Augmentation of Apoptosis and Interferon-γ Production at Sites of Active *Mycobacterium tuberculosis* Infection in Human Tuberculosis

C. S. Hirsch,[1] Z. Toossi,[2] J. L. Johnson,[1] H. Luzze,[4] L. Ntambi,[6] P. Peters,[1] M. McHugh,[1] A. Okwera,[4] M. Joloba,[5] P. Mugyenyi,[6] R. D. Mugerwa,[5] P. Terebuh,[1] and J. J. Ellner[3]

[1]*Case Western Reserve University, University Hospitals of Cleveland, and* [2]*Veterans Administration Medical Center, Cleveland, Ohio;* [3]*University of Medicine and Dentistry, Newark, New Jersey;* [4]*National Tuberculosis and Leprosy Programme, Ugandan Ministry of Health,* [5]*Makerere University, and* [6]*Joint Clinical Research Centre, Kampala, Uganda*

Pleural tuberculosis (TB) was employed as a model to study T cell apoptosis at sites of active *Mycobacterium tuberculosis* (MTB) infection in human immunodeficiency virus (HIV)–coinfected (HIV/TB) patients and patients infected with TB alone. Apoptosis in blood and in pleural fluid mononuclear cells and cytokine immunoreactivities in plasma and in pleural fluid were evaluated. T cells were expanded at the site of MTB infection, irrespective of HIV status. Apoptosis of CD4 and non-CD4 T cells in the pleural space occurred in both HIV/TB and TB. Interferon (IFN)–γ levels were increased in pleural fluid, compared with plasma. Spontaneous apoptosis correlated with specific loss of MTB-reactive, IFN-γ–producing pleural T cells. Immunoreactivities of molecules potentially involved in apoptosis, such as tumor necrosis factor–α, Fas-ligand, and Fas, were increased in pleural fluid, compared with plasma. These data suggest that continued exposure of immunoreactive cells to MTB at sites of infection may initiate a vicious cycle in which immune activation and loss of antigen-responsive T cells occur concomitantly, thus favoring persistence of MTB infection.

Despite advances in diagnosis and treatment, active tuberculosis (TB) remains a major cause of morbidity and mortality worldwide, especially in developing countries in sub-Saharan Africa and Asia, where infection with *Mycobacterium tuberculosis* (MTB) is endemic and infection with human immunodefieciency virus (HIV) compounds the natural history of TB. Recently, significant progress has been made in the understanding of the immunopathogenesis of TB, through examination of the interaction between MTB and peripheral blood mononuclear cells (PBMC) from patients with active pulmonary TB [1–8]. However, whether findings in PBMC from patients with TB provide a true picture of MTB-specific immune responses at sites of active MTB infection is mostly unknown. An in-depth evaluation of immunologic responses at sites of active MTB infection, such as the lung and pleural space, therefore, may be a prerequisite for the design of new therapeutic approaches for preventive and adjunctive anti-TB therapy (including vaccines and/or immunotherapy).

Initial studies assessing the phenotype and activation status of bronchoalveolar lavage cells obtained from patients with active pulmonary TB demonstrated that TB is characterized by a T lymphocytic and immature macrophage alveolitis [9]. Subsequent work indicates that there is compartmentalization of MTB-specific interferon (IFN)–γ–producing cells in the lungs of patients with active TB [10]. These results corroborate earlier findings, which showed that both IFN-γ and interleukin (IL)–2 are compartmentalized at the site of active MTB infection in TB pleuritis [11].

Studies of PBMC from patients with pulmonary TB indicate that excessive activation of mononuclear phagocytes and T cells may lead to loss of antigen-responsive and bystander T cells through apoptosis [12, 13]. Furthermore, rates of both spontaneous and MTB-induced apoptosis of CD4 and non-CD4 T cells in TB patients correlated inversely with depressed T cell reactivity, and T cell apoptosis declined after completion of anti-TB therapy [13]. On the other hand, Li et al. have shown that apoptosis results in selective depletion of $\nu\gamma 2/\delta 9$, but not $\alpha\beta$, T cells in the lung during active pulmonary TB [14]. Also, apoptosis of macrophages in the tuberculous granuloma [15] and of alveolar macrophages obtained from patients with culture-positive pulmonary TB [16] has been demonstrated. Overall, these data indicate that the microenvironment of an MTB-infected focus is conducive to apoptosis of mononuclear cells. Although this view is still controversial, apoptosis of infected

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Informed written consent was obtained from all study participants before their inclusion in this study. The study protocol conforms with human experimentation guidelines of the US Department of Health and Human Services and was approved by the Institutional Review Board at Case Western Reserve University, Cleveland, and the Ugandan National AIDS Research Subcommittee, Kampala, Uganda.

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Reprints or correspondence: Dr. Christina S. Hirsch, Dept. of Medicine, Division of Infectious Diseases, BRB 10W, 10900 Euclid Ave., Cleveland, OH 44106-4984 (cxh40@po.cwru.edu).

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macrophages has been implicated in containment of mycobacterial growth [15, 17–19]. However, little is known about the contribution of T cell apoptosis to disease-specific immune responses at sites of active MTB infection. In the current study, we employed pleural TB as a model to gain a better understanding of the role of T cell apoptosis in immune responses at sites of active MTB infection. For this purpose, we simultaneously evaluated apoptosis and cytokine production in PBMC and pleural fluid mononuclear cells (PFMNC) from patients with pleural TB.

Patients and Methods

Patients. Patients with newly diagnosed moderately sized and large pleural effusions strongly suspected to be due to TB were identified at the National Tuberculosis Treatment Centre, Mulago Hospital, Kampala, Uganda, between November 1997 and September 1999. In sub-Saharan Africa, pleural TB (with or without associated parenchymal pulmonary infiltrates) is diagnosed in ⩽10% of subjects presenting for evaluation of suspected TB, and ~60% of subjects with pleural TB are dually infected with HIV and MTB. After written informed consent for study participation was obtained, patients were evaluated by a history and physical examination; complete blood cell count; electrolyte, renal, and liver function tests; chest x-ray (if not done within 2 weeks prior to evaluation), and HIV testing. Patients aged 18–50 years with clinical signs and symptoms and chest x-ray findings [20] consistent with (pleural) TB were eligible for the study.

Exclusion criteria included a prior history of TB or TB treatment, suspected disseminated or meningeal TB, and a Karnofsky performance scale score <50%. Subjects undergoing therapy with corticosteroids or other major immunosuppressive drugs and patients with diabetes mellitus, cancer, or other major medical illnesses also were ineligible for participation. None of the study subjects dually infected with HIV and MTB were receiving antiretroviral therapy at the time of study. All patients received standard short-course anti-MTB therapy consisting of 2 months of daily isoniazid, rifampicin, ethambutol, and pyrazinamide, followed by 4 months of daily isoniazid and rifampicin. During anti-TB therapy, study subjects were followed up at monthly intervals.

Only patients in whom a definite diagnosis of pleural TB could be established, by a positive culture of either pleural fluid or pleural biopsy material for MTB or by documenting caseating granulomata on pleural biopsy specimens by histologic examination, were included in the final analysis.

Thoracocentesis and pleural biopsy. Thoracocentesis and closed pleural biopsy (Abrams needle) were performed, as described elsewhere [21]. In brief, following local anesthesia of the skin and subcutaneous tissues, ~300 mL of pleural fluid was aspirated under sterile conditions, using an 18G needle, which minimizes contamination of the pleural fluid with peripheral blood. Most (250 mL) of the pleural fluid was dispensed into 50-mL polystyrene tubes, each containing 1000 U of heparin. The remainder of the fluid was inoculated into 2 13A BACTEC radiometric culture vials (5 mL each; Becton Dickinson) for MTB culture and into a vacutainer tube containing citrate as preservative. After thoracocentesis was completed, closed pleural biopsy was performed, and 3 or 4 biopsy specimens were obtained from each subject and submitted for MTB culture and histologic examination.

Plasma and pleural fluid. Cell-free plasma and pleural fluid were stored frozen at −70°C until cytokines and molecules associated with apoptosis were assessed.

Preparation of cells. PBMC were prepared from whole heparinized blood by Ficoll-Paque (Pharmacia) density gradient centrifugation, as described elsewhere [13]. To obtain PFMNC, heparinized pleural fluid was first centrifuged for 10 min at 300 *g* to sediment the cellular constituents. Cell pellets then were resuspended in RPMI 1640 (BioWhittaker) and were sedimented over Ficoll-Paque gradients to remove contaminating red blood cells. PBMC and PFMNC were diluted (2×10^6 cells/mL) in RPMI 1640 supplemented with 10% fetal calf serum. Viability of both PBMC and PFMNC was >98%, as assessed by trypan blue exclusion.

Reagents. The laboratory strain of MTB, H37Ra, was grown to log-phase, and the number of mycobacteria contained in the suspension was determined by a colony-forming unit assay, as described elsewhere [13]. Avirulent mycobacteria were chosen as stimulus, because a biosafety level 3 facility is not available on site in Uganda. However, it is unlikely that the outcome of the current study would differ significantly had virulent rather than avirulent MTB been used as stimulus, because results of a study recently published [22] indicate that H37Ra and H37Rv are equally potent in inducing apoptosis in MTB-responsive γδ T cells. Lipopolysaccharide contamination of the MTB suspension was determined by the limulus amebocyte lysate assay (BioWhittaker) to be <0.01 ng/mL. Phytohemagglutinin (PHA) was obtained from Sigma.

Flow-cytometric assessment of surface markers on PBMC and PFMNC. Cellular composition of PBMC and PFMNC was assessed by 3-color flow cytometry. In brief, 2×10^5 cells were dispensed into multiple polypropylene tubes and were incubated with combinations of fluorochrome-conjugated antibodies (see below) or with isotype-matched control antibodies (all from Becton Dickinson) for 20 min at 4°C in the dark. Cells then were washed, fixed with 1% paraformaldehyde (Sigma), and stored at 4°C. Samples (10^4 cells per variable) were acquired by using a FACScan flow cytometer (Becton Dickinson) and were analyzed with WINMDI 2.8 software (Scripps Institute). To ensure consistency between results of cellular phenotypes of respective PBMC and PFMNC samples, analysis of cell surface markers was performed in 1 setting at the conclusion of the study, using the same electronic gates for all samples analyzed. Combinations of antibodies to CD3, CD4, and CD8 were used to identify T cell subsets. For identification of mononuclear phagocytes in preliminary experiments, PBMC and PFMNC were stained with CD3, CD4, and CD14. We compared the relative frequencies of $CD3^-$ cells that were $CD4^{+low}$ or $CD14^+$ and found that both markers were equally effective in identifying the relative proportions of monocytes and macrophages among freshly isolated PBMC and PFMNC (data not shown). Therefore, monocytes and macrophages were identified subsequently as cells of the $CD3^-/CD4^{+low}$ phenotype.

TUNEL assay to assess apoptosis. The TUNEL assay (terminal deoxynucleotidyl transferase [TdT]–mediated nick end labeling) was used for determination of apoptosis in PBMC and PFMNC, as described elsewhere [13]. In brief, mononuclear cells were stained with fluorochrome-conjugated antibodies to CD3 and CD4 (Becton Dickinson) and were then fixed, permeabilized, and

Table 1. Demographic, clinical, and microbiologic characteristics of patients with *Mycobacterium tuberculosis* infection (MTB) of the pleural space.

Characteristic	HIV/TB coinfection	TB alone
Total patients	16	8
Men/women	9/7	4/4
Mean (range) age, years	32.1 (18–54)	33.2 (18–46)
Mean (range) Karnofsky score	77.5 (70– 90)	80 (70–90)
Mean (range) hemoglobin, g/dL	10.7 (5.0–14.5)	12.1 (7.8–14.7)
Mean (range) CD4 count, μL^{-1}	173 (13–705)	620 (354– 898)
Pleural fluid culture MTB positive	14	2
Pleural fluid culture MTB negative[a]	2	6

NOTE. HIV, human immunodeficiency virus; TB; tuberculosis.
[a] Histology consistent with MTB infection.

incubated with BrdUTP (Sigma) in the presence or absence of TdT (Boehringer Mannheim Biochemicals). TdT facilitates the incorporation of deoxyuridine into exposed 3′-hydroxyl ends of nicked DNA, specific to apoptotic cells. Subsequent incubation with fluorescein isothiocyanate–conjugated antibody to BrdUTP (Becton Dickinson) allows identification of DNA breaks on a single-cell level. Specimens were acquired and were analyzed, as described above, for surface staining.

Immunoassays for cytokines, Fas, and Fas-ligand (FasL). IFN-γ immunoreactivity in biological fluids (plasma and pleural fluid) was assessed by a commercial ELISA (Endogen), which has a lower limit of detection of <15 pg/mL. The ELISA kits for tumor necrosis factor (TNF)–α and soluble Fas (sFas) were purchased from R&D Systems and were used according to the manufacturer's instructions. These assays are sensitive to <4.4 pg/mL of TNF-α and <20 pg/mL of sFas immunoreactivity, respectively. Soluble FasL (sFasL) in plasma and pleural fluid was assessed with an ELISA kit from MBL, which has a lower limit of detection of <80 pg/mL.

ELISASPOT assay to assess the relative frequencies of IFN-γ–producing T cells. To evaluate the relative frequencies of T cells producing IFN-γ, PBMC or PFMNC were seeded in serial 10-fold dilutions (200 μL/well in duplicate, and starting at 2×10^5 cells/well) into microtiter ELISASPOT plates (Polyfiltronics) previously coated with antibody to IFN-γ (Endogen). Cells either remained unstimulated or received MTB (4×10^5 cfu/mL) or PHA (10 μg/mL) and were incubated overnight at 37°C, with 5% CO_2. On the next morning, the cells were washed off, and the plates were incubated with a biotinylated capping antibody (Endogen) overnight. On the next day, the plates were washed extensively and were incubated with streptavidin–horseradish peroxidase (Dako). The plates then were developed with AEC substrate (Pierce), after a final washing step. The number of spots in each well, which corresponds to the frequency of antigen-responsive T cells, was counted with an ELISASPOT reader (available to us in the Department of Pathology at CWRU).

Statistics. Results were analyzed by Student's t test, paired t test, analysis of variance (ANOVA) (where indicated), and linear correlation and regression analysis. $P \leq .05$ was considered significant.

Results

Patient characteristics. We enrolled 30 subjects suspected to have pleural TB; 20 were HIV coinfected (HIV/TB), and 10 had TB alone. All study participants had newly diagnosed moderately sized or large unilateral pleural effusions. After initial evaluation, 4 HIV/TB and 2 TB enrollees were excluded from further analysis, because a diagnosis of pleural TB infection could not be established by routine microbiologic methods or histologic examination. Presenting symptoms were similar between the patients with TB and HIV/TB included in the final analysis (data not shown).

Selected clinical and laboratory data and microbiological profiles of TB and HIV/TB patients are shown in table 1. One fourth of HIV-uninfected patients with pleural TB had positive pleural fluid cultures for MTB, indicating ongoing infection. In the remaining subjects, a diagnosis of TB pleuritis was established on the basis of histologic examination of pleural biopsy material. By contrast, 14 of 16 of the HIV/TB patients had positive pleural fluid cultures for MTB, whereas the remaining 2 patients had histologic findings consistent with TB pleuritis on pleural biopsy (table 1). These data confirm previous findings in a cohort of HIV/TB and TB patients with pleural disease in Seville, Spain [23]. There was no correlation between absolute CD4 T cell counts in the peripheral blood and a positive MTB culture result in pleural fluid in either HIV/TB or TB patients (data not shown).

Cellular composition of PFMNC and PBMC from TB and HIV/TB patients with pleural TB. The relative cellular composition of PFMNC and PBMC from TB and HIV/TB patients was examined by FACS analysis. Results of cellular phenotyping of PBMC and PFMNC (expressed as number of electronic events per 10^4 cells acquired) are shown in table 2. As noted, there is compartmentalization of T cells in the pleural space of both patients with HIV/TB and those with TB alone. This finding likely is a result both of active recruitment and of local expansion of CD4 and CD8 T cells at sites of active MTB

Table 2. Cellular profile of peripheral blood mononuclear cells (PBMC) and pleural fluid mononuclear cells (PFMNC) in patients with human immunodeficiency virus/*Mycobacterium tuberculosis* coinfection (HIV/TB) and patients with TB alone.

	Cell phenotype			
Disease, cells	$CD3^+/CD4^+$	$CD3^+/CD8^+$	$CD3^+CD4^-/CD8^-$	$CD3^-/CD4^{+low}$
TB				
PBMC	1900 ± 361	1174 ± 239	186 ± 74	641 ± 85
PFMNC	4439 ± 467[a]	1796 ± 221	264 ± 84	412 ± 157
HIV/TB				
PBMC	559 ± 35	1637 ± 258	69 ± 27	1024 ± 198
PFMNC	1898 ± 291[b,c]	3389 ± 321[d,e]	90 ± 45	488 ± 120[f]

NOTE. Freshly isolated PBMC and PFMNC were stained with antibodies to CD3, CD4, and CD8, and the relative frequencies of T cell subsets and those of mononuclear phagocytes (per 10^4 events accrued) were assessed by flow cytometry.
[a] $P \leq .0001$, compared with CD4 T cells among PBMC.
[b] $P \leq .0001$, compared with CD4 T cells among PBMC.
[c] $P \leq .003$, compared with PFMNC from subjects with TB only.
[d] $P \leq .0001$, compared with CD8 T cells among PBMC.
[e] $P \leq .001$, compared with PFMNC from patients with TB alone.
[f] $P \leq .02$, compared with number of mononuclear phagocytes among PBMC.

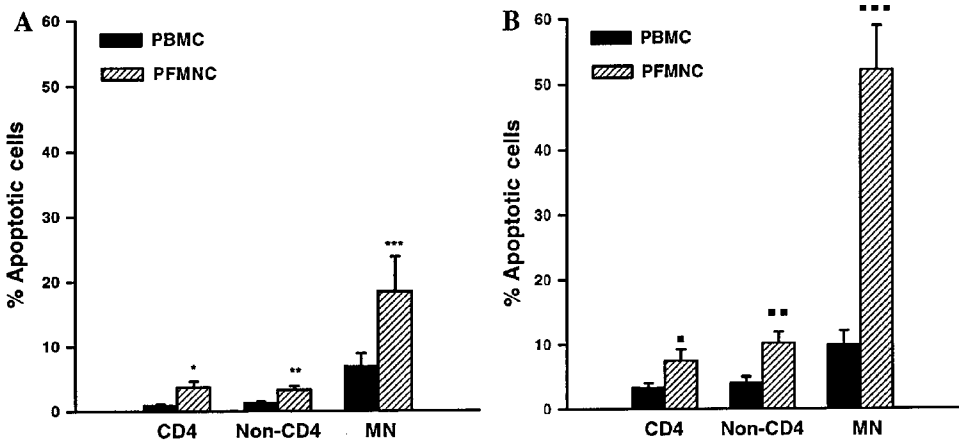

Figure 1. Apoptosis among pleural fluid mononuclear cell (PFMNC) and peripheral blood mononuclear cell (PBMC) T cell subsets and in mononuclear phagocytes (MN) from human immunodeficiency virus (HIV)–uninfected *(A)* and HIV–infected *(B)* patients with pleural *Mycobacterium tuberculosis* infection. Freshly isolated PBMC and PFMNC first were stained with antibodies to CD3 and CD4 and were processed according to the TUNEL (terminal deoxynucleotidyl transferase [TdT]–mediated nick end labeling) protocol, and then the relative proportions of apoptotic cells were assessed by flow cytometry. $*P \leq .009$, compared with the numbers of apoptotic CD4 T cells among PBMC. $**P \leq .007$, compared with the percentage of apoptotic non-CD4 T cell apoptosis among PBMC. $***P \leq .05$, compared with the relative proportion of apoptotic mononuclear phagocytes among PBMC. $■P \leq .03$, compared with the numbers of apoptotic CD4 T cells among PBMC. $■■P \leq .005$, compared with the percentage of apoptotic non-CD4 T cell apoptosis among PBMC. $■■■P \leq .001$, compared with the relative proportion of apoptotic mononuclear phagocytes among PBMC.

infection. However, the absolute numbers of CD4 T cells among PFMNC from HIV/TB patients still remained >2-fold lower than those found among PFMNC from patients with TB alone ($P \leq .003$, ANOVA). By contrast, frequencies of CD8 T cells among PFMNC from HIV/TB patients exceeded those among PFMNC from patients with TB alone ($P \leq .001$, ANOVA). Furthermore, the relative proportion of macrophages ($CD3^-$ $CD4^{+low}$) in PFMNC from both study groups was reduced when compared with that of monocytes among PBMC. However, differences in results were statistically significant only in subjects with HIV/TB ($P \leq .02$, *t* test; table 2).

Apoptosis at sites of active MTB infection. We have shown previously that freshly isolated circulating T cells (both CD4 and non-CD4) from patients with advanced active pulmonary TB undergo apoptosis at increased rates, compared with T cells from healthy control subjects, and that exposure to MTB induces apoptosis in T cells from TB patients, but not from healthy purified protein derivative–positive control subjects [13]. To examine whether T cells at sites of active MTB infection were differentially susceptible to apoptosis when compared with circulating T cells, we first examined apoptosis (using the TUNEL method) in freshly isolated PFMNC and PBMC from patients with pleural TB.

In both TB and HIV/TB, a greater proportion of freshly isolated PFMNC was apoptotic, when compared with the corresponding population in PBMC, and, in addition to CD4 and non-CD4 T cells, apoptosis also affected other cell types (macrophages and $CD3^-CD4^-$ cell populations). In TB patients, rates of spontaneous apoptosis were 4.1-fold higher among CD4 T cells ($P \leq .009$), 2.8-fold higher among non-CD4 T cells ($P \leq .007$), and 2.7-fold higher among mononuclear phagocytes ($P = .05$) in PFMNC than in PBMC (*t* test; figure 1*A*). In HIV/TB patients, apoptosis among PFMNC was 2.3-fold higher in CD4 T cells ($P \leq .03$), 2.6-fold higher in non-CD4 T cells ($P \leq .005$), and 5.3-fold higher in mononuclear phagocytes ($P \leq .001$) than in PBMC (*t* test; figure 1*B*). Thus, relative differences in spontaneous apoptosis of pleural fluid CD4 T cells (compared with that among PBMC) were higher in TB than in HIV/TB, whereas apoptotic cell death among non-CD4 T cells was similar. In contrast, apoptosis of pleural macrophages relative to blood monocytes was accentuated in HIV/TB, as compared with TB.

To examine whether bacterial load (as evidenced by positive or negative pleural fluid BACTEC culture for MTB) correlated with the extent of mononuclear cell apoptosis in TB and HIV/TB patients, we also stratified PFMNC apoptosis by pleural fluid culture status. Apoptosis in both CD4 (figure 2*A*) and non-CD4 (figure 2*B*) T cells was higher among PFMNC from TB and HIV/TB patients who had culture-positive pleural TB, compared with PFMNC from those with culture-negative pleural TB. In contrast, macrophage apoptosis predominated in culture-negative as compared with culture-positive pleural TB, regardless of HIV status (data not shown).

Expression of molecules associated with apoptosis. Both Fas/FasL and TNF-α/TNF-RII interactions have been implicated in mononuclear cell apoptosis [24–29]. Therefore, next

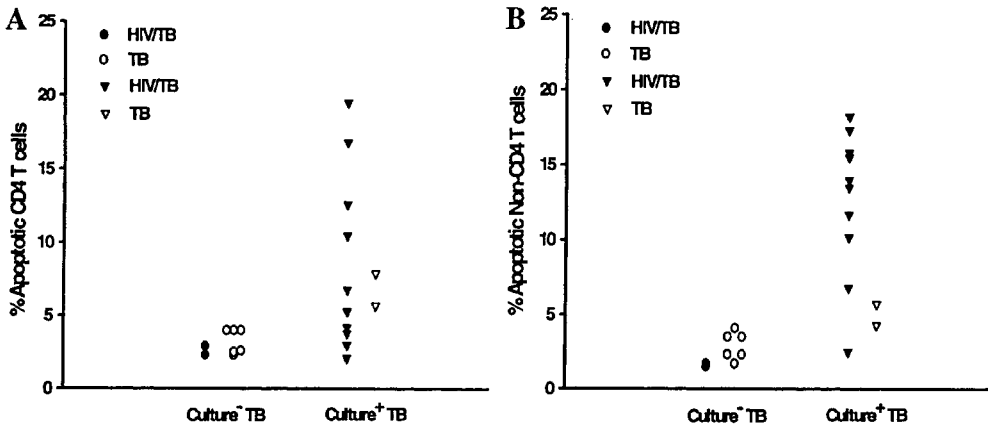

Figure 2. Correlation of apoptosis among CD4 and non-CD4 T cells from patients with human immunodeficiency virus (HIV)/*Mycobacterium tuberculosis* (MTB) coinfection (HIV/TB) and patients with TB alone, with MTB culture status in pleural TB. Apoptosis of CD4 *(A)* and non-CD4 *(B)* T cells from HIV/TB and TB patients, as assessed by the TUNEL (terminal deoxynucleotidyl transferase [TdT]–mediated nick end labeling) method, was correlated with pleural fluid culture status. The culture-negative (−) group included subjects in whom a diagnosis of pleural TB could be established only on the basis of histologic evaluation of pleural biopsy specimens. In contrast, all persons with culture-positive (+) pleural TB had positive pleural fluid cultures for MTB, by the BACTEC method.

we examined immunoreactivities of these molecules in pleural fluid and plasma obtained from patients with pleural TB and correlated them with apoptosis of PFMNC.

Levels of both sFas and sFasL were increased in pleural fluid, compared with plasma, of TB and HIV/TB patients. In patients with HIV/TB, sFasL immunoreactivity in pleural fluid was 7-fold ($P \leq .03$) and that of Fas 1.6-fold ($P \leq .04$) higher than those in plasma (*t* test and ANOVA; figure 3*B*). Also, there was a positive correlation between the extent of apoptosis in PFMNC (both CD4 and non-CD4 T cells) and amounts of sFasL present in pleural fluid in patients with HIV/TB (CD4 T cells, $r = 0.986$, $P = .0001$; non-CD4 T cells, $r = 0.990$, $P = .0001$; linear correlation and regression). A positive correlation between sFasL immunoreactivity in pleural fluid and apoptosis in PFMNC T cell subsets also was observed in patients with TB only (CD4 T cells, $r = 0.780$, $P = .05$; non-CD4 T cells, $r = 0.709$, $P = .05$; linear correlation and regression). However, this association between T cell apoptosis and levels of sFasL was less striking than that in patients with HIV/TB. These findings suggest that sFas and sFasL are compartmentalized to sites of active MTB infection and may be involved in apoptosis in vivo, particularly in patients with HIV/TB.

We also examined TNF-α immunoreactivities in pleural fluid and plasma. Levels of TNF-α were higher in pleural fluid than in plasma, in both TB and HIV/TB patients (TB, 9-fold higher, $P \leq .01$ [$n = 8$]; HIV/TB, 3.4-fold higher, $P \leq .001$ [$n = 12$]; *t* test). However, in contrast to the findings for sFasL, absolute levels of TNF-α in pleural fluid did not differ significantly between subjects with HIV/TB and those with TB alone (figure 3). The correlation of pleural fluid TNF-α with rates of spontaneous apoptosis of PFMNC (both CD4 and non-CD4 T cells) was similar in HIV/TB and TB patients (data not shown). Thus, the apoptosis-inducing capacity of TNF-α may be independent of HIV coinfection.

IFN-γ production at sites of active MTB infection. To evaluate how apoptosis at sites of active MTB infection correlated with IFN-γ production, which has been used as a marker of protective immunity [30–32], we next examined the immunoreactivity of this cytokine in pleural fluid and plasma. IFN-γ levels were increased in pleural fluid from both TB and HIV/TB patients, compared with plasma values (HIV/TB, 35.8-fold higher, $P \leq .001$ [$n = 12$]; *t* test; TB, 64.5-fold higher, $P \leq .04$ [$n = 8$]; *t* test). Surprisingly, however, the amounts of IFN-γ in pleural fluid from patients with HIV/TB exceeded those in pleural fluid of subjects with TB alone (mean ± SE, 1535 ± 351.4 vs. 484 ± 238.7 pg/mL; $P \leq .02$; ANOVA; data not shown), despite lower mean CD4 T cell counts. To investigate whether differences in amounts of IFN-γ in plasma as opposed to pleural fluid could be explained by differential expression of Th2 cytokines in the blood versus the pleural compartment, we also examined levels of IL-4 and IL-5. No IL-4 or IL-5 was detected in either plasma or pleural fluid (data not shown). Finally, we also determined whether levels of IFN-γ correlated with MTB culture results in HIV/TB and TB patients by examining IFN-γ immunoreactivities in pleural fluid of MTB culture–positive and –negative subjects. IFN-γ levels were higher in pleural fluid from HIV/TB and TB patients with culture-positive pleural TB, compared with all culture-negative subjects ($P \leq .002$, ANOVA; figure 4). However, we did not find a correlation between IFN-γ levels and T cell apoptosis.

Spontaneous and MTB-induced apoptosis after in vitro culture. Data presented thus far indicate that both in vivo apop-

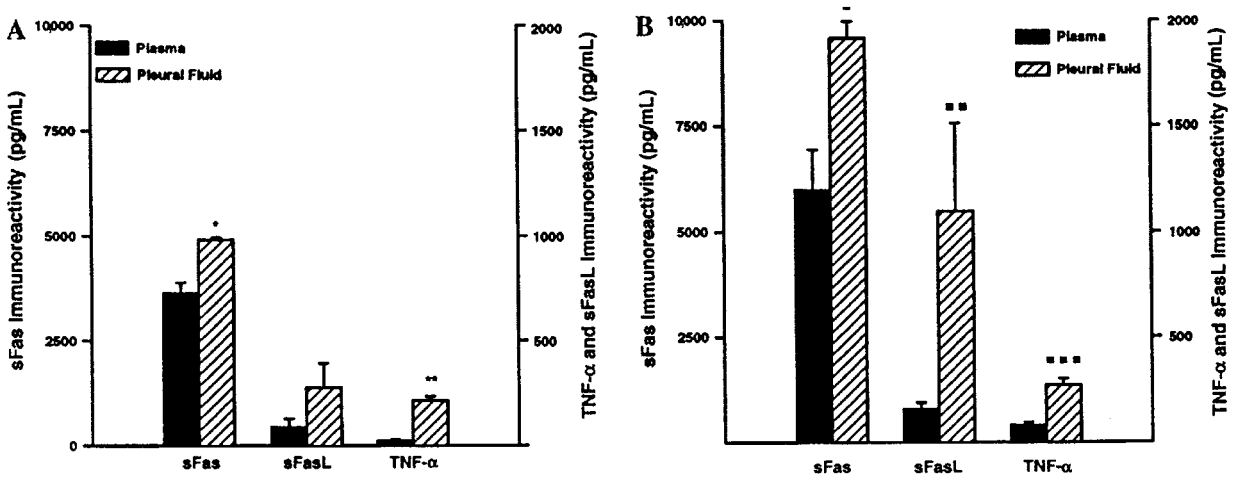

Figure 3. Immunoreactivities of soluble Fas (sFas), sFas-ligand (sFasL), and tumor necrosis factor (TNF)–α in pleural fluid and plasma from patients with pleural *Mycobacterium tuberculosis* infection alone (TB) *(A)* and patients coinfected with TB and human immunodeficiency virus (HIV/TB) *(B),* as assessed by ELISA. $*P \leq .001$, compared with sFas immunoreactivity in plasma. $**P \leq .01$, compared with TNF-α levels in plasma. $\blacksquare P \leq .04$, compared with sFas immunoreactivity in plasma. $\blacksquare\blacksquare P \leq .03$, compared with sFasL levels in plasma. $\blacksquare\blacksquare\blacksquare P \leq .001$, compared with TNF-α immunoreactivity in plasma.

tosis and production of IFN-γ by PFMNC are increased in patients with culture-positive pleural TB. To examine whether mononuclear cells from sites of active MTB infection could be stimulated to undergo further apoptosis, we next evaluated spontaneous and MTB-induced apoptosis in PFMNC from patients with culture-positive pleural TB ($n = 12$; HIV/TB, 10; TB, 2) after in vitro culture. The percentage of T cells (both CD4 and non-CD4 subsets) undergoing apoptosis spontaneously increased steadily throughout the culture period (figure 5). In fact, the numbers of apoptotic CD4 and non-CD4 T cells were 5.7- and 4.9-fold higher ($P \leq .003$ and $P \leq .006$, respectively; paired *t* test) at 72 h than at T0. Apoptosis also increased over time among T cells cocultured with MTB (figure 5). However, there were no significant differences between spontaneous and MTB-induced apoptosis among either CD4 or non-CD4 T cells at any of the time points studied (figure 5). This finding may relate to the fact that PFMNC are already maximally stimulated through exposure to MTB in situ, as evidenced by the high rate of apoptosis among freshly isolated (T0) PFMNC and the persistently high rates of spontaneous apoptosis after in vitro culture.

Functional significance of apoptosis. Data presented in the previous section indicate that rates of spontaneous apoptosis increase significantly during in vitro culture. However, how this phenomenon affects responsiveness of T cells to subsequent stimulation by antigens, or whether it has any functional consequences, is still unclear. Therefore, next we assessed whether the accentuated spontaneous apoptosis seen among PFMNC from patients with culture-positive pleural TB preferentially involved antigen-specific T cells. For this purpose, we performed ELISASPOT assays on both freshly isolated (T0) PFMNC and PFMNC precultured in the absence of MTB for 72 h (T72 cells) in a subset of patients (HIV/TB, $n = 4$; TB, $n = 1$). The relative frequencies of IFN-γ– producing cells among T0 and T72 PFMNC incubated in medium alone or after stimulation with MTB (4×10^5 cfu/mL) or PHA (10 μg/mL) were assessed. Selection of the 72 h time point was based on the finding that maximal spontaneous apoptosis was observed after 72 h of culture (figure 5). Total numbers of cells at T72 were 30%–40% lower than at the initiation of culture. To compensate for loss of cells during preincubation, equal numbers of viable T0 and T72 PFMNC (as assessed by trypan blue exclusion) were seeded into ELISASPOT plates at the onset of the assay. MTB-stimulated T0 PFMNC contained 2.9-fold greater numbers of IFN-γ –producing cells than did T72 PFMNC ($P \leq .02$, $n = 5$; *t* test; figure 6). In contrast, PHA stimulation of T0 and T72 PFMNC resulted in comparable frequencies of IFN-γ–producing cells (figure 6). Thus, the loss of IFN-γ–producing cells was limited specifically to MTB-responsive cells. Furthermore, when we compared the degree of spontaneous apoptosis of CD4 (but not CD8) T cells among T72 PFMNC (figure 1) and the relative frequency of MTB-stimulated IFN-γ – producing cells among T72 PFMNC, we found a negative correlation between these parameters ($r = -0.865$, $P = .05$; $n = 5$; linear correlation and regression analysis). Therefore, it appears that, at sites of active MTB infection, T cell apoptosis and loss of MTB-responsive T cells occur concurrently.

Discussion

Understanding the interaction between host immunoreactive cells and MTB at sites of active infection, such as the lung or

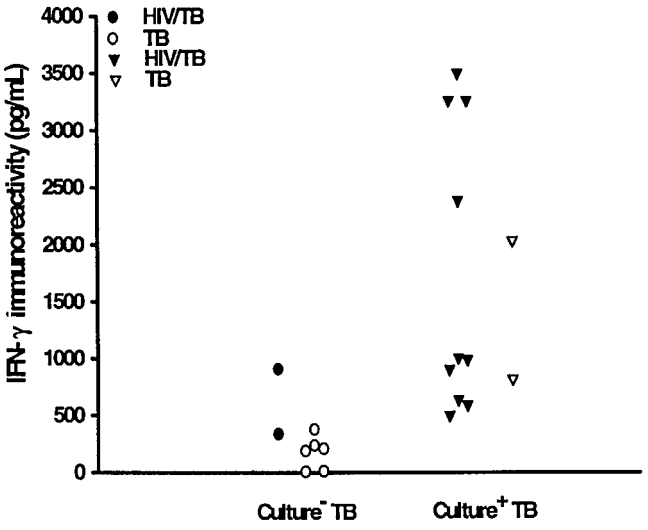

Figure 4. Interferon (IFN)–γ immunoreactivities (culture positive [+] or culture negative [−]) in pleural fluid from patients with human immunodeficiency virus/*Mycobacterium tuberculosis* coinfection (HIV/TB) and patients with TB alone (TB). Levels of IFN-γ were assessed by ELISA and were correlated with pleural fluid/biopsy culture status, as described above.

pleural spaces, is critical to the design of new strategies for prevention and treatment of TB. In the current study, we examined apoptosis and cytokine production in pleural TB. Our findings indicate that T cells are compartmentalized to sites of active MTB infection, regardless of HIV status. Notably, the "immunologic milieu" in pleural TB is characterized both by accentuated T cell apoptosis and by a vigorous Th1 T cell response. In addition, immunoreactivities of TNF-α, sFasL, and sFas were increased in pleural fluid, compared with plasma, from patients with pleural TB, and, therefore, these molecules may be involved in regulation of the local apoptotic process. Importantly, apoptosis was associated with loss of MTB-responsive T cells. Thus, during active MTB infection, in situ T cell apoptosis may contribute to the local immunopathogenesis of TB.

Apoptosis is a genetically determined process, whereby activation of an intrinsic cascade of cellular responses culminates in the death and efficient disposal of cells [24, 25]. Apoptotic cell death is tightly regulated both by intracellular and extracellular signals and by cytokines. Dysregulation of this physiologic process appears to be involved in the pathogenesis of several human diseases: lack of apoptosis plays a causative role in (hematologic) malignancies and autoimmune disease [24, 25], whereas excess apoptosis has been associated with infectious processes including HIV infection [27–29], malaria [33], and trypanosomiasis [34]. Recent evidence also suggests that, during active TB, apoptosis of CD4 and non-CD4 T cells may contribute to the T cell hyporesponsiveness observed in PBMC from patients with active pulmonary disease [13].

In the current study, apoptosis of PFMNC (T cells, mononuclear phagocytes, and others) was augmented at the site of active MTB infection in patients with pleural TB, regardless of HIV status. This finding may be indicative of the general immune activation inherent to TB, and excess apoptosis, therefore, may be primarily directed at regulating the exuberant inflammatory response during active MTB infection. Furthermore, in situ apoptosis was increased, particularly among pleural T cells from patients with HIV/TB, compared with those from patients with TB alone. This observation could be explained by the fact that, in HIV disease, mononuclear cells are

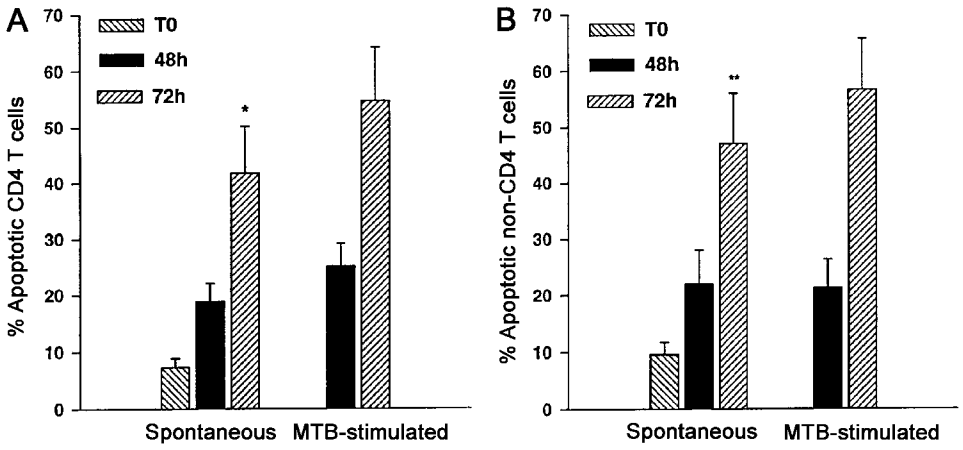

Figure 5. Spontaneous and *Mycobacterium tuberculosis* infection (MTB)–induced apoptosis after in vitro culture of pleural fluid mononuclear cells (PFMNC). PFMNC were incubated for ≤72 h in vitro in the absence (spontaneous apoptosis) or presence (MTB-stimulated apoptosis) of MTB. At the end of the respective culture periods, apoptosis among CD4 *(A)* and non-CD4 *(B)* T cells was assessed by the TUNEL (terminal deoxynucleotidyl transferase [TdT]–mediated nick end labeling) method. *$P \leq .003$, compared with the relative proportion of freshly isolated (T0) apoptotic CD4 T cells. **$P \leq .006$, compared with the numbers of apoptotic non-CD4 T cells at T0.

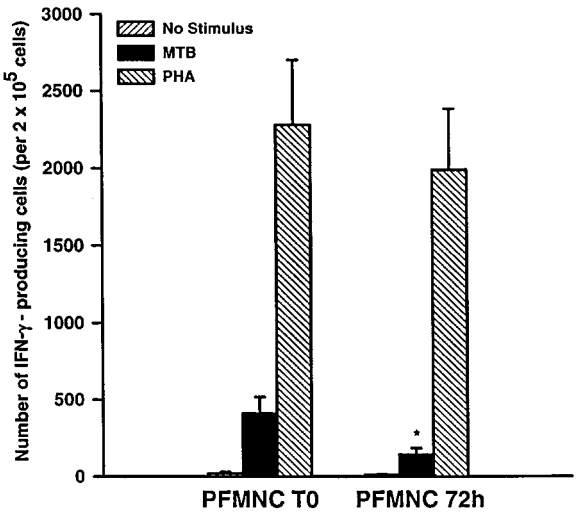

Figure 6. Functional significance of apoptosis. The relative frequencies of cells producting interferon (IFN)–γ were assessed by the ELISASPOT method in freshly isolated (T0) pleural fluid mononuclear cells (PFMNC) and in PFMNC that were preincubated for 72 h in vitro, in a subset of patients ($n = 5$) with pleural *Mycobacterium tuberculosis* infection (MTB). Phytohemagglutinin (PHA) stimulation resulted in similar frequencies of IFN-γ–producing cells. $^*P \leq .02$, compared with the relative frequency of IFN-γ–producing PFMNC at T0.

already primed for apoptosis through exposure to constituents of HIV. However, it is also possible that susceptibility to apoptosis may be further augmented by the presence of MTB, as HIV replication is enhanced at sites of MTB infection [35]. Nevertheless, the extent to which the underlying HIV disease contributes to apoptosis among PFMNC of HIV/TB patients needs to be further investigated.

Recent studies indicate that macrophage apoptosis is a feature of tuberculous granulomata and is seen (in addition to T cell apoptosis) in bronchoalveolar lavage cells from patients with active pulmonary TB [15, 16]. Furthermore, MTB-infected alveolar macrophages from healthy subjects have been shown to undergo apoptosis at increased rates [15, 18], a phenomenon that may be involved in limiting the intracellular replication of MTB. Therefore, it is possible that the increased number of apoptotic macrophages among PFMNC, compared with PBMC, seen in this study (figure 1) is indicative of activation of macrophages to contain MTB.

A number of recent studies indicate that molecules such as FasL and TNF-α on activated mononuclear cells may provide direct apoptotic signals on engagement of their receptors Fas and TNF-RI/II. Furthermore, down-regulation of expression of the anti-apoptotic protein Bcl-2 [36, 37] also may contribute to increased susceptibility to apoptotic cell death. Observations in the current study and those of others indicate that both sFasL and TNF-α may play a role in the accentuated apoptosis observed at sites of active MTB infection. In support of this notion, Li et al. [14] showed that increased apoptosis of $\gamma\delta$ T cells obtained by bronchoalveolar lavage from patients with active pulmonary TB involved the Fas/FasL pathway. Another study [22] also demonstrated that stimulation with MTB antigens induces expression of FasL on chronically stimulated $\gamma\delta$ T cells and on peripheral blood T cells from patients with active pulmonary TB. Furthermore, our own data indicate that coculture with MTB induces excess surface expression of Fas and FasL on PBMC from patients with newly diagnosed TB, compared with control subjects (C.S.H, unpublished observation), and that levels of both molecules and those of TNF-α are higher in culture supernatants of MTB-stimulated PFMNC than those of autologous PBMC (data not shown). Finally, in recent experiments ($n = 4$), we found that coculture with neutralizing antibodies to FasL and TNF-α (both alone or in combination) results in a reduction of T cell apoptosis among PFMNC by 40%–60% both in HIV/TB and in TB alone (C.S.H., unpublished observation). However, levels of sFasL and sFas were much higher in the pleural fluid from HIV/TB patients than in pleural fluid from patients with TB alone, whereas pleural fluid TNF-α immunoreactivities were comparable between HIV/TB and TB patients. As Fas/FasL interactions have been found to play a prominent role in apoptotic cell death during HIV infection [27–29], it is possible that the excess sFasL accounts for the increased apoptosis seen among T cells from these patients with HIV/TB, compared with that among T cells from persons with TB alone. In contrast, TNF-α may be a more global marker for MTB-induced T cell apoptosis.

Increased T cell apoptosis among PFMNC was associated with increased local production of IFN-γ in pleural fluid of patients with TB and HIV/TB. Furthermore, significantly higher levels of IFN-γ were seen in culture-positive than in culture-negative pleural TB patients, regardless of HIV status, and stimulation of PFMNC with MTB in vitro resulted in augmentation of IFN-γ immunoreactivities in culture supernatants from HIV/TB versus TB patients (data not shown). Most murine [30, 31] and human [32] studies to date indicate a critical role for IFN-γ in the capacity of the host to contain MTB, which, in general, is believed to be mediated through macrophage activation. However, the association between high levels of IFN-γ and excess apoptosis of pleural T cells in the current study may indicate that IFN-γ plays a dual role at sites of active MTB infection. It may be immunoprotective in the sense that it increases the ability of macrophages to contain MTB, but, on the other hand, it also may contribute to apoptosis in MTB-responsive T cells. The latter premise is supported by recent publications indicating a role for IFN-γ in up-regulating the transcription of a number of genes (including Fas, FasL, and Bak) involved in apoptosis [34, 38–41]. In addition, IFN-γ also induces TNF-α production.

In conclusion, data presented here indicate that both apoptosis and levels of IFN-γ are increased at sites of active MTB infection, both in HIV/TB and TB patients. Furthermore, both Fas/FasL and TNF-α/TNF-R pathways seem to be involved

in apoptotic cell death during pleural TB. Additional studies are required to establish the significance of T cell apoptosis and of the role of IFN-γ in anti-MTB immunity and/or to the pathogenesis of TB.

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