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 co-exist 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 intracytoplasmic 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.

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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 × 10⁶ 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 10⁶ 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 then was 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 trypsin 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 μM; 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% NaN₃. 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 Mab111, IgG1; Becton Dickinson) suspended in PBS, 0.5% BSA, 0.1% NaN₃, and 0.5% saponin. Finally, cells were washed twice with PBS, 0.5% BSA, 0.1% NaN₃, and 0.01% saponin; suspended in PBS; and analyzed on a FACScan flow cytometer (Becton Dickinson).

Cytokine and LDH analysis by ELISA. Proinflammatory cytokine production was determined in the supernatants of MTB-infected M/M. IL-1β, TNF-α, and IL-6 levels were determined by means of ELISA, according to the manufacturer’s instructions. IL-1β determination was performed by use of an ELISA kit assay (R&D systems); TNF-α and IL-6 levels were determined by use of antibody pairs, as recommended by the supplier (Endogen). The detection limit was 3.9 pg/mL for IL-1β and 15.6 pg/mL for TNF-α and IL-6.

MTB-induced cell death was monitored by measuring the presence of LDH in the cell-culture supernatant with the CytoTox96 kit (Promega), according to the manufacturer’s instructions. The percentage of cytotoxicity was calculated as follows: 100 × (experimental release – spontaneous release)/(total release – spontaneous release), where spontaneous release is the amount of LDH activity in supernatants of uninfected cells and total release is the activity in M/M lysates.

Maturation of IL-1β in MTB-infected macrophages. SDS-PAGE was performed on 15% gel under reducing conditions by use of a MiniProtean II apparatus (BioRad). For each sample, 0.8 μg of total protein concentration (assessed at 280 nm) was analyzed simultaneously after boiling for 10 min in SDS-reducing sample buffer that contained a final concentration of 2% SDS. After separation, the proteins were transferred to nitrocellulose paper (Amersham) by use of a MiniProtein II transfer apparatus, according to the manufacturer’s instructions. Membranes then were washed with PBS and were treated for 1 h at room temperature with blocking buffer that consisted of PBS, 0.05% Tween 20, and 5% milk. The blots then were incubated overnight at 4°C with the primary mouse monoclonal antibody against IL-1β (clone 8516.311; R&D Systems) diluted in PBS, 0.5% BSA, and 0.05% Tween 20. Membranes then were washed with PBS and were treated with 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
Discussion was subsequent to that observed in annexin V–negative cells. The analysis, performed at 1 and 5 h 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.

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. 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.

The very early phase of interaction between MTB and human 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
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 and were obtained by testing supernatants from MTB-infected monocytes/macrophages (M/M) derived from 4 different donors. * and ** , vs. MTB-infected M/M.

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


