

Apoptosis and T Cell Hyporesponsiveness in Pulmonary Tuberculosis

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Mycobacterium tuberculosis (MTB)-induced T cell responses are depressed in peripheral blood mononuclear cells of persons with newly diagnosed pulmonary tuberculosis (TB), and levels of interferon (IFN)- γ remain low even after completion of antituberculous therapy. Loss of MTB-reactive T cells through apoptotic mechanisms could account for this prolonged T cell hyporesponsiveness. T cell apoptosis was studied in TB patients and healthy control subjects. Both spontaneous and MTB-induced apoptosis (in CD4 and non-CD4 T cells) from TB patients was increased when compared with healthy control subjects, whereas coculture with control antigen (candida) had no effect on T cell apoptosis in either group of study subjects. An inverse correlation existed between increased MTB-induced T cell apoptosis and IFN- γ and interleukin (IL)-2 immunoreactivities. Successful antituberculous chemotherapy resulted in a 50% reduction in both spontaneous and MTB-induced apoptosis, which coincided with 3- and 8-fold increases in levels of MTB-stimulated IL-2 and IFN- γ , respectively. These data indicate that apoptotic pathways are operant during active MTB infection and may contribute to deletion of MTB-reactive T cells and the immunopathogenesis of this disease.

T cell function in patients with newly diagnosed pulmonary tuberculosis (TB) is depressed as evidenced by depressed T cell proliferative responses to *Mycobacterium tuberculosis* (MTB) antigens and suppression of production of the cytokines interferon (IFN)- γ and interleukin (IL)-2 [1–7]. In parallel, production of transforming growth factor (TGF)- β , a cytokine associated both with T cell growth arrest and suppression of T cell function, is increased [8, 9]. Results of a recent longitudinal study in Kampala, Uganda, suggest that depression of MTB-induced T cell responses is not only immediate, at the time of TB diagnosis, but also protracted. Despite significant improvement, purified protein derivative (PPD)-induced IFN- γ production in peripheral blood mononuclear cells (PBMC) from patients with pulmonary TB did not recover fully to levels found in healthy tuberculin-reactive control subjects even 1 year after completion of successful combination chemotherapy (18 months of follow-up; C.S.H., unpublished data). These findings

indicate that mechanisms other than or in addition to cytokine-mediated suppression of T cell function, such as loss of antigen-responsive T cells by mechanisms including apoptosis, may be operant at the time of TB diagnosis and may persist for prolonged periods thereafter.

Programmed cell death, or apoptosis, is a process whereby developmental or environmental stimuli activate a genetically determined cascade of intrinsic cellular responses that culminate in cell death and result in the efficient disposal of a cell. The apoptotic process is tightly regulated by intra- and extracellular signals and by cytokines. Dysregulation of this process has been associated with disease states [10]. The lack of physiologic apoptosis appears to be involved in the pathogenesis of some hematologic malignancies and autoimmune diseases, such as systemic lupus erythematosus, whereas inappropriately high levels of spontaneous T cell apoptosis are encountered during malaria, trypanosomiasis, and viral infections, such as human immunodeficiency virus (HIV) disease [10–16]. Whether T cell apoptosis occurs during chronic mycobacterial infection, and how it relates to the immunopathogenesis of TB is not known. However, as PBMC and lung T cells from patients with TB express stigmata indicating in vivo “immune activation” [17, 18] and other data indicate that activated T cells are predisposed to apoptosis [19–22], it is plausible that increased susceptibility of MTB-responsive T cells to undergo apoptosis may contribute to the low T cell responses found in persons with active MTB infection. To resolve some of the questions posed above, we studied spontaneous and MTB-induced apoptosis and cytokine production in T cells from Ugandans with smear-positive pul-

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The study protocol was approved by institutional review boards at Case Western Reserve University and Makerere University. All subjects gave informed written consent for study participation and for human immunodeficiency virus testing and received pre- and posttesting counseling.

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monary TB (but not infected with HIV) and healthy PPD skin test-reactive control subjects.

Materials and Methods

Study subjects. We studied 20 patients with sputum smear-positive, culture-confirmed, newly diagnosed pulmonary TB (no prior TB chemotherapy) and 15 healthy tuberculin reactors. A diagnosis of TB was established by routine radiographic, clinical, and bacteriologic criteria at the Uganda-Case Western Reserve University (CWRU) Research Collaboration in Kampala, Uganda. By assessment using standardized radiographic criteria [23], 5 patients had minimal disease, 3 had moderately advanced, and 12 far advanced TB. We excluded from the study persons ≥ 50 years of age, those with concomitant debilitating diseases such as cancer or diabetes, individuals being treated with immunosuppressive drugs, and subjects with drug-resistant TB. All patients were treated with standard short-course antituberculous (anti-TB) chemotherapy (started on the day of enrollment) consisting of 2 months of self-administered daily isoniazid, rifampicin, ethambutol, and pyrazinamide (intensive phase of treatment), followed by 4 months of daily isoniazid and rifampicin (consolidation phase of treatment). Healthy tuberculin skin test-reactive (control) subjects ($n = 15$), age- and sex-matched to patients, were recruited from the laboratory and from New Mulago Hospital (Kampala) nursing staff. No patients or control subjects had serologic evidence of HIV infection (by EIA and Western blot [if the HIV EIA was inconclusive]). Ten patients with TB were restudied after completion of anti-TB treatment.

Antigens used. The avirulent laboratory strain of MTB (H37Ra) was grown in the laboratory and quantified in a colony forming-unit assay before use. 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; results of a recent study [24] indicate that H37Ra and H37Rv are equally potent in inducing apoptosis in MTB-responsive $\gamma\delta$ T cells. Candida antigen was purchased from Greer Laboratories (Lenore, NC). The lipopolysaccharide content of the MTB suspension and candida antigen, tested by limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD), was < 0.01 ng/mg protein for both.

Preparation of cells and cell culture conditions. After 30 mL of whole heparinized blood were collected from each patient and control subject, PBMC were obtained by sedimentation over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). To induce cytokines and to assess apoptosis following in vitro culture (as described below), PBMC (2×10^6 /mL) suspended in RPMI 1640 (Biowhittaker) containing 10% heat-inactivated fetal calf serum were cultured with or without MTB (H37Ra, infection ratio 10:1 MTB-to-target cell [anticipated number of blood monocytes]) or candida antigen (20 μ g/mL; Greer Laboratories) for ≤ 96 h. At the end of respective culture periods, culture supernatants were collected and stored frozen until use. PBMC were processed according to the TUNEL method to enumerate the proportion of apoptotic cells.

TUNEL assay for assessment of T cell apoptosis. We used the

TUNEL assay to evaluate spontaneous and MTB-induced apoptosis in select T cell subsets. To assure that TUNEL positivity represented apoptosis, in initial experiments we performed gel analysis for DNA fragmentation on DNA extracted from whole PBMC in parallel with the TUNEL method (data not shown). PBMC from patients with TB or healthy subjects were either processed immediately (T0) or cultured as described above. At T0 and at 48 and 96 h, PBMC were initially stained with fluorochrome-conjugated antibodies to CD3 (PerCP) and CD4 (PE; both from Becton Dickinson, San Jose, CA), fixed, permeabilized with 0.1% Triton X-100 (Sigma, St. Louis) in 0.1% citrate (Sigma), and then incubated with bromodeoxyuridinetriphosphate (BrdUTP; Sigma) in the presence or absence of terminal deoxynucleotidyl transferase (TdT; Boehringer, Indianapolis). TdT specifically facilitates the incorporation of deoxyuridine into the exposed 3'-hydroxyl ends of nicked DNA specific to apoptotic cells. Subsequent incubation with fluorescein isothiocyanate (FITC)-conjugated antibody to BrdUTP (Becton Dickinson) allows identification of DNA breaks. Specimens were assessed by flow cytometry (FACSScan; Becton Dickinson), and data were analyzed by the Lysis II software program (Becton Dickinson), which allows analysis of samples stained with 3 fluorochrome-conjugated antibodies simultaneously.

Assessment of cytokine immunoreactivity in culture supernatants. IFN- γ immunoreactivity was assessed by a commercial ELISA (Endogen, Boston), which has a lower limit of detection of 15 pg/mL. The ELISA for IL-2 uses a pair of antibodies (R&D Systems, Minneapolis). This assay is sensitive to 15 pg/mL of IL-2 activity. Tumor necrosis factor (TNF)- α immunoreactivity was assessed with a pair of monoclonal antibodies to TNF- α (PharMingen, San Diego). The lower level of sensitivity of this assay is 32 pg/mL of TNF- α activity. Soluble Fas (sFas) in culture supernatants was assessed by a commercial kit (Amersham/Calbiochem, Arlington Heights, IL).

Statistical analysis. Results were analyzed by Student's *t* test, paired *t* test, and linear correlation and regression analysis. $P \leq .05$ was considered significant.

Results

Spontaneous and MTB-induced T cell apoptosis is increased in PBMC of persons with newly diagnosed TB as compared with healthy control subjects. Previous evidence suggests that in PBMC from persons with newly diagnosed pulmonary TB both MTB-stimulated T cell proliferation and production of IL-2 and IFN- γ in vitro are depressed [5, 8]. To examine whether during active TB programmed cell death may contribute to low T cell responses, we first examined the rate of apoptosis in freshly isolated T cells and in T cells cultured for prolonged periods in the presence or absence of MTB. For this purpose, PBMC from healthy tuberculin-reactive subjects or from patients with TB were either processed immediately (T0) or cultured in the presence or absence of MTB for ≤ 96 h. At the end of respective culture periods, apoptosis was examined in individual T cells (CD4 and non-CD4) by the TUNEL method, which allows identification of DNA breaks, the hallmark of apoptosis.

At T0, an insignificant number of CD4 and non-CD4 T cells from control subjects ($n = 15$) were apoptotic. By contrast, in TB patients ($n = 20$) at T0, the proportion of apoptotic T cells was increased 10- (CD4 cells) and 15-fold (non-CD4-cells), respectively, compared with the proportion of apoptotic T cells at T0 in PBMC of control subjects ($P \leq .001$ for both T cell subsets; figure 1A, 1B). Spontaneous apoptosis in both CD4 and non-CD4 T cells from healthy subjects increased during culture: The proportion of apoptotic T cells (both CD4 and non-CD4) was increased 20-fold after 96 h of culture when compared with the proportion of apoptotic cells at T0 ($P \leq .001$, $n = 15$, for both; figure 1A, 1B). However, coculture with MTB for 48 or 96 h did not result in any further significant increases in the proportion of apoptotic cells in either CD4 or non-CD4 T cell subsets (figure 1C, 1D). By contrast, the percentage of T cells (both CD4 and non-CD4) undergoing apoptosis increased over time both in unstimulated and MTB-stimulated cultures of PBMC from persons with TB (figure 1). Stimulation of PBMC from TB patients with MTB led to a further 2-fold increase in the proportion of apoptotic CD4 and non-CD4 T cells compared with unstimulated T cells ($P \leq .001$; $n = 20$ for both).

Next, spontaneous and MTB-induced apoptosis of CD4 and non-CD4 T cells from patients with radiographically defined minimal disease ($n = 5$) was compared with that of subjects with radiographically defined moderately and far advanced TB ($n = 15$). Interestingly, the percentage of apoptotic T cells from patients with minimal TB at T0 was significantly lower than in PBMC of patients with advanced TB (CD4 cells: 1.1 ± 0.2 vs. 6.1 ± 0.7 ; non-CD4 cells: 1.1 ± 0.3 vs. 5.4 ± 0.67 ; $P \leq .03$ for both) and more closely resembled values found in healthy control subjects. In contrast, both CD4 and non-CD4 T cells from patients with minimal TB were as susceptible as T cells from persons with advanced TB to both spontaneous and MTB-induced apoptosis after *in vitro* culture (data not shown).

Antigen specificity of T cell apoptosis during active TB. Next we studied the antigenic specificity of this observation in a subset of 10 TB patients and 10 control subjects. Candida antigen was used as control antigen, as previous studies indicated that many Ugandans are sensitized to this antigen (J.L.J., unpublished data). Results presented here (figure 2) show a less striking difference in the percentage of spontaneous apoptosis between TB patients and control subjects, but confirm findings outlined above (figure 1) that coculture of PBMC with MTB leads to increased apoptosis among T cell subsets from TB patients only. In contrast, stimulation of PBMC from TB patients and control subjects with candida antigen failed to induce apoptosis of CD4 and non-CD4 cells beyond baseline levels, regardless of whether PBMC were cultured for 48 or 96 h (figure 2).

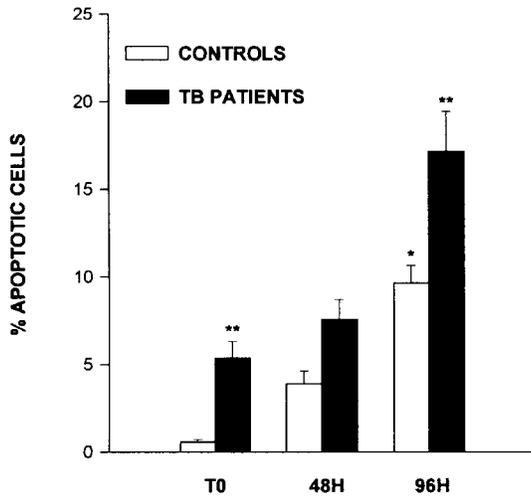
Relationship between T cell apoptosis and cytokine content in culture supernatants. To examine the relationship between

apoptosis and (depressed) T cell responses, cytokine immunoreactivities in culture supernatants from patients with TB and healthy control subjects were assessed. IL-2 (at 48 h of culture) and IFN- γ (at 96 h of culture) immunoreactivities were measured in culture supernatants of unstimulated and MTB or candida-stimulated PBMC from patients and control subjects. These time points were chosen on the basis of optimal induction of these cytokines in previous work. Insignificant IFN- γ and IL-2 immunoreactivities were observed in culture supernatants from unstimulated PBMC of patients and control subjects. MTB-induced IFN- γ immunoreactivity in PBMC culture supernatants from TB patients was 9-fold lower ($P \leq .001$) than IFN- γ concentrations in supernatants from control subjects (mean \pm SE: controls, 5.3 ± 1.1 ng/mL, $n = 15$; patients, 0.59 ± 0.16 ng/mL, $n = 20$; figure 3). Further, MTB-induced apoptosis in T cells from patients correlated inversely with MTB-induced production of IFN- γ ($r = -0.8$; $P \leq .0001$, $n = 20$). Also, by use of an IFN- γ ELISA spot assay, we found that the frequency of MTB-stimulated IFN- γ -producing cells in PBMC from patients with newly diagnosed TB was decreased compared with PBMC from healthy tuberculin-reactive control subjects (C.S.H., unpublished data). Candida-induced IFN- γ production also was 6-fold lower in PBMC of TB patients when compared with control subjects (controls, 0.33 ± 0.13 ng/mL; patients, 0.05 ± 0.03 ng/mL, $P = .06$, $n = 10$; data not shown).

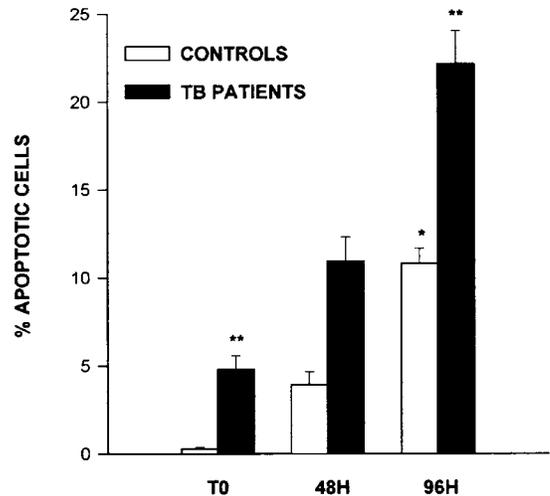
MTB-induced IL-2 immunoreactivity in PBMC culture supernatants from patients was reduced by 60% and candida antigen-induced IL-2 production was reduced by 80% compared with findings in culture supernatants of healthy control subjects (MTB: $P \leq .001$, $n = 10$; figure 3; candida: $P \leq .02$, $n = 10$; data not shown). In contrast, levels of sFas (CD95) and TNF- α , molecules implicated in T cell apoptosis, were increased 2-fold in MTB-stimulated culture supernatants from patients collected at 96 and 48 h, respectively, compared with immunoreactivities in supernatants from control subjects (table 1). Coculture with candida antigen, however, induced only insignificant amounts of sFas and TNF- α in PBMC of both TB patients and control subjects (data not shown).

Alterations in T cell apoptosis and in production of immunoreactive cytokines after successful chemotherapy for MTB infection. The above observations show that T cells from patients with newly diagnosed TB undergo apoptosis at increased rates both spontaneously and after stimulation with MTB. Further, MTB-induced T cell apoptosis is associated with a low frequency of IFN- γ -producing cells and low levels of IFN- γ and IL-2 in culture supernatants. Also, there is a correlation between radiographic severity of disease and degree of apoptosis. Therefore, we next examined the effect of successful anti-TB therapy on spontaneous and MTB-induced apoptosis. Ten patients with far advanced TB, who were first studied before initiation of treatment, were recalled after successfully completing short-course chemotherapy (6 months of follow-up). At reevaluation, all had improved clinically, had negative sputum

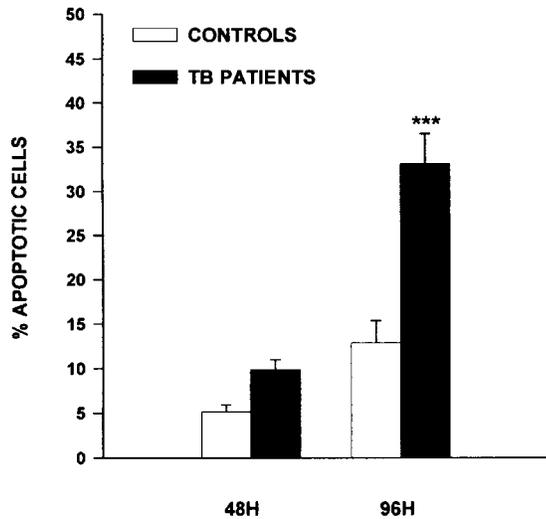
A Spontaneous Apoptosis, CD4 T cells



B Spontaneous Apoptosis, non-CD4 T cells



C MTB-induced Apoptosis, CD4 cells



D MTB-induced Apoptosis, non-CD4 cells

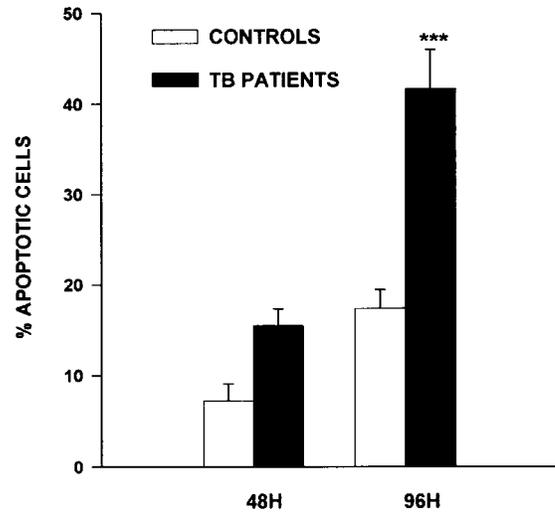
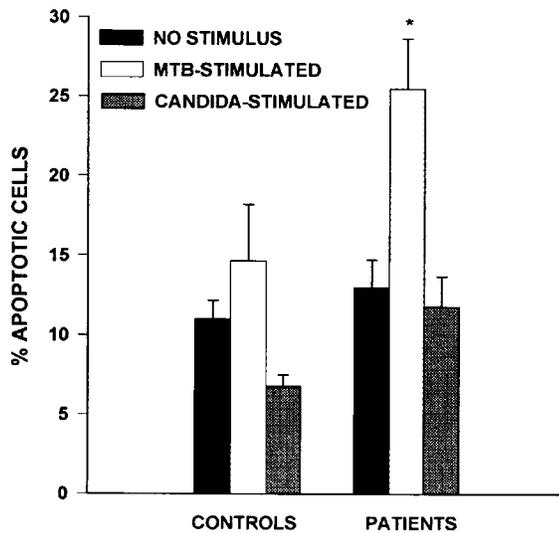


Figure 1. Spontaneous and *Mycobacterium tuberculosis* (MTB)-induced apoptosis in CD4 and non-CD4 T cells of TB patients ($n = 20$) and healthy controls ($n = 15$). Peripheral blood mononuclear cells (PBMC) from TB patients and controls were processed immediately (T0) or incubated ≤ 96 h in presence or absence of MTB. Proportion of apoptotic cells was assessed by 3-color flow cytometry (TUNEL method). Spontaneous apoptosis in CD4 (A) and non-CD4 (B) cells both at T0 and after in vitro culture for ≤ 96 h was increased in T cells from TB patients compared with controls. MTB-stimulated T cells from TB patients contained 2-fold (CD4 cells, C) and 3-fold (non-CD4 cells, D) more apoptotic cells than cells of controls. Bars show mean \pm SE apoptotic cell %. * $P \leq .0001$ ($n = 15$) for spontaneous apoptosis among T cell subsets (CD4 and non-CD4) of controls at baseline (T0) and after 96 h of culture in absence of MTB. ** $P \leq .0001$, spontaneous apoptosis in freshly isolated (T0) and cultured (96 h, no MTB) T cells (CD4 and non-CD4 subsets) of TB patients and controls. *** $P \leq .002$, MTB-induced apoptosis (after 96 h of culture) in CD4 and non-CD4 T cells from TB patients and controls.

A Apoptosis, CD4 T cells



B Apoptosis, non-CD4 T cells

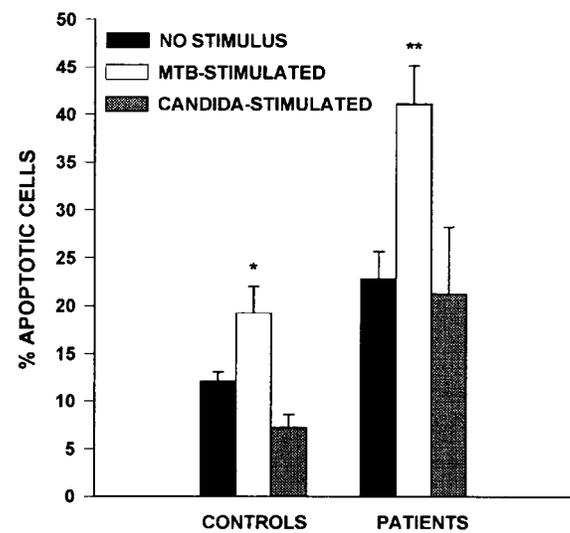


Figure 2. *Mycobacterium tuberculosis* (MTB)- and candida antigen-stimulated apoptosis in T cells of TB patients and healthy controls. Peripheral blood mononuclear cells (PBMC) from TB patients ($n = 10$) and controls ($n = 10$) were cultured ≤ 96 h in presence or absence of MTB or candida antigen. Stimulation with MTB was associated with increased apoptotic CD4 (A) and non-CD4 T cells (B) in PBMC from patients but not from controls. % of apoptotic CD4 and non-CD4 T cells from patients or controls cultured with candida antigen did not differ significantly from background levels (A, B). Bars show mean \pm SE of % of apoptotic cells. * $P \leq .002$ vs. apoptosis in CD4 or non-CD4 cells cultured in presence of candida antigen. ** $P \leq .001$ vs. candida-stimulated apoptosis in non-CD4 cells from TB patients.

acid-fast smears and cultures for at least the preceding 2–3 months, and had improved radiographic findings.

Apoptosis in CD4 and non-CD4 T cells and IFN- γ , IL-2, TNF- α , and sFas immunoreactivities in culture supernatants were assessed as described. Compared with the baseline evaluation, both spontaneous and MTB-induced apoptosis in CD4 and non-CD4 T cells decreased by $\sim 50\%$ (figure 3) when patients were restudied at 6 months. However, despite a significant reduction compared with baseline values, the fraction of apoptotic T cells at T0 still differed significantly between patients ($n = 10$) restudied after completion of anti-TB therapy and PBMC of control subjects ($n = 15$) ($P \leq .001$). In contrast, significant differences no longer were seen between the proportion of apoptotic CD4 and non-CD4 cells from patients and controls cultured for 48 (data not shown) or 96 h in the absence or presence of MTB (figure 3). In parallel with reductions in MTB-induced apoptosis in PBMC from persons with TB, levels of sFas in culture supernatant declined, and TNF- α immunoreactivity returned to baseline (table 1). Conversely, MTB-stimulated production of IL-2 by PBMC from persons treated for 6 months increased to a level comparable to that produced by PBMC from healthy control subjects (figure 3). IFN- γ immunoreactivity in PBMC culture supernatants from TB patients in the presence of MTB also increased (10-fold; figure 3). However, in contrast to findings for IL-2 immunoreactivity, IFN- γ levels did not reach values comparable to those found

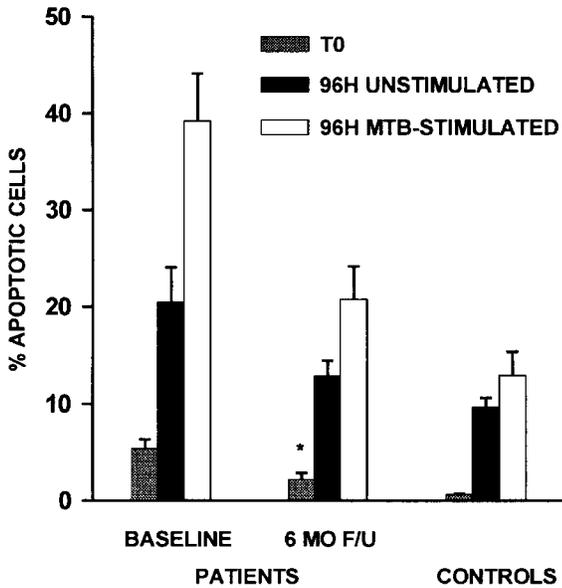
in supernatants from healthy control subjects. Differences, however, were no longer statistically significant (figure 3).

Discussion

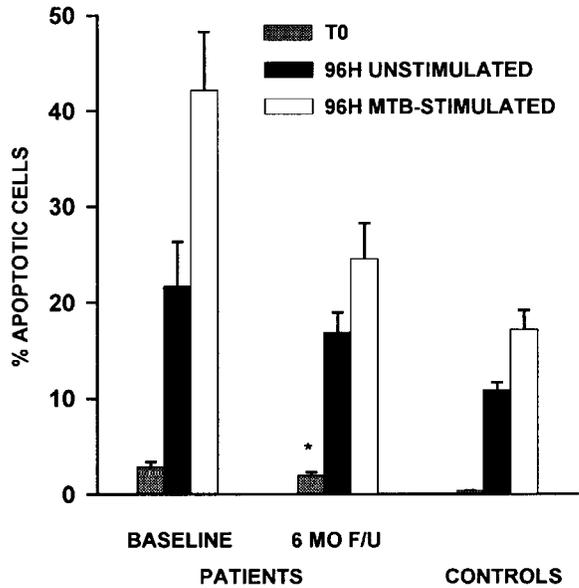
During active TB, exposure of T cells to MTB in situ at sites of active MTB replication may lead to T cell activation and prime MTB-responsive T cells to die through apoptotic mechanisms. This scenario provides a likely explanation for the increased numbers of apoptotic T cells seen among freshly isolated T cells from patients with TB compared with cells from healthy control subjects and their predisposition to apoptosis after in vitro culture as observed in this study. This notion is further substantiated by two recent studies that reported a direct relationship between depressed T cell proliferative responses and apoptotic cell death of $\gamma\delta$ T cells during active TB [25, 26]. However, while $\gamma\delta$ T cells clearly are one of the T cell subsets contributing to enhanced T cell apoptosis among the non-CD4 T cell population described in this study, they represent a very small proportion of circulating T cells. Therefore, it is likely that both CD8 and double-negative T cells, which reportedly play a role in anti-TB immunity [27, 28], also are affected. As a result, studies aimed at further characterizing non-CD4 T cell subsets particularly prone to MTB-induced apoptosis are currently in progress.

In contrast to T cell apoptosis, which may lead to T cell

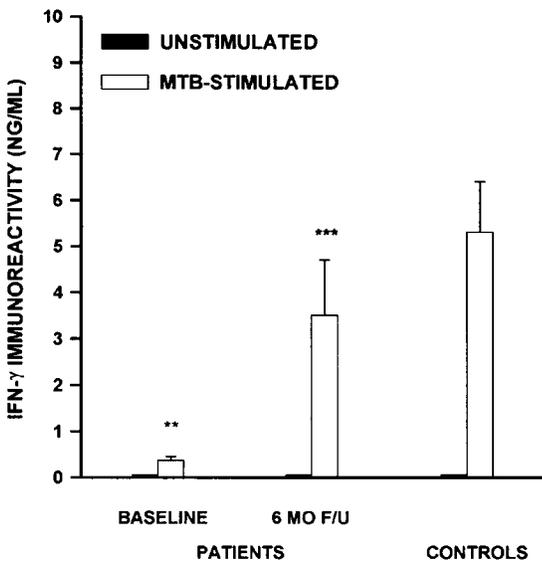
A Apoptosis, CD4 T cells



B Apoptosis, non-CD4 T cells



C IFN- γ



D IL-2

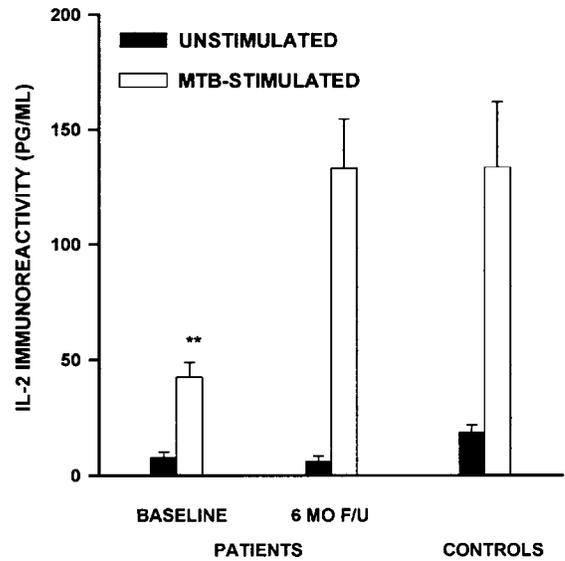


Figure 3. Effects of anti-TB chemotherapy on spontaneous and *Mycobacterium tuberculosis* (MTB)-induced apoptosis in CD4 and non-CD4 T cells from TB patients and on cytokine immunoreactivity in peripheral blood mononuclear cell (PBMC) culture supernatants. 10 patients recalled after successful completion of anti-TB chemotherapy. % of apoptotic CD4 (A) and non-CD4 (B) T cells and spontaneous and MTB-induced production of interferon (IFN)- γ (C) and interleukin (IL)-2 (D) at baseline and after 6 months follow-up were compared with spontaneous and MTB-induced apoptosis in T cell subsets and with cytokine immunoreactivity in PBMC culture supernatants of control subjects. Bars show mean \pm SE of apoptotic cell %, IFN- γ (ng/mL), and IL-2 (pg/mL) immunoreactivity. * $P \leq .001$ ($n = 10$) vs. spontaneous apoptosis in freshly isolated T cells (CD4 and non-CD4) from TB patients at baseline. ** $P \leq .0001$, baseline production of IFN- γ and IL-2 by PBMC from TB patients vs. cytokine immunoreactivities in culture supernatants from controls. *** $P \leq .02$, MTB-stimulated IFN- γ production by PBMC from TB patients at baseline and after 6 months of follow-up.

Table 1. Mycobacterium tuberculosis–induced tumor necrosis factor- α (TNF- α) and sFas production in peripheral blood mononuclear cell (PBMC) culture supernatants from TB patients at baseline and at 6 months follow-up compared with cytokine immunoreactivities in PBMC culture supernatants of healthy control subjects.

Parameter tested	Patients (<i>n</i> = 20)		Control subjects (<i>n</i> = 15)
	Baseline	6 months	
TNF- α (ng/mL)	14.1 \pm 1.5 ^a	5.3 \pm 0.3 ^b	7.3 \pm 1.3
sFas (EU/mL)	3.1 \pm 0.7 ^c	1.5 \pm 0.3 ^d	1.2 \pm 0.1

NOTE. PBMC from patients and controls were cultured for \leq 96 h. TNF- α levels were measured in supernatants collected at 48 h; concentrations of sFas were assessed in culture supernatants collected at 96 h. Results represent mean \pm SE of cytokine immunoreactivity in respective PBMC culture supernatants.

^a $P \leq .009$ vs. TNF- α levels in PBMC culture supernatants from controls.

^b $P \leq .0001$ vs. baseline cytokine immunoreactivity.

^c $P \leq .002$ vs. sFas immunoreactivity in PBMC supernatants from controls.

^d $P \leq .05$ vs. T0 determination of sFas.

hyporesponsiveness, MTB-induced apoptosis of mononuclear phagocytes may be an important mechanism in limiting the intracellular growth of this organism [29–31]. In fact, a recent study by Keane et al. [30] suggests that the caseating granuloma, the hallmark of effective anti-MTB immunity *in vivo*, contains large numbers of apoptotic cells (macrophages). Therefore, induction of apoptotic cell death of MTB-infected (alveolar) macrophages may facilitate effective anti-MTB host effector function.

From results in the current study, it is not clear whether apoptosis only affects antigen-specific T cells or if it also leads to destruction of other (bystander) T cells. The fact that \geq 30% of both CD4 and non-CD4 cells from patients with TB in the current study were apoptotic after 96 h of culture in the presence of MTB suggests that apoptosis may involve both non-MTB-specific and MTB-specific T cells. Thus, T cells with specificity to respond to nondisease-specific antigens may be predisposed to apoptosis in a “bystander” manner. This may account for depressed responses to non-MTB antigens, such as candida. However, as increased apoptosis was seen in response to stimulation with MTB and not with candida antigen, it appears that at least induction of apoptosis in TB is disease-specific. Alternatively, despite the extreme sensitivity of the TUNEL assay in detection of apoptosis, lack of candida antigen-induced apoptosis in PBMC of patients may be due to low precursor frequencies of candida antigen-responsive T cells, thus underestimating the degree of apoptosis induced by this antigen.

To address some of the issues raised above, we recently studied both the rate of spontaneous apoptosis in tuberculin skin test–negative subjects (to evaluate the question of “nonspecific” background reactivity) and the effect of polyclonal stimulation with pokeweed mitogen (to assess whether T cells from patients with TB are globally more sensitive to induction of apoptosis). The results indicate that the percentage of T cells undergoing apoptosis spontaneously is higher in PPD-reactive healthy per-

sons and in patients with active pulmonary TB than in tuberculin skin test–negative subjects (C.S.H., unpublished data). As TB is associated with general immune activation [17], it is conceivable that the increased susceptibility of T cells from patients with active TB to apoptotic cell death is a consequence of activation *in vivo*, presumably affecting both bystander and antigen-specific T cell subsets. However, experiments using pokeweed mitogen as stimulus confirm that induction of apoptosis is disease-specific, as the percentage of apoptotic T cells in cultures from patients containing pokeweed mitogen does not differ significantly from the rate of apoptosis among PBMC cultured in media only (G.V., unpublished data).

Recent research has significantly improved our understanding of apoptotic cell death and identified key molecules that induce and modulate this process. Overproduction of FasL and TNF- α provide direct apoptotic signals for activated T cells through interactions with their receptors, Fas and TNF-RI/II [10, 32]. It is possible that as part of the general immune activation during TB, in addition to up-regulation of expression of surface molecules such as HLA-DR [17], there is an up-regulation in expression of Fas, FasL, and TNF-RI/II. In support of this notion, concentrations of sFas were increased in PBMC culture supernatants from patients with newly diagnosed TB in the present study and decreased after successful anti-TB chemotherapy. Further, in preliminary experiments, we found that FasL is expressed, albeit in small quantities, on the surface of freshly isolated T cells from patients with TB and that coculture of PBMC from patients with MTB *in vitro* further up-regulates the expression of FasL (C.S.H., unpublished data).

Manfredi et al. [24] recently reported that engagement of the $\gamma\delta$ T cell receptor by MTB antigens induces expression of FasL on chronically stimulated $\gamma\delta$ T cells. Results of additional experiments established that the ensuing apoptotic cell death in Fas-expressing bystander cells was triggered by Fas/FasL interactions [24]. Like FasL, TNF- α can induce apoptosis in activated murine T cells [33] and appears to utilize downstream mechanisms similar to FasL [34, 35]. In the present study, we found that TNF- α immunoreactivity was increased in PBMC culture supernatants of patients with newly diagnosed TB. Also, recent data indicate that levels of TNF-RII are increased in the plasma of patients with TB (C.S.H., unpublished data) [36]. Therefore, similar to FasL, TNF- α may play a role in the increased susceptibility of T cells from patients with TB to undergo apoptosis both spontaneously and upon restimulation with MTB *in vitro*. Experiments are ongoing in an attempt to identify the apoptosis mechanisms involved. Preliminary results from experiments utilizing coculture with neutralizing antibodies to both FasL and TNF- α indicate that both FasL and (cell associated) TNF- α are involved in T cell apoptosis during active TB (C.S.H., unpublished data).

Results of the current study provide preliminary evidence of a relationship between low T cell responses, as evidenced by

depressed production of IFN- γ and IL-2, and T cell apoptosis. The observation that both phenomena occur simultaneously may lead to the conclusion that apoptosis is directly involved in the immunopathogenesis of TB. However, previous studies also have established a role for immunosuppressive cytokines produced by macrophages (e.g., TGF- β and IL-10) in depressed T cell responses during human TB [8, 9, 37], and levels of TGF- β were increased in PBMC culture supernatants from patients with TB but not from healthy control subjects in the current study (data not shown). Whether the immunosuppressive properties of TGF- β during active TB are due at least in part to induction of T cell apoptosis is not known. However, since TGF- β induces apoptosis in B cells [38, 39] and causes growth arrest in activated T cells [40–42], this is a distinct possibility.

Thus, it is likely that during human TB both enhanced T cell apoptosis and T cell hyporesponsiveness occur as a consequence of a complex mechanism that involves excessive immune activation, production of deactivating cytokines, and inflammation. Further studies are needed to establish the relevance of apoptosis in the pathogenesis of TB and to identify the mediators and mechanisms involved.

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