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Mycobacterium tuberculosis Induces Apoptosis in γ/δ T Lymphocytes from Patients with Advanced Clinical Forms of Active Tuberculosis

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Antigens from inactivated *Mycobacterium tuberculosis* H37Ra induce activation in a subpopulation of gamma/delta (γ/δ) T lymphocytes in a manner that resembles that of superantigens from α/β T cells. After culture in vitro with H37Ra proteins, γ/δ T lymphocytes from patients with advanced clinical forms of active tuberculosis (ACF-TBC) display cytotoxic activity against homotypic target cells exposed to H37Ra. Cytotoxicity by γ/δ T lymphocytes from ACF-TBC patients occurs in a range similar to that observed in healthy subjects. Following activation, H37Ra-stimulated γ/δ T lymphocytes from healthy subjects did proliferate in the presence of exogenous recombinant human interleukin 2. However, under the same conditions, γ/δ T lymphocytes from ACF-TBC patients not only did not proliferate but died by apoptosis. These results suggest that in γ/δ T lymphocytes from patients with ACF-TBC, antigens from *M. tuberculosis* may induce cell activation that leads to apoptotic cell death.

Gamma/delta (γ/δ) T lymphocytes constitutively express a heterodimeric T-cell receptor (TcR) which contains rearranged γ and δ chains. This receptor is different from the heterodimeric α/β TcR expressed in the majority of T lymphocytes from human peripheral blood (11, 14).

The antigen specificity and function of γ/δ T lymphocytes have not been totally characterized. However, there is increasing evidence showing that γ/δ T lymphocytes are involved in immune responses to mycobacterial antigens. In the presence of antigen-presenting cells, antigens from *Mycobacterium tuberculosis* (H37Ra) stimulate γ/δ T lymphocyte proliferation (12) and promote both specific target-cell cytolytic activity and cytokine production (3, 8, 13, 25). This response to *M. tuberculosis* is restricted to γ/δ T lymphocytes expressing the Vγ9 and Vδ2 segments of the TcR, called δ_2 T lymphocytes (8, 18, 22, 23). The response induced by H37Ra in γ/δ T lymphocytes resembles the response induced by bacterial superantigens in α/β T lymphocytes (9). This finding suggests that H37Ra may contain a component(s) that acts as a superantigen(s) for γ/δ T lymphocytes (4, 24).

The clinical outcome of tuberculosis (TBC) may be a consequence of the immune response to infection. While in most asymptomatic, infected subjects an effective immune response to mycobacteria is observed, the development of miliary, extrapulmonary, and advanced pulmonary forms of TBC reflect immune incompetence (7). Since a role for γ/δ T lymphocytes in the clinical evolution of TBC has not been established, Barnes et al. have shown that the basal percentage of γ/δ T lymphocytes in the peripheral blood of patients with advanced pulmonary and extrapulmonary TBC is similar to that observed in healthy subjects. However, after in vitro exposure to H37Ra, the rate of increase in terms of γ/δ T lymphocytes in patients with protective immunity was significantly higher than that in patients with advanced clinical forms of TBC (ACF-TBC) (2).

The aim of this study was to evaluate the in vitro response of γ/δ T lymphocytes from ACF-TBC patients to H37Ra. Our results indicate that γ/δ T lymphocytes from ACF-TBC patients respond to *M. tuberculosis*; this is reflected by the cytolytic activity against homotypic target cells cultured with H37Ra antigens. However, in contrast to those observed in normal subjects, H37Ra-activated γ/δ T lymphocytes from ACF-TBC patients failed to proliferate; furthermore, these cells displayed the process of apoptosis. These results suggest that γ/δ T lymphocytes from patients with ACF-TBC may undergo apoptosis after activation by *M. tuberculosis*.

MATERIALS AND METHODS

Patient population. The study included 11 patients with miliary or advanced pulmonary TBC, diagnosed according to standard criteria (21). The control group consisted of 10 healthy volunteers, who were age matched and did not have a known history of TBC infection. No patient or healthy donor had a positive serologic test or risk factors for human immunodeficiency virus infection. The study was approved by the local ethics committee, and both patients and controls voluntarily agreed to participate in the study and signed a consent form.

Cell preparation. Venous blood was collected in heparin, and peripheral blood mononuclear cells (PBMC) were separated by centrifugation on a Ficoll-Hypaque discontinuous gradient (Rafer, s.l.). PBMC were cultured (37°C in 6% CO_2) at 10^6 cells/ml in complete medium composed of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, HEPES (25 mM), L-glutamine (2 mM), pyruvate (1 mM), streptomycin (0.4 μg/ml), penicillin (0.2 μg/ml), and amphotericin B (0.5 μg/ml; Bio-Wittaker, Walkersville, Md.).

Staining for phenotypic markers. Phenotypic analysis of cells was done by flow cytometry (FACScan; Becton Dickinson) with the following fluorescein isothiocyanate-conjugated monoclonal antibodies (MAbs): TCRδ1, δV2(a), and δV1(a), which respectively recognize pan-γ/δ TcR, δ_2 TcR, and δ_1 TcR (T Cell Diagnostics, Cambridge, United Kingdom) as well as Leu-4 (anti-CD3), Leu-2a (anti-CD8), and Leu-3a+b (anti-CD4) (Becton Dickinson). Background nonspecific fluorescence was determined with an isotype-matched murine antibody to an irrelevant antigen or with no antibody.

Proliferation assay. PBMC (10^5) were cultured in triplicate in 100 μl of complete medium in 96-well flat-bottom plates with 10 μg of sonicated, heat-killed *M. tuberculosis* (H37Ra) per ml, 1 μg of 2,5-diphenyl-1-3-4-oxaciazole (PPD) per ml, 1 μg of tetanus toxoid (TT) (Bacto; DIFCO Laboratories, Detroit, Mich.) per ml, or 100 IU of recombinant human interleukin 2 (rhIL-2) (British Biotechnology Products Ltd.). After 2 days, 100 IU of rhIL-2 was added to all

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samples, and under these conditions, cells were cultured for 10 days (37°C in 6% CO_2). Eighteen hours before the end of the culture period, 1 μCi of [^{3}H]thymidine (specific activity, 5 mCi/mmol; Amersham) was added. The cultures were harvested onto glass-fiber filters with an automated cell harvester; the counts per minute were determined in a liquid scintillation counter.

Preparation of effector cells. Freshly isolated PBMC were stimulated with H37Ra, PPD, or TT (see doses described above), and subsequently, monocytes/macrophages were removed by plastic adherence. We also used control samples without specific antigenic stimulation. Then, γ/δ TcR$^+$ cell populations were negatively selected by immunomagnetic depletion of B and α/β T lymphocytes. Briefly, this negative selection was done by incubating (37°C for 2 h) peripheral blood lymphocyte suspensions with murine MAb anti-CD19 (Immunotech, Marseille, France) and anti-α/β TcR (T Cell Diagnostic) in a first step and with magnetic beads coated with rabbit-anti-mouse immunoglobulin G (Dynal, Mountain View, Calif.) in a second step. A magnetic particle concentrator for microtubes of Eppendorf type (MPC-E; Dynal) was used to remove B and α/β T lymphocytes. The resulting cell population consistently contained more than 80% γ/δ TcR-expressing cells, as assessed by staining with the TCRδ1 MAb, and was used as the source of effector cells for cytotoxic assays.

Preparation of target cells. Monocytes/macrophages were separated by plastic adherence for 2 h at 37°C from freshly isolated PBMC. After 2 h of incubation with H37Ra, PPD, or TT (see doses described above), adherent cells were washed twice with phosphate-buffered saline (PBS) and used as target cells in the cytotoxic assays. We also used control samples without specific antigenic stimulation.

Cytotoxic assay. Target cells were incubated with 250 μCi of $Na^{51}CrO_4$ for 1 h and then washed three times in PBS. Effector cells ($5 \cdot 10^5$) were added to $5 \cdot 10^3$ ^{51}Cr-labeled target cells in 200 μl of complete medium in 96-well flat-bottom plates for 4 h at 37°C. Fresh complete medium or 1% Triton X-100 was also added to target cells to measure, respectively, spontaneous and total chromium release. One hundred microliters of supernatant was collected and counted in a gamma counter (LKB) after incubation for 4 h at 37°C. Percent specific lysis was calculated as [(sample counts per minute − spontaneous counts per minute)/(total counts per minute − spontaneous counts per minute)] × 100. The spontaneous release of ^{51}Cr was consistently lower than 10%.

Cell apoptosis. The degree of apoptosis was assessed by flow cytometry. Cells (10^5) were resuspended at room temperature in 200 μl of PBS supplemented with bovine serum albumin (0.1%), sodium azide (0.1%), and ethidium bromide (EBr; 200 ng/ml), and 20,000 cells were analyzed immediately with a FACScan (Becton Dickinson) with logarithmic amplification of fluorescence detection and linear amplification of forward scatter. Cell debris and clumps were excluded from analysis by single-cell gating in the forward and side light scatters. The simultaneous analysis of cell size and EBr permeability allowed the differentiation of reduced cell sizes according to stages of DNA fragmentation, stages demarcated by the following categories of cell appearance after EBr staining: (i) EBr-low, (ii) EBr-intermediate, and (iii) EBr-bright cells. Lysis II software (Becton-Dickinson) was used for data acquisition and analysis. Only EBr-bright cells were considered apoptotic cells (20).

Statistical analysis. Differences between means were analyzed by analysis of variance followed by the Duncan test. Results are presented as means ± standard errors.

RESULTS

Proportions of γ/δ T lymphocytes in peripheral blood. Cytofluorometric analysis of our two study groups' peripheral blood lymphocytes revealed a statistically significant increase in the percentage of ACF-TBC patients' γ/δ T lymphocytes (16.09% ± 1.52% versus 7.35% ± 0.41% in controls; $P < 0.01$). However, relative percentages of δ_1 and δ_2 T subsets over total γ/δ T lymphocytes were not statistically different between patients and controls. These cell percentages as well as their absolute numbers are reported in Table 1.

Specific target-cell lysis by H37Ra-stimulated γ/δ T lymphocytes. Soon after the identification of γ/δ T lymphocytes, it was shown that these cells express broad killer activity (12). The cytolytic potential of H37Ra-activated γ/δ T lymphocytes from ACF-TCB patients and controls was tested against homotypic major histocompatibility complex class II$^+$ monocytes incubated with H37Ra or other unrelated antigens. γ/δ T lymphocytes from both controls and ACF-TBC patients exhibited effective cytolytic activity on target cells coated with H37Ra (Fig. 1). In addition, both control and ACF-TBC-stimulated γ/δ T lymphocytes demonstrated a low cytotoxic capacity against uncoated or unrelated antigen-coated cells (Fig. 1).

TABLE 1. Percentages and absolute numbers of γ/δ T lymphocytes and their main subsets (δ_1 and δ_2) in peripheral blood samples from ACF-TBC patients and healthy controls

Patient[a]	%[b]			Absolute no.[c]		
	γ/δ	δ_1	δ_2	γ/δ	δ_1	δ_2
C1	7	28	70	149	42	104
C2	9	30	68	179	54	122
C3	9	30	67	235	70	157
C4	6	24	71	146	35	104
C5	7	31	64	130	40	83
C6	10	27	68	204	55	139
C7	7	29	66	149	43	98
C8	7	27	69	184	47	127
C9	5	28	63	120	34	76
C10	9	25	65	189	47	123
T1	23	20	67	600	120	360
T2	18	26	61	380	99	232
T3	18	38	53	356	135	189
T4	20	36	52	462	166	240
T5	16	36	51	400	144	204
T6	18	37	56	356	132	199
T7	12	28	63	292	82	184
T8	9	38	48	251	95	120
T9	16	33	56	382	126	214
T10	8	31	52	225	70	117
T11	14	34	58	364	124	211

[a] C, healthy control; T, ACF-TBC patient.
[b] Values for γ/δ T lymphocyte-positive cells are percentages of total CD3$^+$ cells. Values for δ_1- and δ_2-positive cells are percentages of total γ/δ T lymphocytes.
[c] Number of cells per milliliter.

The effect of H37Ra on rhIL-2-dependent γ/δ T lymphocyte proliferation. The proliferative capacity of γ/δ T lymphocytes cultured in the presence of exogenous rhIL-2 with H37Ra and other bacterial antigens was measured. As previously shown by others, in the presence of rhIL-2, H37Ra induced proliferation of γ/δ T lymphocytes in samples from healthy subjects (Fig. 2). In contrast, γ/δ T lymphocytes from ACF-TCB patients failed to proliferate in response to H37Ra; this was not due to a reduced basal number of γ/δ T lymphocytes, since before stimulation with H37Ra, the percentages of these cells in ACF-TBC patients were even higher than in controls. This poor proliferative response of γ/δ T lymphocytes from ACF-TBC patients to H37Ra contrasted with the normal proliferation observed after stimulation with TT or PPD, which was similar to that obtained in samples from healthy subjects (Fig. 2).

H37Ra stimulation leads to activation-driven cell death of γ/δ T lymphocytes from ACF-TBC patients. To explore the possibility that the previously exposed, missing proliferative response may have been due to the induction of a process of activation-driven cell death, nuclear chromatin of H37Ra-activated γ/δ T lymphocytes from ACF-TBC patients was studied. Apoptotic cells were studied by a method by which degradation of chromatin into internucleosomal fragments could be detected by reduced fluorescence of the DNA binding dye EBr. As shown in Fig. 3, after a 72-h culture in the presence of rhIL-2, H37Ra-stimulated cells (42% ± 9% of cells) from ACF-TBC patients showed a concomitant loss of chromatin material, a reduction in cell size, and an inability to exclude vital dye. These are characteristic changes associated with a pattern of apoptotic cells.

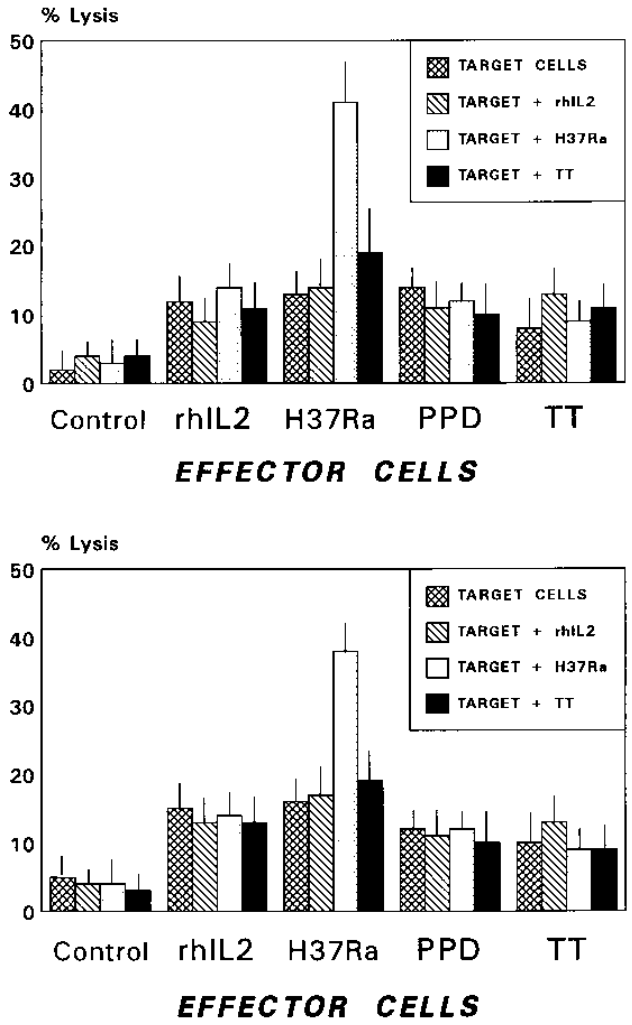

FIG. 1. H37Ra-dependent lysis of homotopic monocytes by γ/δ T lymphocytes. Freshly isolated PBMC were obtained from every healthy donor (top) or ACF-TBC patient (bottom). Then, effector γ/δ T lymphocytes and target homotypic monocytes were stimulated with different or no (control sample) bacterial antigens and prepared as we described in Materials and Methods. Target-cell lysis percentages were measured by ^{51}Cr release. The cytotoxic assay conditions are described in Materials and Methods as well. The result for every patient or control is the mean of an assay in triplicate. Results are means ± standard deviations.

DISCUSSION

Previous reports have shown that the mycobacterial antigenic pool H37Ra induces activation and proliferation in a human γ/δ T-lymphocyte population which expresses mainly the Vγ9 or Vδ2 phenotype (3, 8, 13, 25). Our results show an increased proportion of γ/δ T lymphocytes in the peripheral blood of patients with ACF-TBC, compared with levels of controls. However, the relative percentages of δ_1 and δ_2 T subsets found in ACF-TBC patients remained similar to those in healthy controls. These results are in accordance with a previous report (16) that demonstrated an increased proportion of peripheral blood γ/δ T lymphocytes in TBC patients and support a role for these cells in the immune response against *M. tuberculosis*. Furthermore, in this study we report that γ/δ T lymphocytes from patients with ACF-TBC were also activated by H37Ra, as their ability to lyse homotypic target cells cultured with H37Ra reflected. However, these H37Ra-activated cytolytic γ/δ T lymphocytes from ACF-TBC patients could not proliferate in the presence of rhIL-2, in contrast to their normal responses to PPD and TT. Actually, they died by apoptosis, suggesting that γ/δ T lymphocytes from patients with ACF-TBC exposed to *M. tuberculosis* antigens (H37Ra) undergo an activation that may lead to apoptotic cell death.

These results support previous findings reported by Barnes et al. (2) which indicate that H37Ra-activated γ/δ T lymphocytes from patients with protective immunity proliferate more than those from patients with ineffective immunity. These authors suggested that the lack of proliferation may be due to specific suppressor cells present in patients in TBC. However, we have observed that cells from tuberculous patients died by apoptosis when activated with H37Ra plus rhIL-2. This indicates that the missing proliferative response to H37Ra may be due mainly to apoptotic cell death.

It has been reported that chronic infection or reinfection with a superantigen-producing virus or bacterium may induce

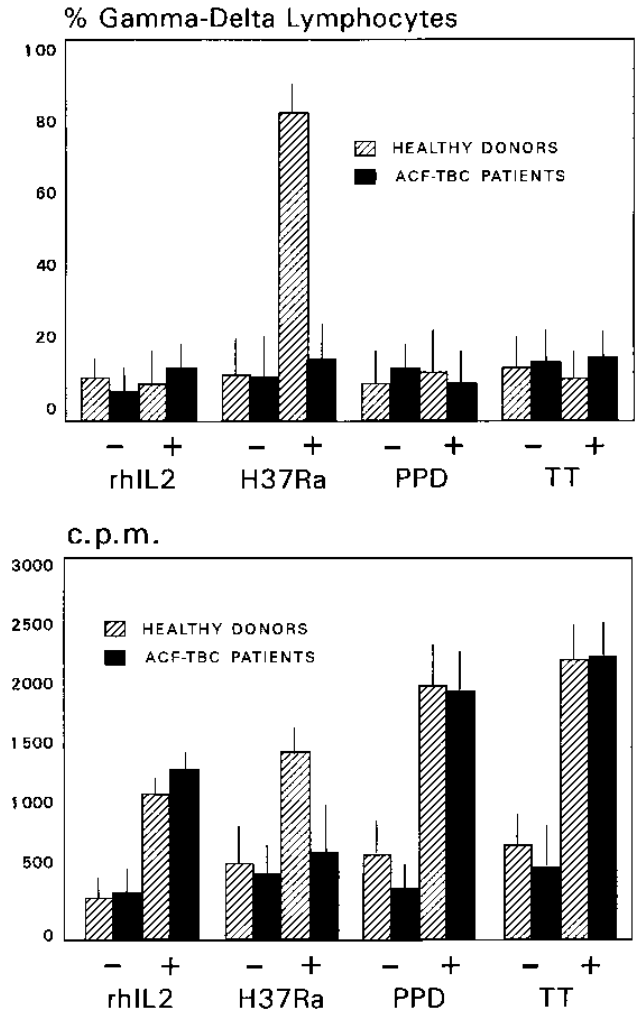

FIG. 2. Proliferation of γ/δ T lymphocytes cultured with bacterial antigens. PBMC (10^5) from every healthy donor or ACF-TBC patient were cultured in triplicate with different bacterial antigens plus rhIL-2. Control samples stimulated only with rhIL-2 were also incubated. After culture, a cytofluorometric analysis (top) as well as a proliferation test based on [^{3}H]thymidine incorporation (bottom) was performed on stimulated cells. The adopted conditions and elements for these techniques are described in Materials and Methods. Results are means ± standard deviations. The result for every patient or control is the mean of an assay in triplicate.

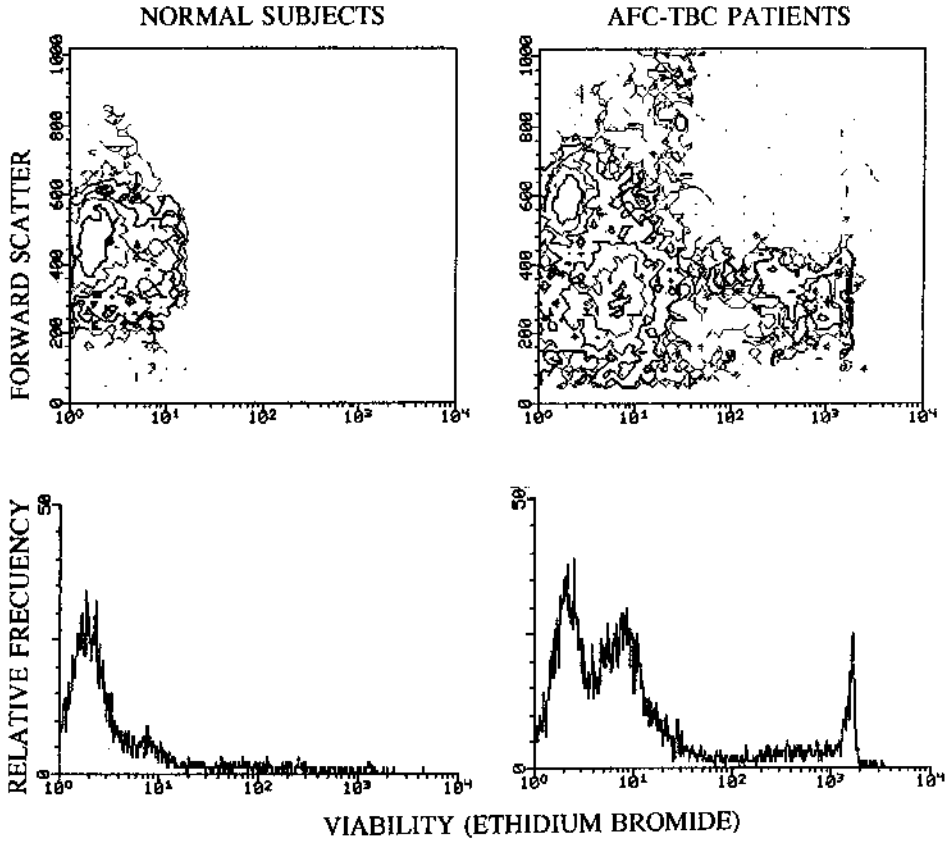

FIG. 3. H37Ra-induced apoptosis of γ/δ T lymphocytes from ACF-TBC patients. DNA fragmentation induced by H37Ra plus rhIL-2 was assessed with a FACScan flow cytometer. Programmed cell death was determined at day 3 of culture by cell cycle analysis. Aliquots of cells treated as mentioned above were fixed in ethanol followed by staining with EBr (see Materials and Methods). Data are representative of results from three independent experiments.

α/β T-lymphocyte apoptosis that leads to peripheral tolerance by cell anergy (5, 6, 15, 17). Our results are in accordance with results of previous studies suggesting that H37Ra may act as a superantigen for γ/δ T lymphocytes (4, 24). Like other superantigens, H37Ra may induce tolerance by anergy of reactive γ/δ T lymphocytes.

Apoptosis induced by mycobacterial antigens in γ/δ T lymphocytes from ACF-TBC patients and the increased proportion of γ/δ T lymphocytes in the peripheral blood of these patients represent an apparent paradox. However, there are some clues that help us to understand it. First, several findings support a role for γ/δ T lymphocytes in early local response, immune resistance, and the clinical outcome of TBC (2, 10, 12, 13, 19). Second, it is known that γ/δ T lymphocytes, and especially the mycobacterium-reactive δ_2 T subset, are not only present in peripheral blood but predominant in the pulmonary microenvironment, as well as in other epithelia (1, 14). Third, it is in this pulmonary epithelium that the potential progression of *M. tuberculosis* infection is controlled or not controlled (7). Fourth, if this potential progression of TBC is controlled in the lungs and if the function of mycobacterium-reactive γ/δ T lymphocytes develops mainly in this microenvironment, as their selective presence indicates, a slightly higher or lower proportion of circulating γ/δ T lymphocytes does not seem to be the point. Actually, the γ/δ T lymphocyte proportion in peripheral blood samples of our ACF-TBC patients is about the upper limit of normality in some studies (7, 12). In our opinion, this slight peripheral increase could even be simply reactive to local functional impairment.

In summary, our data support a role for γ/δ T lymphocytes in local resistance to mycobacterial infections. Impaired proliferation with programmed cell death of a large fraction of γ/δ T lymphocytes in response to mycobacterial proteins may help to explain some of the phenomena of local anergy in patients with ACF-TBC.

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