Differential Induction of Apoptosis and Necrosis in Monocytes from Patients with Tuberculosis and Healthy Control Subjects

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Background. Mycobacterium tuberculosis and purified protein derivative (PPD) induce apoptosis in murine macrophages and apoptosis and necrosis in human monocytes and alveolar epithelial cells. Macrophages from bronchoalveolar lavages and granulomas from patients with tuberculosis (TB) present both types of cell death; however, the significance of the type of cell death in TB remains uncertain.

Methods. Monocytes from PPD-positive control subjects and from patients with TB were exposed to PPD or M. tuberculosis. Apoptosis, necrosis, and the percentage of tumor necrosis factor (TNF)-α–positive and interleukin (IL)-10–positive cells were determined cytofluorometrically. Levels of lactate dehydrogenase, TNF-α, and IL-10 were measured in culture supernatants. The role of TNF-α and IL-10 was tested by blockade experiments.

Results. PPD and M. tuberculosis induced apoptosis in monocytes from PPD-positive control subjects, whereas cells from patients with TB presented apoptosis and necrosis. Cells from PPD-positive control subjects produced mainly TNF-α, whereas cells from patients with TB produced mainly IL-10. Blockade experiments suggest that TNF-α and IL-10 regulate the type of cell death occurring in response to M. tuberculosis.

Conclusions. Results suggest that apoptosis of monocytes exposed to mycobacteria may partly explain the protective immune response found in PPD-positive control subjects, whereas necrosis may be determinant of the bacterial dissemination and tissue damage that occur in patients with active TB.

The declaration of tuberculosis (TB) as a global emergency by the World Health Organization [1] resulted in renewed interest in the study of the biology of Mycobacterium tuberculosis, interactions between the host and M. tuberculosis, and pathogenesis of TB. During early phases of M. tuberculosis infection, macrophages play a key role in controlling the infection, by phagocytosing mycobacteria, producing cytokines that have modulatory effects on macrophages themselves and on other cells (e.g., T lymphocytes), and regulating induction of cell-mediated specific immune response [2].

The initial macrophage response depends on its innate capacity to recognize mycobacteria and activate antimycobacterial biochemical mechanisms [3, 4]. In recent years, accumulating evidence suggests that the fate of infected macrophages may be crucial for the subsequent events that occur within the infected host [5–8]. According to results of our previous studies, apoptosis of M. tuberculosis–infected murine macrophages depends on both bacterial and phagocytic factors. Although live M. tuberculosis H37Rv and purified protein derivative (PPD) are able to induce apoptosis [7, 9, 10], killed M. tuberculosis and mannosylated lipoarabinomannan [7, 11] inhibit apoptosis, suggesting that mycobacteria have developed mechanisms to modulate the apoptotic response of the host cells. On the phagocytic-cell side, apoptosis is influenced by the balance between tumor necrosis factor (TNF)-α and interleukin (IL)-10 produced in response to mycobacterial stimuli, suggesting that apoptosis may be involved in the innate antimycobacterial mechanisms [9, 12, 13]. However, other authors, using human monocytes and macrophages, have reported that apoptosis is induced mainly by nonvir-
ultent mycobacteria [14–18] and that even small inocula may inhibit apoptosis of infected macrophages [19]. Although apoptotic macrophages have been found in tissue granulomas and bronchoalveolar lavages from patients with TB [6, 20, 21], the role of apoptosis in the pathogenesis of the disease is still poorly understood.

Results from our laboratory, by use of the human promonocytic U-937 cell line, have shown that exposure of nondifferentiated cells to PPD results in changes compatible with necrosis, whereas differentiated cells undergo apoptosis [22], suggesting that human mononuclear phagocytes may exhibit both types of cell death after exposure to mycobacterial products. This observation is relevant, since apoptosis of infected phagocytes may contain the infection and prevent the inflammatory response, whereas necrosis of such cells, with disruption of cell membrane, may lead to release of intracellular bacteria and lysosomal enzymes [23, 24], resulting in bacterial dissemination and tissue damage. Furthermore, apoptotic infected macrophages may be determinants of the induction of CD8+ T cell–dependent responses [25, 26]. Thus, the type of cell death that occurs in infected macrophages may be crucial in the outcome of the infection.

The results presented here show that monocytes obtained from tuberculin-positive (i.e., PPD-positive) healthy control subjects undergo apoptosis when exposed to PPD or infected with M. tuberculosis, whereas monocytes from patients with TB present both apoptosis and necrosis. Both types of cell death were modulated by TNF-α and IL-10 produced in response to mycobacteria. These observations suggest that apoptosis of infected macrophages may be an important antimycobacterial defense mechanism and that, during active TB, there is a dysregulation of the mechanisms involved in the death of mononuclear phagocytes, which may partly explain the tissue damage and bacterial dissemination that occur in patients with active TB.

SUBJECTS, MATERIALS, AND METHODS

Patients and control subjects. Twenty-five patients with bacteriologically proven pulmonary TB were recruited from the Tuberculosis Clinic at Hospital La María (Medellín, Colombia). Bacteriological confirmation of TB was done by sputum acid-fast staining and culture. All patients were studied within 2 weeks of diagnosis. Thirty PPD-positive control subjects were selected from the medical and laboratory personnel who voluntarily agreed to participate in the study. None of the control subjects had previously received anti-TB treatment. All subjects studied signed an informed consent form previously approved by the Ethics Committee of the Centro de Investigaciones Médicas, Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia. All participants were HIV negative and underwent tuberculin skin testing with 1 TU of Tubersol (PPD CT68; Connaught). Patients with TB included 19 men and 6 women whose ages ranged from 19 to 65 years. Control subjects included 20 men and 10 women whose ages ranged from 20 to 53 years.

Sample collection and cell isolation. Fifty milliliters of venous blood was obtained under aseptic conditions and defibrinated by continuous agitation with glass beads. Defibrinated blood was centrifuged, and theuffy coat was resuspended 1:3 (vol:vol) in PBS (Gibco BRL Life Technologies). Mononuclear cells (MNCs) were obtained by centrifugation on Lymphocyte Separation Media (BioWittaker). Cell viability was determined by use of trypan blue exclusion, and the cells were resuspended at 1 × 10⁸ cells/mL in RPMI 1640 medium (Gibco BRL) without antibiotics and were supplemented with 10% heat-inactivated fetal calf serum (FCS; BioWittaker). In selected experiments, 2 × 10⁶ MNCs/well were plated in 24-well flat-bottomed culture plates (Corning), in complete medium containing 0.5% FCS, for 2 h at 37°C. Thereafter, nonadherent cells were removed by repeatedly washing with prewarmed PBS plus 10% FCS. The number of adherent cells was determined by mechanical removal and counting the cells in a replicate well. The purity of monocytes in the adherent cell population was determined by use of flow cytometry using anti–CD14–fluorescein isothiocyanate (FITC; clone M5E2), anti–CD3–CyCh (clone 145-2C11), anti–CD19–peridinin chlorophyll protein (PerCP; clone 2H7), and anti–CD14–phycoerythrin (PE; clone M312), and anti–CD14–phycoerythrin (PE; clone M312), and anti–CD19–peridinin chlorophyll protein (PerCP; clone 2H7), and anti–CD6–fluorescein isothiocyanate (FITC; clone M6002). Cell viability was determined by use of trypan blue exclusion, and the cells were resuspended at 1 × 10⁸ cells/mL in RPMI 1640 medium (Gibco BRL) without antibiotics and were supplemented with 10% heat-inactivated fetal calf serum (FCS; BioWittaker). In selected experiments, 2 × 10⁶ MNCs/well were plated in 24-well flat-bottomed culture plates (Corning), in complete medium containing 0.5% FCS, for 2 h at 37°C. Thereafter, nonadherent cells were removed by repeatedly washing with prewarmed PBS plus 10% FCS. The number of adherent cells was determined by mechanical removal and counting the cells in a replicate well. The purity of monocytes in the adherent cell population was determined by use of flow cytometry using anti–CD14–fluorescein isothiocyanate (FITC; clone M5E2), anti–CD3–CyCh (clone 145-2C11), anti–CD19–peridinin chlorophyll protein (PerCP; clone 2H7), and anti–CD14–phycoerythrin (PE; clone M312), and anti–CD14–phycoerythrin (PE; clone M312), and anti–CD6–fluorescein isothiocyanate (FITC; clone M6002).

Analysis of hypoploidy by flow cytometry. The number of hypoploidic cells was determined as described elsewhere [7]. In brief, 3 × 10⁵ PPD-stimulated and nonstimulated MNCs from PPD-positive control subjects were fixed with 70% ethanol and resuspended in 1.5 mL of 50 μg/mL propidium iodide (PI) and 100 U/mL RNase A (Sigma Chemical). The cells were incubated for 30 min at room temperature in the dark before flow cytometric analysis. The PI fluorescence of 10⁵ nuclei was determined within the monocyte gate on the basis of the forward-scatter/side-scatter dot plot. The percentage of nuclei with...
DNA content smaller than the normal diploid complement (higher peak in histograms) was considered to be hypoploidic.

**TUNEL assay.** The MEBSTAIN apoptosis kit (Medical & Biological Laboratories) was used for the TUNEL assay. In brief, cells were washed with PBS containing 2% FCS and 0.1% NaN₃ and fixed with 4% paraformaldehyde. The cells were permeabilized with 200 μL of 0.5% Tween 20, containing 0.2% bovine serum albumin (BSA), and the terminal deoxynucleotidyl transferase reaction was performed in the presence of FITC-dUTP. Cells were resuspended in 100 μL of PBS, and 5 μg/mL anti–CD14-PE (clone M5E2), anti–CD3-CyCh, or the respective isotype controls were added. Counting of TUNEL-positive cells was performed by use of flow cytometry by gating the CD14+CD3+ region.

**Differentiation of apoptotic and oncocytic/necrotic monocytes.** To simultaneously identify oncocytic/necrotic and apoptotic monocytes, 3 × 10⁶ cells were incubated in 100 μL of PBS containing 0.1 μmol/L DIOC₁₂ (Molecular Probes) and 5 μg/mL EB for 30 min. Cytosfluorometric analysis of mitochondrial PT and membrane integrity was performed in 10,000 cells. This technique allows for a differentiation between live (DIOC₁₂ highEB low), apoptotic (DIOC₁₂ lowEB high), and necrotic cells that were defined as cells with membrane damage (EB+) [28, 29].

**Measurement of lactate dehydrogenase (LDH) in cell culture supernatants.** Since membrane leakage observed during necrosis results in release of LDH into the culture medium [18, 30], measurement of this enzyme was used to confirm induction of this type of cell death. Cells were cultured for 48 h, in the presence or absence of PPD, and 50 μL of the supernatant fluid was collected to determine LDH concentration by use of a commercial kit (Promega). In brief, supernatants were mixed with the substrate mix buffer for 30 min at room temperature in the dark. The reaction was stopped by the addition of 1:10 volume of 1 N HCl to each sample. Finally, absorbance was determined at 490 nm.

**Detection of production of TNF-α and IL-10.** Adherent cells were cultured in the presence or absence of PPD or were infected with *M. tuberculosis* (5:1) for 18 h before treatment with 1 μg/mL Brefeldin A for 6 h at 37°C. Then, cells were washed and fixed with 4% paraformaldehyde in 0.1 mol/L KH₂PO₄ for 20 min at room temperature and permeabilized with 200 μL of 0.5% Tween 20, with 0.2% BSA in PBS, for 30 min at 4°C. Five microliters of anti–TNF-α–FITC or anti–IL-10–FITC was added to the cell suspension for 30 min at 4°C. Cells were washed with cold PBS containing 1% pooled human serum. Specificity of antibodies was determined by incubating the cells with 10X excess of anti–TNF-α–anti–TNF-α–FITC or anti–IL-10–anti–IL-10–FITC. Counting of stained cells was performed by use of flow cytometry. Concentrations of TNF-α and IL-10 in culture supernatants were determined by use of commercial ELISA kits (Endogen). The TNF-α:IL-10 ratio was calculated for each subject studied as follows: (TNF-α in stimulated cultures – TNF-α in nonstimulated cultures)/(IL-10 in stimulated cultures – IL-10 in nonstimulated cultures).

**Blockade of TNF-α and IL-10.** Adherent cells were plated at 3 × 10⁵ cells/well in 24-well, flat-bottomed culture dishes and were cultured in the presence of PPD or were infected with *M. tuberculosis* in the presence of 2.5 μg/mL anti–TNF-α or 3.0 μg/mL anti–IL-10 for 24 h. Thereafter, the cells were split for studies of apoptosis and necrosis and for intracellular determination of TNF-α, IL-10, and TUNEL.

**Statistical analysis.** All experiments were performed in triplicate and independently repeated at least 3 times. Data were analyzed by analysis of variance–type III of square sum. Interactions above the second level were excluded. Correlation analysis was done by use of Pearson’s product moment. *P* < .05, calculated by the interactions between the factors, was considered to be statistically significant. Data are presented as the mean ± 95% confidence interval for the mean. For all analyses, we used Statgraphics Plus software (release 2; Statgraphics).

**RESULTS**

To determine whether exposure to PPD induces apoptosis in human monocytes, MNCs obtained from PPD-positive control subjects were stimulated with PPD for 0–72 h. Thereafter, the cells were stained with PI, and the percentage of hypoploidic cells within the monocyte gate was determined by use of flow cytometry. PPD induced a significant time-dependent increase in the number of hypoploidic cells, a phenomenon that was not observed in nonstimulated cultures (figure 1). Significant differences between stimulated and control cultures were observed at 48 and 72 h (*P* < .001).

That monocytes were indeed undergoing apoptosis was verified in adherent cell cultures. Adherence resulted in 91% ± 5% of CD14+ cells with ≤5% CD3+ cells. There were no CD19+ or CD56+ cells, and 95% of the cells exhibited the same forward- and side-scatter cytometric characteristics as monocytes (data not shown). To compare the apoptotic response of adherent cells (from both PPD-positive control subjects and patients with TB) to PPD, adherent cells were stained with TUNEL (figure 2). In both groups, PPD induced a significant increase in the percentage of apoptotic cells (*P* < .003). However, this increase was higher in PPD-positive control subjects (69.27% ± 13.81%) than in patients with TB (32.47% ± 9.96%), suggesting that apoptosis is a normal response of monocytes from PPD-positive control subjects, whereas, in patients with TB, there may be alterations in monocyte death response to mycobacterial stimuli.

Whether the diminished apoptotic response observed in patients with TB may be due to a differential death response between apoptosis and necrosis was then explored. MNCs, cultured in the presence or absence of PPD, were analyzed by use
Figure 1. Kinetics of purified protein derivative (PPD)-induced monocyte apoptosis. Mononuclear cells from tuberculin skin test–positive individuals were cultured in the presence or absence of 10 μg/mL PPD for 0–72 h. DNA was stained with propidium iodide, and monocytes were gated to determine the no. of hypoploidic cells by flow cytometry. Histograms are from a representative experiment. Nos. indicate mean ± 95% confidence interval (n = 6). A significant increase in the no. of apoptotic monocytes was found at 48 and 72 h (P < .001).

Figure 2. Purified protein derivative (PPD)-induced monocyte apoptosis in PPD-positive control subjects and patients with tuberculosis (TB). Adherence-enriched monocytes stimulated with PPD (10 μg/mL) for 48 h were stained with anti–CD14-phycoerythrin and TUNEL (dUTP–fluorescein isothiocyanate) or the respective isotype controls. Figure shows the percentage of TUNEL-positive (TUNEL+) cells after culture of adherent cells in the presence or absence of PPD, from 15 patients with TB and from 15 tuberculin skin test–positive control subjects (P < .003).

Induction of necrosis by PPD in MNCs from patients with TB was confirmed by measuring the release of LDH into culture supernatants [30]. Nonstimulated cultures from both patients with TB and PPD-positive control subjects and PPD-stimulated cells from PPD-positive control subjects had negligible levels of LDH (table 1). However, PPD induced a significant release of LDH (P < .001) in MNC cultures from patients with TB.

To establish whether live M. tuberculosis also is able to induce a differential pattern of apoptosis and necrosis, adherent cells from PPD-positive control subjects and patients with TB were infected with M. tuberculosis (5:1) and stained with TUNEL, for detection of apoptotic cells. Infection with M. tuberculosis resulted in 60.9% ± 10.4% TUNEL-positive cells from PPD-positive control subjects and 26.0% ± 5.0% TUNEL-positive cells from patients with TB (P < .002) (table 2). Necrosis was present in 1.1% ± .06% of infected cells from PPD-positive control subjects and in 25.6% ± 2.5% of infected cells from patients with TB (P = .001), verifying that, similar to PPD exposure, monocytes undergo a differential pattern of apoptosis and necrosis after infection with M. tuberculosis. Furthermore, M. tuberculosis induced release of LDH only in cell cultures from patients with TB (P < .01).

It has been observed, in different models, that mycobacteria-
induced apoptosis is modulated by TNF-α and IL-10 [6, 9, 12, 15, 17]. Thus, we assessed whether these cytokines may also be involved in the modulation of the death of monocytes from patients with TB. For this purpose, we compared the percentage of adherent cells isolated from patients with TB and from PPD-positive control subjects producing TNF-α and IL-10 under our experimental conditions (table 1). In both groups of subjects, stimulation with PPD induced a significant increase in the percentage of cells producing these cytokines (P<.002). However, there was a differential response between PPD-positive control subjects and patients with TB. Although PPD-stimulated cultures from PPD-positive control subjects had 55.0% ± 2.3% TNF-α-positive cells, cultures from patients with TB had 25.0% ± 5.3% TNF-α-positive cells (P<.002).

In contrast, adherent cells from PPD-positive control subjects stimulated with PPD exhibited 20.1% ± 5.3% of IL-10–positive cells, compared with 70.6% ± 5.5% of IL-10–positive cells from patients with TB (P<.001). Similar results were obtained in culture supernatants from PPD-stimulated monocytes. PPD induced a higher release of TNF-α in cultures of cells from PPD-positive control subjects (20.5 ± 28.4 pg/mL) than in cultures of cells from patients with TB (50.4 ± 8.3 pg/mL) (P<.001) (table 1). In contrast, when stimulated with PPD, monocyte supernatants from patients with TB had a higher concentration of IL-10 (202.7 ± 19.0 pg/mL), compared with similar cultures from PPD-positive control subjects (95.5 ± 16.8 pg/mL) (P<.001) (table 1). The TNF-α:IL-10 ratio in supernatants from PPD-positive control subjects was 5.0 ± 1.5, whereas, in su-

### Table 1. Induction of apoptosis and necrosis and tumor necrosis factor (TNF)–α and interleukin (IL)–10 by monocytes of purified protein derivative (PPD)–positive control subjects and patients with tuberculosis (TB), stimulated with 10 μg/mL PPD.

<table>
<thead>
<tr>
<th>Cell outcome</th>
<th>PPD-positive control subjects (n = 15)</th>
<th>Patients with TB (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonstimulated</td>
<td>Stimulated</td>
</tr>
<tr>
<td>Live, %</td>
<td>91.8 ± 3.9</td>
<td>29.8 ± 11.2</td>
</tr>
<tr>
<td>Apoptotic, %</td>
<td>3.6 ± 1.7</td>
<td>72.9 ± 11.5</td>
</tr>
<tr>
<td>Necrotic, %</td>
<td>3.4 ± 1.8</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>LDH level, optical density</td>
<td>0.03 ± 0.001</td>
<td>0.01 ± 0.002</td>
</tr>
<tr>
<td>TNF-α+, %</td>
<td>2.0 ± 2.3</td>
<td>55.0 ± 2.3</td>
</tr>
<tr>
<td>IL-10+, %</td>
<td>5.0 ± 2.3</td>
<td>20.1 ± 5.3</td>
</tr>
<tr>
<td>TNF-α level, pg/mL</td>
<td>22.6 ± 2.0</td>
<td>210.5 ± 28.4</td>
</tr>
<tr>
<td>IL-10 level, pg/mL</td>
<td>25.7 ± 1.5</td>
<td>95.5 ± 16.8</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean ± 95% confidence interval. P values compare samples from patients with TB and healthy control subjects, stimulated with PPD. The TNF-α:IL-10 ratio was 5.0 ± 1.5 for PPD-positive control subjects and 0.25 ± 0.04 for patients with TB (P<.001, F ratio). LDH, lactate dehydrogenase.

### Table 2. Induction of apoptosis and necrosis and tumor necrosis factor–α and interleukin–10 by monocytes obtained from purified protein derivative (PPD)–positive control subjects and patients with tuberculosis and infected (5:1) with Mycobacterium tuberculosis H37Rv.

<table>
<thead>
<tr>
<th>Cell outcome</th>
<th>PPD-positive control subjects (n = 15)</th>
<th>Patients with TB (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonstimulated</td>
<td>Stimulated</td>
</tr>
<tr>
<td>Live, %</td>
<td>92.3 ± 6.3</td>
<td>30.1 ± 4.5</td>
</tr>
<tr>
<td>Apoptotic, %</td>
<td>5.0 ± 3.6</td>
<td>60.9 ± 10.4</td>
</tr>
<tr>
<td>Necrotic, %</td>
<td>3.4 ± 1.8</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>LDH level, optical density</td>
<td>0.01 ± 0.001</td>
<td>0.03 ± 0.001</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean ± 95% confidence interval. P values compare samples from patients with TB and healthy control subjects, infected with M. tuberculosis. LDH, lactate dehydrogenase.

a. 3,3′-dihexyloxacarbocyanine iodide and ethidium bromide assay.
b. P<.01.
c. TUNEL assay.
d. P<.001.
pernatants from patients with TB, it was 0.25 ± 0.04. More important, the TNF-α:IL-10 ratio correlated positively with the induction of apoptosis in PPD-positive control subjects (Pearson’s correlation coefficient, 0.86; \( P < .001 \)) and in patients with TB (Pearson’s correlation coefficient, 0.67; \( P < .01 \)) and correlated negatively with necrosis in patients with TB (Spearman’s rank correlation coefficient, −0.86; \( P < .001 \)).

Monocyte cultures had a small degree of contamination (≈5%) with CD3+ T lymphocytes that may also produce cytokines able to modulate macrophage cell death. Therefore, cells from PPD-positive control subjects, cultured in the presence or absence of PPD, were tested for intracellular TNF-α and IL-10 within the CD3+ and CD14+ gates (figure 3). It was not possible to detect cells producing either cytokine in nonstimulated cultures (data not shown). In PPD-stimulated cultures, there were 67% CD14+, TNF-α-positive cells with high fluorescence intensity and 10% CD3+, TNF-α-positive cells with a lower fluorescent intensity. The latter percentage is equivalent to 0.5% of un gated cells. With respect to IL-10–producing cells, no CD3+, IL-10–positive cells were detected, whereas there were 75% CD14+, IL-10–positive cells. These results suggest that, in adherent cell cultures stimulated with PPD, production of TNF-α and IL-10 is attributable mainly to monocytes.

Finally, we further assessed the roles of TNF-α and IL-10 in PPD-induced monocyte apoptosis and necrosis by use of blockade experiments with monoclonal antibodies specific for these cytokines. Apoptosis (figure 4A) was observed in 70% ± 5% and 45% ± 3% of monocytes from PPD-positive control subjects and patients with TB, respectively, stimulated with PPD in the presence of an irrelevant isotypic antibody (\( P < .002 \)). Blockade of TNF-α by anti–TNF-α antibodies completely inhibited apoptosis (2% ± 5% and 5% ± 5% in monocytes from PPD-positive control subjects and patients with TB, respectively; \( P < .002 \)). In contrast, treatment with anti–IL-10 resulted

Figure 3. Tumor necrosis factor (TNF)–α and interleukin (IL)–10 secretion by CD14+ cells. Purified protein derivative (PPD)–stimulated adherent cell cultures from PPD-positive control subjects were stained with anti–CD14–phycoerythrin (PE) plus anti-CD3 CyCh after intracellular staining of TNF-α–positive and IL-10–positive cells. Cells were gated for CD3+ or CD14+ and, thereafter, were analyzed for the expression of both cytokines. CD3+ cells are represented by filled histograms, and CD14+ cells are represented by open histograms. Figure shows a representative experiment (\( n = 5 \)). FITC, fluorescein isothiocyanate.

Figure 4. Modification of the necrotic and apoptotic events in monocytes stimulated with purified protein derivative (PPD) by blockade of anti–tumor necrosis factor (TNF)–α and anti–interleukin (IL)–10. The percentage of apoptotic (A) and necrotic (B) monocytes from patients with tuberculosis (TB; \( n = 5 \)) and control subjects (\( n = 5 \)) was determined after stimulation of adherent cells with PPD, in the presence of an isotypic control antibody or anti–TNF-α or anti–IL-10 antibodies. *\( P < .01 \), vs. isotypic control cultures.
in a significant increase of apoptotic monocytes in both PPD-positive control subjects (80% ± 5%) and patients with TB (65% ± 5%) (P < .01). With regard to necrosis (figure 4B), stimulation with PPD in the presence of an isotypic control antibody showed 3% ± 1.5% and 30% ± 2.0% (P < .001) necrotic monocytes from PPD-positive control subjects and from patients with TB, respectively. Blockade of TNF-α increased the number of necrotic monocytes to 30% ± 5% in cultures from PPD-positive control subjects and 42% ± 5% in cultures from patients with TB (P < .001). Blockade of IL-10 did not significantly change the percentage of necrotic monocytes in cultures from PPD-positive control subjects but reduced the number of necrotic macrophages (from 32% to 17% ± 5%; P < .003) in cultures from patients with TB, compared with the isotype control. Together, these results suggest that differential production of TNF-α and IL-10 may modulate necrosis and apoptosis in monocytes from patients with TB and from PPD-positive control subjects.

**DISCUSSION**

The results presented here clearly demonstrate that monocytes from patients with TB undergo apoptosis, as well as necrosis, after infection with virulent *M. tuberculosis* or exposure to PPD, whereas monocytes from PPD-positive control subjects undergo only apoptosis. Apoptosis was consistently demonstrated by quantification of hypoploidia, TUNEL, and disruption of mitochondrial PT, whereas necrosis was demonstrated by cell membrane damage and release of LDH into culture media. Previous reports from our laboratory, as well as from other laboratories, using murine and human macrophages have shown that infection with *M. tuberculosis* leads to apoptosis of infected cells [5–22]. There is also evidence that soluble mycobacterial products are able to modulate apoptosis. PPD induces apoptosis of murine macrophages [7, 10], and a sonicate from *Mycobacterium avium* [31], as well as the 19-kDa lipoprotein of *M. tuberculosis* [32–34], have been reported to induce apoptosis of human monocytes. In addition, there are reports indicating that apoptotic macrophages may be found in bronchoalveolar lavages and pulmonary granulomas from patients with TB [6, 20, 21], but little is known about the significance and the mechanisms of macrophage cell death in patients with active TB. Classic descriptions of the histology of tuberculous granuloma were performed before a clear distinction between necrosis and apoptosis, as different forms of cell death, was established. Thus, those descriptions refer to caseous necrosis at the tissue level, rather than at the individual cellular level. However, it was already accepted that the “burst” of bacilli-laden macrophages is involved in bacterial dissemination and tissue damage [35]. It is worth noting that one of the first descriptions of apoptosis in human monocytes infected with *Mycobacterium bovis* bacille Calmette-Guérin demonstrated that cells treated with ATP became apoptotic and showed a reduction in bacillary viability, but, after exposure to H₂O₂, monocytes underwent necrosis with no inhibition of intracellular mycobacterial replication [5].

The demonstration that a significant percentage of monocytes from patients with TB undergo necrosis after infection with virulent *M. tuberculosis* or exposure to PPD may be of great importance in understanding the pathogenesis of TB. Necrosis of infected macrophages may result in the release of bacteria, lysosomal enzymes, and other proinflammatory mediators, leading to bacterial dissemination, tissue damage, and active disease. In contrast, apoptosis of infected macrophages has been considered as a defense mechanism that creates a nonpermissive environment for bacterial replication with no release of intracellular enzymes into the surrounding milieu [36, 37]. Bystander macrophages are able to kill *M. tuberculosis* after ingestion of apoptotic cells containing bacilli, thus avoiding spread of mycobacteria [36]. In addition, TNF-α–induced apoptosis of *M. tuberculosis*–infected macrophages correlated with a reduction of mycobacterial viability, whereas complement-dependent cell lysis had no effect on bacterial viable counts [13]. In the Salmonella model, apoptosis, but not necrosis, of infected monocytes and macrophages limited growth of pathogens and retained bacilli within the apoptotic bodies [38].

Protective immunity against *M. tuberculosis*, as expected to occur in PPD-positive control subjects, is the result of innate and acquired defense mechanisms. Recent evidence indicates that apoptotic vesicles derived from *M. tuberculosis*–infected macrophages are ingested by dendritic cells, resulting in cross-priming by major histocompatibility complex I– and CD1b-restricted CD8⁺ T cells [26]. There is a growing acceptance that activated CD8⁺ T cells play an important role in anti-*M. tuberculosis* immune response, by secretion of IFN-γ and by their cytotoxic activity over infected macrophages [39]. Thus, apoptosis of infected macrophages may directly enhance protective immunity by limiting intracellular mycobacterial replication and, indirectly, by facilitating a strong CD8⁺ T cell response, as described elsewhere for PPD-positive individuals [40–43]. Thus, apoptosis of infected phagocytes may be regarded as a direct correlate of the protective immune response that occurs in ~90% of the infected individuals who never develop active TB during their lifetime.

Regulation of the mechanisms of phagocyte death by infection with *M. tuberculosis* is more complex than expected. Other authors have observed that apoptosis of human monocytes is preferentially induced by nonvirulent *M. tuberculosis* [14–18] and that, at low inoculum doses, *M. tuberculosis* suppress apoptosis [19]. Infected macrophages also showed a reduced susceptibility to FasL–induced apoptosis, correlating with a reduced level of Fas expression [13]. In addition, we have re-
ported that structural components from the mycobacterial cell wall or heat-inactivated bacteria inhibited cell death, indicating that the metabolic activity of *M. tuberculosis* is required to induce cell death. It is possible that, for induction of apoptosis, in addition to host factors (such as the TNF-α:IL-10 ratio), a balance between different mycobacterial metabolic products and structural components is required to modulate host cell death and survival. Thus, at low infecting doses, antiapoptotic structural components may be more relevant, whereas, at higher doses, the threshold for the activity of proapoptotic metabolic compounds is attained more readily.

Previous studies from our laboratory have shown that induction of apoptosis in murine macrophages depends on the TNF-α:IL-10 balance [9]. In the experiments described above, the role of TNF-α and IL-10 was further confirmed and extended to the human disease. First, monocytes from PPD-positive control subjects preferentially produced TNF-α, whereas cells from patients with TB produced more IL-10 than TNF-α, resulting in a higher TNF-α:IL-10 ratio in patients with TB that correlates with induction of apoptosis. Second, anti–TNF-α almost completely inhibited apoptosis, whereas it increased necrosis in both PPD-positive control subjects and patients with TB. In contrast, anti–IL-10 did not have such a dramatic effect but significantly increased apoptosis in patients with TB. Although TNF-α and IL-10 may be produced by different types of cells, such as T lymphocytes and NK cells, our results suggest that, in our system, monocytes are the main source of these cytokines. These results are in agreement with those of other authors, who showed that macrophages are the main producers of these cytokines in patients with TB [44] and leprosy [45]. Regulation of mycobacterial-exposed macrophage cell death by TNF-α and IL-10 also is consistent with the role of TNF-α in formation of granulomas and protection against mycobacterial infections [46], as well as with the deactivating and suppressive role of IL-10 [47, 48].

With regard to induction of necrosis, it must be noted that blockade of TNF-α dramatically increased the percentage of oncotic/necrotic cells, whereas anti–IL-10 significantly decreased this percentage in patients with TB. It may be speculated that, in the absence of apoptosis, which creates a nonpermissive environment for mycobacterial growth, the bacilli continue replicating, and the host cell eventually explodes as a result of membrane damage. However, the time period studied in our experiments (48 h) is not enough to attain such a number of intracellular bacilli; thus, there must be an active mechanism in the cells of patients with TB that makes infected phagocytes more prone to this type of cell death. We hypothesize that induction of necrosis might involve an increased calcium flux, which has been observed during mycobacterial infection [11]. Calcium fluxes can activate enzymes, such as lipases or proteases [49], other than caspases, able to digest the cytoplasmic membrane.

Elucidation of the fate of infected macrophages, the type of macrophage death that occurs during *M. tuberculosis* infection, and the biochemical and molecular mechanisms underlying such cell death may increase our understanding of fundamental mechanisms of protective anti-TB immune response and open new possibilities to modulate antimycobacterial and inflammatory responses in patients with active TB.

**Acknowledgments**

We thank Maggy E. Muñoz (Hospital la María, Medellín, Colombia), for her invaluable contribution to the recruitment of patients with tuberculosis, and Mauricio Arias, for critical reading of the manuscript.

**References**

15. Balcewicz-Sablinska MK, Keane J, Kornfeld H, Remold HG. Pathogenic *Mycobacterium tuberculosis* evades apoptosis of host macrophages by...
36. Poulet M, Gil et al.