Proinflammatory Cytokines in the Course of Mycobacterium tuberculosis–Induced Apoptosis in Monocytes/Macrophages

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Mycobacterium tuberculosis (MTB) can induce apoptosis in monocytes/macrophages both in vitro and in vivo, and this phenomenon is associated with mycobacterial survival. The present study addresses the possibility that apoptotic and inflammatory pathways could coexist through a caspase-1–mediated mechanism. In this context, a caspase-1 inhibitor (YVAD), but not caspase-3 (DEVD) or caspase-4 (LEVD) inhibitors, was able to strongly inhibit MTB-induced apoptosis. Moreover, caspase-1 activity was confirmed by the increased maturation of interleukin (IL)–1β. Of interest, IL-1β and tumor necrosis factor (TNF)-α were produced massively in the course of infection, and both were inhibited by YVAD pretreatment. To determine whether TNF-α was produced actively by apoptotic cells, the intracytoplasmatic cytokine content and apoptotic phenotype were analyzed at the single-cell level. Results showed a progressive increase of TNF-α production in annexin V–positive cells. These results indicate that MTB-induced apoptosis is associated with proinflammatory cytokine production.
course of infection, and both were inhibited by YVAD. Finally, a progressive increase of TNF-α production in apoptotic cells was observed, showing that proinflammatory cytokine production is associated strictly with MTB-induced apoptosis.

Materials and Methods

Isolation and culture of blood monocytes. Peripheral blood mononuclear cells were isolated from human buffy-coat blood preparations by means of centrifugation on ficoll-hypaque and were suspended at $5 \times 10^6$ cells/mL in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 5 mM l-glutamine, and 5 μg/mL gentamicin). Then, they were incubated for 1 h in 10 mL of complete medium at 37°C in polystyrene T75 cm² tissue-culture flasks (Corning). After incubation, nonadherent cells were removed by washing with warm RPMI 1640. In each experiment, cells from a representative flask were analyzed in a flow cytometer by CD14 staining and morphological parameters (forward scatter vs. side scatter) to assess monocyte purity, which was always >85%. Finally, adherent monocytes were collected by gently detaching with a cell scraper after a 15-min incubation at 4°C in the presence of 5 mM EDTA in PBS.

Culture conditions and MTB infection. Adherence-purified monocytes/macrophages (M/M) were suspended at $10^6$ cells/mL and were plated with complete medium in 24-well plates. To evaluate the role played by caspase-1, caspase-3, and caspase-4 in the apoptotic process, M/M were preincubated with 1, 50 and 100 μM acetyl (Ac)–YVAD-chloromethylketone, 50 μM of Ac-caspase-3 inhibitor (DEVD)–CHO cells, or 50 μM of Ac-caspase-4 inhibitor (LEVD)–fluoromethylketone (all provided by Calbiochem) for 60 min before MTB H37Rv infection. Incubation was then performed by exposing M/M to 20 bacilli/cell for 1, 5, and 24 h. Thereafter, supernatants were collected for cytokine and lactate dehydrogenase (LDH) determination. At the indicated time points, adherent M/M were detached by a previous incubation with cold PBS at 4°C for 10 min, were counted by trypan blue exclusion, and were analyzed by use of flow cytometry and Western blotting.

Annexin V and intracellular staining of TNF-α by flow cytometry. MTB-induced apoptosis was monitored by annexin V staining, as described elsewhere [23]. Moreover, to investigate whether apoptotic cells can actively produce TNF-α, M/M were exposed to MTB H37Rv for either 1 or 5 h at an MOI of 20. Brefeldin A ($10 \mu$M; Calbiochem) was added together with the pathogen for the 1-h time point and in the last 4 h for the analysis performed at 5 h after MTB exposure. M/M then were collected and stained for 10 min at room temperature with fluorescein isothiocyanate–labeled annexin V (Boehringer) suspended in an annexin V buffer that consisted of 10 mM HEPES, 10 mM NaCl, and 2.5 mM CaCl₂. After incubation, cells were washed twice, first with annexin V buffer and then with PBS, 0.5% bovine serum albumin (BSA), and 0.1% Na₂SO₄. Cells then were fixed by a 5-min incubation with 4% paraformaldehyde, washed twice, and incubated for 1 h at room temperature with phycoerythrin–labeled anti–TNF-α monoclonal antibody (clone 8516.311; R&D Systems) diluted in blocking buffer at 1 mg/mL, followed by a peroxidase-conjugated goat anti–mouse IgG (BioRad). The signal was developed by enhanced chemiluminescence (ECL) and was exposed to ECL-Hyperfilm (Amersham).

Statistical analysis. Student’s t test was used to compare the differences between the means. $P < .05$ was considered to be statistically significant.

Results

MTB-induced apoptosis is caspase-1 but not caspase-3 and caspase-4 dependent. To evaluate whether caspase-1 is involved in the process of MTB-induced apoptosis, we performed experiments by pretreating cells with YVAD, DEVD, or LEVD [25]. Figure 1 shows that preincubation with YVAD, but not with either DEVD or LEVD, significantly inhibits MTB-induced apoptosis at 1 h after MTB exposure ($P < .005$). To evaluate the extent of MTB-induced cell death considered over a longer incubation time, we analyzed the accumulation of LDH in the supernatant of M/M at 5 h after MTB exposure. Figure 2A shows that YVAD pretreatment almost completely inhibits MTB-induced cytotoxicity ($P < .05$). Similar results also were obtained at the same time point by monitoring MTB-induced apoptosis in terms of annexin V positivity ($P < .05$; data not shown). Finally, caspase-1 activity was analyzed by use of West-
M/M is crucial for the outcome of the infection. During the course of this interaction, M/M can be activated [2, 3, 26, 27] or undergo apoptosis, depending on the MOI [4]. The results reported here were obtained by infecting cells with high amounts of virulent mycobacteria. The present experimental model can resemble the macrophage response that occurs in the presence of high bacterial load, such as that observed during the course of human immunodeficiency virus (HIV) infection. In fact, the number of bacilli increases in the lungs of HIV-infected patients with tuberculosis [28], and an increase in apoptotic alveolar macrophages was observed in patients with AIDS with disseminated pulmonary tuberculosis, compared with that in patients who only had tuberculosis [4].

Recently, evidence has been reported showing that MTB-induced apoptosis in M/M at early stages of infection is mediated by the cell wall–associated mycobacterial 19-kDa protein [17]. Such a lipoprotein has been described to induce both proinflammatory signals [13, 14] and apoptosis [15, 16] after binding to TLR2. The present study addresses the possibility that, in the course of apoptosis induced by MTB in human M/M, apoptotic and inflammatory pathways could coexist through a common caspase-1–mediated mechanism. In this context, caspase-1 activation has been reported in the course of apoptosis induced by bacterial lipoprotein and mediated by TLR2 [16], and its increase in TNF-α production in the course of MTB-induced apoptosis. Caspase-1 activation leads to the processing and release of mature IL-1β. Thus, we investigated the presence of IL-1β in the supernatant of MTB-infected M/M. Figure 2 shows the increased production of IL-1β at 5 h after MTB exposure, which is inhibited by YVAD pretreatment in a dose-dependent fashion. Moreover, we also investigated the inflammatory response in terms of TNF-α and IL-6 production. Figure 3 shows a strong increase in TNF-α production at 5 h after MTB exposure, which was dose-dependently inhibited by YVAD pretreatment. IL-6 production also was increased, but no YVAD-mediated inhibitory effect was observed (data not shown). Finally, TNF-α production then was monitored over the course of time. Figure 4 shows that TNF-α production progressively increased during the 24-h incubation time, a period in which most of the cells died [23]. Pretreatment of M/M with 50 μM YVAD inhibits such production, with a peak of activity at 5 h after mycobacterial exposure.

Apopotic cells produce TNF-α. Because dying cells can passively release cytoplasmic content, we investigated whether apoptotic cells actively produce TNF-α, by analyzing, at the single-cell level, the simultaneous expression of both annexin V–binding sites and intracytoplasmatic cytokine. The analysis, performed at 1 and 5 h (figure 5) after MTB exposure showed a progressive increase in TNF-α production in annexin V–positive cells that was subsequent to that observed in annexin V–negative cells.

Discussion

The very early phase of interaction between MTB and human M/M is crucial for the outcome of the infection. During the

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**Figure 1.** Monocytes/macrophages (M/M) were exposed for 1 h to *Mycobacterium tuberculosis* (MTB) H37Rv at an MOI of 20 after incubation with the indicated irreversible caspase inhibitors, used at a concentration of 50 μM. Apoptosis was determined by use of flow cytometry, using annexin V staining. Data are mean ± SD of separate experiments performed on M/M derived from 3 different donors. DEVD, caspase-3 inhibitor; LEVD, caspase-4 inhibitor; YVAD, caspase-1 inhibitor. P < .005, vs. control infected M/M. NS, not significant.

![Figure 2](https://academic.oup.com/jid/article-abstract/186/9/1277/941186)

**Figure 2.** A, Cell survival was monitored in terms of lactate dehydrogenase release in the supernatant of monocytes/macrophages (M/M) at 5 h after *Mycobacterium tuberculosis* (MTB) H37Rv exposure (MOI, 20). Data are expressed as mean ± SD of percentage of cytotoxicity by monitoring enzymatic activity in the supernatants derived from experiments performed on M/M obtained from 3 different donors. P < .05, vs. MTB-infected M/M. B, Caspase-1 activity was monitored by use of Western blotting analysis of mature and immature forms of interleukin (IL)-1β. Cell lysates of either uninfected (lanes 1 and 3) or MTB H37Rv–infected (lanes 2 and 4) M/M at 1 (lanes 1 and 2) and 5 (lanes 3 and 4) h after MTB exposure (MOI, 20) were resolved by SDS-PAGE and were blotted for IL-1β. Data are representative of 3 different experiments. YVAD, caspase-1 inhibitor.
Mycobacterium tuberculosis (MTB)–induced apoptosis is associated with interleukin (IL)–1β (A) and tumor necrosis factor (TNF)–α (B) proinflammatory cytokine production. Cells were treated with caspase-1 inhibitor (YV AD; 5, 50, and 100 μM) and were exposed to MTB H37Rv (MOI, 20). Cell-culture supernatants were collected at 5 h after MTB exposure and were tested for cytokines by use of ELISA. Data are mean ± SD and were obtained by testing supernatants from MTB-infected monocytes/macrophages (M/M) derived from 4 different donors. * and **, vs. MTB-infected M/M.

Volvement has been demonstrated in a murine model of MTB-induced apoptosis [29]. The results reported here show that caspase-1, but not caspase-3 or caspase-4, mediates the apoptosis induced by MTB. Moreover, proinflammatory cytokine production is strictly associated with the apoptotic program. In fact, high amounts of IL-1β and TNF-α are produced when massive cell death is induced by MTB, and both were inhibited by YVAD pretreatment. Single-cell analysis revealed that TNF-α is actively produced in apoptotic cells. Moreover, such production appears to be subsequent to that observed in nonapoptotic cells. The different kinetics of production suggest that TNF-α can be regulated initially by TLR2 through the involvement of the adaptor molecule, MyD88, which can activate both NF-κB [13] and caspase cascade [16]. However, the evidence that YVAD almost completely inhibits TNF-α production suggests that it can be maintained at a later time by caspase-1, through IL-1β-mediated signals. In this regard, caspase-1–deficient mice are defective in producing IL-1α, IL-6, TNF-α, and IFN-γ in response to lipopolysaccharide (LPS) [30].

The role of apoptosis in the context of human tuberculosis is still controversial. Some authors have suggested that macrophage apoptosis could represent a protective mechanism of MTB killing [18–21]. In this context, the inverse relationship between the ability to induce apoptosis and the pathogenicity of different mycobacterial strains has been reported, which suggests that macrophage apoptosis is a protective phenomenon [31]. However, evidence reported elsewhere that showed that (1) Fas-mediated apoptosis did not have any effect on mycobacterial viability [20, 22] and (2) MTB-induced apoptosis was associated with mycobacterial survival [23] strongly suggests that apoptosis may play different roles, depending on the nature of the inducing stimulus. It could represent a way of controlling the growth of intracellular pathogens when associated with mycobacterial killing in the presence of low bacterial burden [18–21]. On the other hand, when the bacterial load exceeds a particular threshold, mycobacteria can kill macrophages by apoptosis [4, 16, 23] and induce proinflammatory cytokines. In this context, TNF-α has been implicated in the pathologic response of the host to MTB infection and is often cited as a major factor in host-mediated destruction of lung tissue [1]. Recently, evidence also has been reported showing that TNF-α can promote the growth of virulent MTB in human monocytes [32] and in alveolar macrophages [33]. Thus, MTB-induced apoptosis may result in a direct tissue injury [34] and, through the production of proinflammatory cytokines, have detrimental effects by promoting intracellular mycobacterial replication and dissemination.

Immunotherapeutic approaches aimed at reducing caspase activity have been described successfully in a murine model of LPS-induced septic shock [35] and in a model of rat cerebral ischemia [36], in which both increased survival and reduced IL-1β and TNF-α production, and apoptosis inhibition were ob-
served after zVAD and YVAD treatment, respectively. To this regard, the present study suggests that caspase-1 can represent a novel molecular target for immunotherapeutic approaches aimed at reducing both inflammation and macrophage cell death in the course of human tuberculosis.

References


