mtb-induced apoptosis in neutrophils.

Mtb-induced activation of caspase-3 is dependent on ROS

Having established the role of ROS and caspase-3 in Mtb-stimulated neutrophil apoptosis, we examined the possibility that ROS promote apoptosis by targeting caspase-3 activity. DPI-treated neutrophils were exposed to Mtb or medium alone and then cultured for 6 h, after which the caspase-3 activity was measured. The results show that DPI did not alter the DEVD-AMC cleavage in uninfected neutrophils but almost completely blocked the caspase-3 activity induced by infection with Mtb, as indicated by DEVD-AMC cleavage with a value of 84 ± 4 with Mtb alone and 37 ± 3 with Mtb plus DPI (Fig. 5*B*).

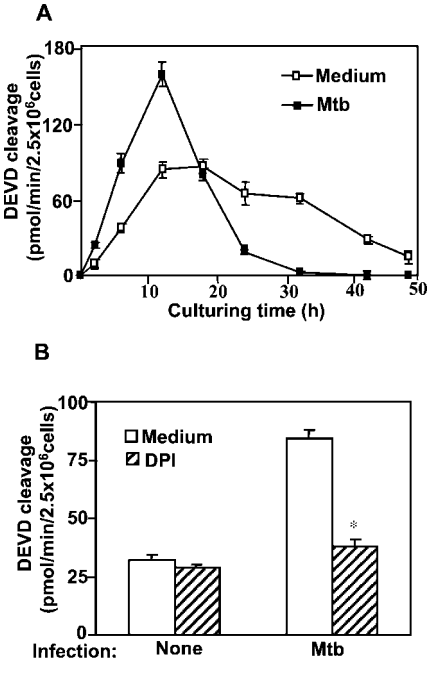

FIGURE 5. The activity of caspase-3 in neutrophils undergoing spontaneous or Mtb-induced apoptosis. *A*, Neutrophils (5×10^6/sample) that had or had not been exposed to Mtb (1:20, cell-Mtb) for 30 min, were cultured for 0–48 h. Thereafter, the cells were lysed and cleavage of the caspase-3-specific fluorogenic substrate DEVD-AMC was monitored. The maximum rate of AMC release (picomoles per minute) was estimated by linear regression ($r^2 = 0.99$) and the values given represent the mean ± SD of three separate experiments. *B*, Mtb-induced activation of caspase-3 is inhibited by DPI. Neutrophils were pretreated for 5 min at 37°C with 5 μM DPI, and incubated with Mtb or in medium alone, and then the cells were cultured for 6 h. Untreated cells that had or had not been exposed to Mtb were cultured for the same period of time. Thereafter, the cells were lysed and cleavage of DEVD-AMC was monitored. The maximum rate of AMC release (picomoles per minute) was determined and is presented as in *A*. ∗, Significantly different from neutrophils not treated with DPI; $p < 0.01$.

Differential expression of Bax and Bcl-x_L proteins in Mtb-infected neutrophils

It has been shown that proteins of the Bcl-2 family play an essential role in neutrophil apoptosis and that several inflammatory mediators regulate neutrophil apoptosis by altering the expression of antiapoptotic Bcl-x_L and proapoptotic Bax proteins (26–28). To ascertain whether such a mechanism is involved in Mtb-induced apoptosis, we cultured neutrophils with or without ingested Mtb for 2, 6, and 20 h and then determined the amount of Bcl-x_L and Bax expressed in the cells. Freshly isolated neutrophils kept at 4°C were used as a control for basal expression of these proteins. The results show that the expression of antiapoptotic Bcl-x_L decreased with culturing time in both Mtb-infected and uninfected cells, and this decline was more prominent in Mtb-infected cells (Fig. 6*A*, *upper panel*), indicating that loss of functional Bcl-x_L is associated with neutrophil apoptosis. Increased expression of proapoptotic protein Bax was evident in cells undergoing spontaneous apoptosis, and there was a transient, but more pronounced, rise in Bax in neutrophils exposed to Mtb, with a level that peaked at 2 h and then rapidly declined (Fig. 6*A*, *lower panel*). After culturing for 20 h, Mtb-stimulated neutrophils retained only ~14% of the level of Bax detected in freshly isolated cells. Reprobing of the blots with anti-actin Ab confirmed that equal amounts of proteins had been loaded in each lane (not shown).

To further characterize the role of Bax and Bcl-x_L in neutrophil apoptosis, we calculated the Bax-Bcl-x_L ratio, using the mean OD value of three independent experiments for each protein. As shown in Fig. 6*B*, for the uninfected neutrophils (medium), we found a sustained increase in the Bax/Bcl-x_L ratio, which might account for induction of spontaneous apoptosis. Infection with Mtb led to

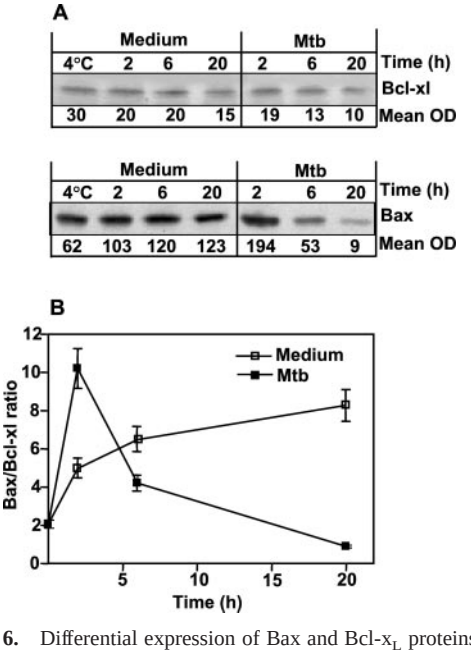

FIGURE 6. Differential expression of Bax and Bcl-x_L proteins by neutrophils undergoing spontaneous or Mtb-induced apoptosis. *A*, Neutrophils (2×10^6/sample) exposed to medium alone or to Mtb (1:20, cell-Mtb) were cultured for the indicated amounts of time. Thereafter, whole cell lysates were prepared and subjected to SDS-PAGE and blotted with anti-Bcl-x_L (*upper panel*) or anti-Bax (*lower panel*) Abs. The OD value for each lane is the mean of three representative and independent experiments. *B*, The Bax-Bcl-x_L ratio from neutrophils undergoing spontaneous (control) and mycobacteria-induced (Mtb) apoptosis was calculated using means ± SD (OD) of the values for expression of Bax and Bcl-x_L, obtained in three separate experiments as described above.

a transient but substantial increase in the Bax-Bcl-x_L ratio at 2 h and a rapid decline thereafter, and this transient increase might represent a "triggering" mechanism for the Mtb-induced apoptosis. In an uninfected cell population, the apoptotic cascade is probably initiated at different time points because the cells are of varying age, resulting in a relatively lower and sustained Bax-Bcl-x_L ratio. By comparison, the apoptotic cascade in Mtb-stimulated cells is triggered at the same time by infection, thereby leading to a substantial increase followed by a rapid decline in the Bax-Bcl-x_L ratio.

Effect of DPI on expression of Bax and Bcl-x_L in Mtb-infected neutrophils

To determine whether the differing expression of Bax and Bcl-x_L in Mtb-infected neutrophils involved the generation of ROS, neutrophils were treated with DPI and then were or were not infected with Mtb and subsequently cultured for 2, 6, and 20 h. DPI had no effect on expression of Bcl-x_L and Bax as well as the Bax-Bcl-x_L ratios by uninfected cells (compare Fig. 7 with Fig. 6). However, the decrease in Bcl-x_L expression in Mtb-infected cells was attenuated by DPI. The down-regulation of Bax was eradicated in the presence of DPI (Fig. 7*A*, *lower panels*), thereby the Bax-Bcl-x_L ratios exhibited during Mtb-induced apoptosis were changed (Fig. 7*B*). In other words, DPI caused a sustained increase in the Bax-Bcl-x_L ratio in Mtb-infected cells, and the kinetics observed were similar to those seen in neutrophils undergoing spontaneous apoptosis.

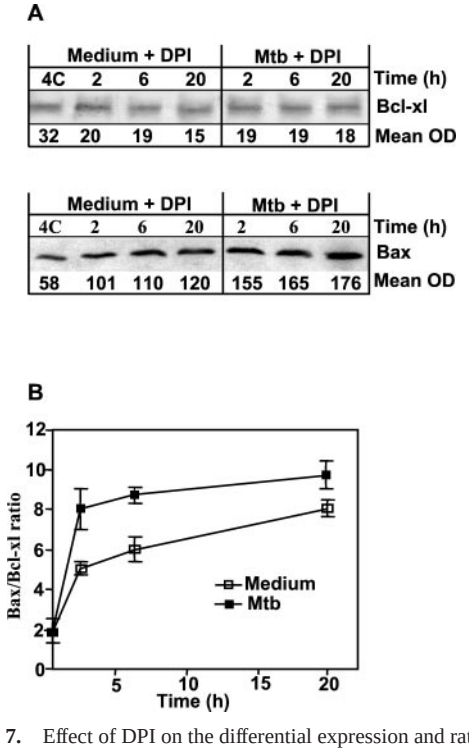

FIGURE 7. Effect of DPI on the differential expression and ratio of Bax and Bcl-x_L in neutrophils undergoing spontaneous or Mtb-induced apoptosis. *A*, DPI-treated neutrophils were exposed to medium alone or to Mtb (1:20, cell-Mtb) and then cultured for the indicated amounts of time. Whole cell lysates were subjected to SDS-PAGE and blotted with anti-Bcl-x_L (*upper panel*) or anti-Bax (*lower panel*) Abs. The OD value for each lane is the mean of three representative and independent experiments. The number indicates the mean OD of each lane obtained from three representative and independent experiments. *B*, Changes in the Bax-Bcl-x_L ratio during apoptosis were calculated using means ± SD (OD) of the values for expression of Bax and Bcl-x_L, obtained in three separate experiments as described above.

Ingestion of Mtb-induced apoptotic neutrophils stimulates TNF-α production in macrophages

Phagocytosis of uninfected apoptotic neutrophils is known to suppress the proinflammatory responses of macrophages (31, 32, 39–41). To find out whether the same is true for uptake of Mtb-infected apoptotic neutrophils, we compared the influence of uninfected- and Mtb-infected-apoptotic neutrophils on the inflammatory responses of human monocyte-derived macrophages. The percentage of macrophages positive for uptake of UV-induced apoptotic neutrophils was 46.8 ± 6.8%, and 44.8 ± 7.2% for uptake of Mtb-induced apoptotic neutrophils. In agreement with previous reports (31, 32, 39, 41), phagocytosis of UV-induced apoptotic neutrophils by macrophages inhibited the production of proinflammatory cytokine TNF-α but increased the anti-inflammatory TGF-β1 production. In contrast, uptake of Mtb-induced apoptotic neutrophils markedly increased the TNF-α production by macrophages (Table II). Mtb alone also stimulated the TNF-α production in macrophages, but at a level much lower than that in macrophages exposed to Mtb-induced apoptotic neutrophils (Table II). There was no detectable cytokine production by the UV- or Mtb-induced apoptotic neutrophils when cultured without macrophages (data not shown), thus the cytokine levels shown reflect macrophage production. These results indicate that phagocytosis of Mtb-induced apoptotic neutrophils stimulate the proinflammatory response in human macrophages.

Discussion

Neutrophils are the first cells to temporarily infiltrate the site of a mycobacterial invasion, and they play a significant protective role during the acute phase of the infection (3, 4). The present study shows that infection with Mtb induced a rapid apoptosis of neutrophils and that a virulent and an attenuated strain of Mtb were equally effective on this process. We found that, in contrast to spontaneous apoptosis, Mtb-induced apoptosis was strictly dependent on the ROS generated by the neutrophils during phagocytosis of the mycobacteria. Furthermore, infection of neutrophils with Mtb resulted in altered expression of Bax and Bcl-x_L, as well as activation of caspase-3 in an oxygen-dependent manner. Inasmuch as apoptotic cells are rapidly removed by macrophages and uptake of Mtb-infected apoptotic neutrophils triggers the proinflammatory responses in macrophages, ROS-dependent apoptosis in Mtb-laden neutrophils represents an important host defense mechanism for selective removal of the infected neutrophils from the site of inflammation, thereby limiting the expansion of the infection.

Previous studies in macrophages have shown that attenuated strains of Mtb are much more efficient than virulent strains in inducing apoptosis in these cells (12) and that lipoarabinomannan

Table II. *Phagocytosis of Mtb-induced apoptotic neutrophils stimulates TNF-α production by human monocyte-derived macrophages*[a]

Stimulus	TNF-α (pg/ml)	TGFβ1 (pg/ml)
Control	45 ± 12	25 ± 8
UV-apoptotic cells	18 ± 6[b]	58 ± 7[b]
Mtb-apoptotic cells	1676 ± 315[c]	66 ± 11[b]
Mtb	255 ± 50[b]	40 ± 9[b]

[a] Human macrophages were incubated with UV-irradiated or Mtb-infected apoptotic neutrophils, or Mtb alone for 1 h and washed. The supernatants were collected 18 h later and cytokine concentrations were determined by ELISA. As a control, macrophages were incubated with medium alone. Data are displayed as mean ± SE from 10 (TNF-α) or 6 (TGFβ1) experiments.

[b] Significantly different from control ($p < 0.05$).

[c] Significantly different from control or macrophages stimulated with Mtb alone ($p < 0.01$).

from virulent Mtb promotes macrophage survival by activating the phosphatidylinositol 3-kinase/Akt pathway (42). However, in our experiments, H37Rv and H37Ra were equally effective at inducing apoptosis in neutrophils. The difference between these Mtb strains regarding their impact on apoptosis in human neutrophils and macrophages may reflect the disparate roles these phagocytes play in the anti-mycobacterial immune response of the host. The virulence of Mtb depends on the ability of the bacteria to survive and replicate within macrophages (11), whereas the main function of neutrophils is to ingest and thereby inhibit the spread of Mtb during the acute phase of infection.

Our observation that both the NADPH oxidase inhibitor DPI and antioxidants effectively blocked Mtb-induced, but not spontaneous, apoptosis in neutrophils, indicates that the generation of ROS elicited by Mtb is a prerequisite for induction of apoptosis. This conclusion is supported by our finding that Mtb-stimulated activation of caspase-3 is also inhibited by DPI. Despite evidence that ROS are common mediators of apoptosis in various types of cells (19, 37, 38, 43), conflicting results have been reported regarding their role in neutrophil apoptosis. For example, phagocytosis of bacteria by neutrophils, which is associated with production of ROS, has been shown to promote and to inhibit apoptosis in these cells (18, 44). One possible explanation for this discrepancy may be due to use of different bacteria and infection models in those studies. Several bacteria components or products (e.g., LPS and enterotoxins) are known to inhibit neutrophil apoptosis (44, 45), hence regulation of apoptosis in these cells my represent the net outcome of the relative balance between proapoptotic and antiapoptotic pathways that are activated simultaneously (e.g., by ROS and LPS, respectively).

The execution of an apoptotic pathway is mediated by caspases. Human neutrophils express several different caspases, but it is mainly caspase-3 and -8 that are activated during apoptosis (23–25). We found that inhibition of caspase-3, but not caspase-8, effectively blocked Mtb-induced apoptosis in neutrophils, and stimulation with Mtb markedly augmented and accelerated the activation of caspase-3. This suggests that Mtb-induced apoptosis in neutrophils is mediated primarily through activation of caspase-3. Moreover, as noted for apoptosis, the Mtb-induced activation of caspase-3 is dependent on ROS. In contrast, Fadeel et al. (20) reported that the production of ROS in PMA-treated neutrophils led to inactivation of caspase-3. This discrepancy is most likely due to use of different stimuli to produce ROS. As suggested by the same group, mild oxidative stress can activate the caspase cascade and induce apoptosis, whereas prolonged or excessive oxidative stress (e.g. stimulated by PMA) prevents caspase activation (46). The precise regulatory mechanisms underlying the ROS-dependent activation of caspase-3 observed in our study are not yet known. In other systems, ROS has been shown to directly or indirectly target mitochondria and release cytochrome *c* from those organelles into the cytosol (43). It is generally assumed that such release is required to activate caspase-3 and it can be regulated by proteins of the Bcl-2 family (27).

Human neutrophils express both the proapoptotic Bax and the antiapoptotic Bcl-x_L and Mcl-1 (28, 29). Bcl-x_L is known to act as an antiapoptotic counterpart that prevents Bax from exerting its proapoptotic effect (26). Therefore, the shift in balance in the Bax-Bcl-x_L ratio, which can be achieved by up- or down-regulation of the interacting partners, determines the fate of neutrophil survival or death. In our study, spontaneous apoptosis was associated with both decreased Bcl-x_L and increased Bax, resulting in a sustained increase in the Bax-Bcl-x_L ratio. These results confirm and extend those reported by Weinmann et al. (27), showing the critical role of Bax-Bcl-x_L in regulation of neutrophil apoptosis. Our observation of a transient, but more pronounced, increase in the Bax-Bcl-x_L ratio of Mtb-stimulated neutrophils implies that such a shift represents a mechanism that regulates apoptosis in infected cells. It seems likely that the rapid decline after the peak in the Bax/Bcl-x_L ratio would occur in a population of Mtb-infected neutrophils when the apoptotic machinery in most of the cells would already have been initiated. This hypothesis is supported by our findings that Mtb-stimulated neutrophils exhibited similar kinetic changes in regard to activation of caspase-3 (i.e., a pronounced increase followed by a rapid decline; Fig. 5*A*).

In Mtb-infected neutrophils, DPI abolished the down-regulation of Bcl-x_L, indicating that ROS is critically involved in the regulation of Bcl-x_L expression in these cells. The transient increased Bax expression observed at 2 h postinfection was also partially suppressed by DPI, although the expression of Bax in these cells was still greater than in controls (Figs. 6 and 7). The underlying mechanism by which DPI completely blocked the Mtb-induced apoptosis, but not Bax expression, is at present unclear. In other systems, it has been shown that the proapoptotic effect of Bax depends not only on its expression but also on its translocation from the cytosol to mitochondria membranes (26, 47). Thus, ROS might regulate the translocation of Bax to mitochondria membranes and exert its proapoptotic activity. This notion is supported by the report that ROS induced the oxidation of mitochondria membrane proteins (48). However, we cannot exclude a possible additional effect of Mtb infection on the Bax expression in neutrophils.

Our results that phagocytosis of uninfected apoptotic neutrophils resulted in a decreased TNF-α and an increased TGF-β production in macrophages are consistent with previous reports (31, 32, 39). This active anti-inflammatory response in macrophages might represent a mechanism for the safe clearance of apoptotic cells under noninfectious conditions. In contrast, uptake of Mtb-infected apoptotic neutrophils by macrophages markedly increased the production of TNF-α, a proinflammatory cytokine which is required for control of acute Mtb infection and granuloma formation (36). These results indicate that the neutrophil apoptosis after Mtb infection is an important host defense mechanism for the control of infection. This conclusion is supported by the report that ingestion of neutrophils containing Mtb increased the activity of mouse peritoneal macrophages against mycobacteria (49). At present, it is unknown about how macrophages distinguish and react differentially to uninfected- or infected-apoptotic neutrophils. Macrophages used a number of surface molecules (pattern recognition receptors) to bind to apoptotic cell-associated molecular patterns (39, 40, 50). However, the pattern recognition receptors involved in apoptotic cell binding overlap with those that recognize pathogens or necrotic cells (39, 40, 50). For example, CD14 or PS receptor has been shown to inhibit macrophage activation upon apoptotic cell binding, but actually activate macrophages once triggered by mycobacteria or necrotic cells, respectively (39–41, 51). In this context, it has been suggested that coengagement of different sets of pattern recognition receptors coupled to distinct signaling pathways results in multiple responses in macrophages (40). Such a mechanism might be responsible for the opposing responses in macrophages after ingestion of uninfected- or infected apoptotic neutrophils.

Although neutrophils are the first cells attracted to the site of an infection, Murray et al. (52) found that, in the absence of macrophage function, massive neutrophilia did not protect IFN-γ-deficient mice from mycobacteria infection. These results indicate that neutrophils participate mainly during the early stages of anti-mycobacterial immunity, when they ingest and inhibit the spread

of Mtb until macrophages accumulate. Ingestion of Mtb by neutrophils is associated with the production of ROS, which not only combat against the bacteria (5–7), but also promote apoptosis (present results). Therefore, the ROS-dependent apoptosis we observed in Mtb-stimulated cells may represent an important host defense mechanism aimed at selective removal of infected neutrophils from an inflamed site, which in turn aids the functional activities of local macrophages.

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