Fas-Fas Ligand (CD95-CD95L) and Cytotoxic T Lymphocyte Antigen–4 Engagement Mediate T Cell Unresponsiveness in Patients with Paracoccidioidomycosis

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The mechanism that leads to the remarkable T cell unresponsiveness to antigens in paracoccidioidomycosis is unknown. We investigated the involvement of cytokines, of Fas-Fas ligand (Fas-FasL)–induced apoptosis, and of cytotoxic T lymphocyte antigen 4 (CTLA-4) engagement, in the mediation of this phenomenon. T cell unresponsiveness was not associated with imbalanced cytokine production or with absence of CD28 expression. Only patient T cells expressed higher levels of CTLA-4, Annexin V+, and FasL. The addition of anti-FasL decreased the levels of apoptosis, suggesting an activation-induced cell death triggered through the Fas-FasL pathway. Blockage of CTLA-4 and FasL resulted in increased production of interferon-γ. Moreover, concomitant inhibition of FasL and of CTLA-4, but not of transforming growth factor–β, resulted in significant T cell proliferation in patients, in response to phytohemagglutinin. Together, these data show that apoptosis mediated by Fas-FasL and engagement of CTLA-4 are involved in modulation of the immune response in patients infected with Paracoccidioides brasiliensis.

Paracoccidioidomycosis, a chronic granulomatous disease caused by the thermomorphogenic fungus Paracoccidioides brasiliensis, is endemic to Latin America, with a high incidence also in Brazil, Colombia, Venezuela, and Argentina [1]. The disease is acquired by inhalation of the infectious form (conidia) that is present in the environment. Initially, the fungi affect the lungs, and, thereafter, they spread to multiple organs via the lymph nodes and bloodstream. There is a wide variety of clinical presentations of the disease, which can be classified into acute, subacute, and chronic forms [2].

Although the clinical heterogeneity of paracoccidioidomycosis has been correlated with the intensity of impairment of host immunity [3], patients with paracoccidioidomycosis usually show cellular immune hyporesponsiveness to several antigens, including fungal antigens [4]. The mechanisms involved in the modulation of this phenomenon are poorly defined but include low interleukin (IL)–2 production [5], low IL-2 receptor expression [6], and an imbalance in cytokine production [7]. Other possible causes include functional T cell inactivation, inactivation or dysfunction

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of other immune cell populations, and elimination of T cells as a consequence of activation-induced cell death (AICD). Of these, AICD, which is a Fas ligand (FasL)–dependent mechanism, is a possibility, because apoptosis of antigen-activated T cells is known to be an important mechanism for control of the T cell response, especially the immune response to parasite infection, as well as for maintenance of homeostasis [8, 9]. Indeed, AICD seems to be associated with the hyporesponsiveness seen after infection with many parasites, including Trypanosoma cruzi, Toxoplasma gondii, Schistosoma mansoni, and Plasmodium falciparum [10–13].

Besides AICD, T cell unresponsiveness might also be regulated through costimulatory receptors. Among these, cytotoxic T lymphocyte antigen 4 (CTLA-4) has been broadly implicated as a critical down-regulator of T cell activation [14]. This coinhibitory receptor is structurally similar to the well-characterized costimulatory molecule CD28, which provides the necessary secondary signal for effective T cell activation. CD28 and CTLA-4 engage the same ligands, B7-1 and B7-2, on antigen-presenting cells; however, unlike the binding of CD28, the binding of CTLA-4 to B7 results in inhibition of IL-2 production, of IL-2 receptor expression, and of T cell proliferation. Consistent with the inhibitory role of CTLA-4, mice deficient for CTLA-4 develop a severe lymphoproliferative disorder and die at 3–4 weeks of age [15, 16]. Because peripheral blood mononuclear cells (PBMCs) from patients with paracoccidioidomycosis proliferate poorly in response to P. brasiliensis antigens [4], produce low levels of IL-2 [5], and exhibit low IL-2 receptor expression [6], we hypothesized that this inhibition of T cell activation could involve CTLA-4. The mechanism that leads to the remarkable T cell unresponsiveness to antigens in paracoccidioidomycosis is unknown; therefore, we investigated the involvement of Fas/FasL–induced apoptosis and of CTLA-4 engagement in the mechanism that mediates the T cell hyporesponsiveness observed in patients with paracoccidioidomycosis.

PATIENTS, MATERIALS, AND METHODS

Patients with paracoccidioidomycosis and healthy control subjects. The study was conducted with PBMCs obtained from healthy control subjects and from patients with paracoccidioidomycosis. The control group consisted of 20 healthy persons (15 men and 5 women; age range, 19–50 years) with no history of pulmonary disease and with a negative result on paracoccidioidin skin testing. The group of patients consisted of 30 persons with active paracoccidioidomycosis, all of them exhibiting clinical and laboratory signs of the disease, such as lung infiltrates, polyadenopathy, mucosal or mucocutaneous lesions, and increased levels of specific antibody. The diagnosis was also confirmed by histopathologic examination or by isolation of P. brasiliensis in culture. The majority of patients were in the chronic stage of the disease. Males outnumbered females (25 vs. 5), and the age range was 15–60 years.

Antigens. For production of fungal antigens, the yeast cells were disrupted by ultrasonic treatment and were centrifuged for 10 min at 150 g. The supernatants were collected, and the total amount of proteins was determined. Fungal cell wall (CW) was obtained as described elsewhere [17]. In brief, yeast forms were sonicated, and lipids from the cell wall were removed by treatment with chloroform-methanol (2:1, vol/vol). Extracts were separated by centrifugation, were dried, and were weighed, and the insoluble cell residue (CW) was used as antigen source. Heat-killed yeast cells were prepared by heating the yeast cells in an autoclave for 20 min at 130°C.

PBMC isolation and culture. Peripheral blood was harvested, by use of heparin (50 U/mL), from healthy subjects and from patients with paracoccidioidomycosis. PBMCs were isolated by Ficoll-Paque (Pharmacia Biotech) density-gradient centrifugation, were washed, and were suspended at 5 × 10^6 cells/mL in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (all from GIBCO BRL). The cell suspensions were distributed (1 mL/well) in 24-well tissue-culture plates (Corning) and were cultured for 24–96 h at 37°C in a humidified 5% CO2 atmosphere, in the presence or absence of CW (30 μg/mL), P. brasiliensis antigen (10 μg/mL), heat-killed yeast forms of P. brasiliensis (1:5 PBMCs), phytohemagglutinin (PHA) (1 μg/mL) (GIBCO BRL), and lipopolysaccharide (LPS) (1 μg/mL) (Sigma). For FasL, CTLA-4, or TGF-β blocking assays, soluble anti-FasL (0.5 μg/mL), anti–CTLA-4 (10 μg/mL), anti–TGF-β (50 μg/mL), or isotype control (mouse IgG; 10 μg/mL) was added to cultures on the day of stimulation with antigen. These cultured and freshly isolated cells (ex vivo) were used to assay expression of Fas, FasL, CD28, and CD152, and the apoptotic index. The supernatants were collected to evaluate cytokine production.

T cell proliferation. PBMC suspension (2 × 10^5 cells) was distributed in 96-well U-bottom tissue-culture plates (Corning)
and was cultured for 120 h at 37°C in a humidified 5% CO₂ atmosphere, in the presence or absence of the following: fungal antigens, LPS or PHA plus soluble anti-FasL, anti-CTLA-4, anti–TGF-β, or isotype control. To address T cell proliferation, 0.5 μCi/well [³H]-thymidine (Amersham) was added during the final 16 h of culture. The cells were harvested, and radioactivity was measured in a liquid scintillation β counter (Beckman Instruments). Data were expressed as mean values of counts per minute of triplicate cultures, and the proliferation index was calculated as mean values of cell proliferation in the presence of antigen, divided by the mean values of cell proliferation in the absence of antigen.

**DNA-labeling technique.** The percentage of apoptotic cells in the samples was estimated by labeling cells by two different methods. The first was based on the use of 7-amino-actinomycin D (Calbiochem-Novabiochem), as described elsewhere [18]. The second method involved the use of FITC-labeled Annexin V, which was used according to the manufacturer’s specifications (Molecular Probes). Stained cells were analyzed by flow cytometry, by use of a FACScan flow cytometer with Cell Quest software (both from Becton Dickinson).

**Flow-cytometry analysis.** Cell surface expression of CTLA-4, CD28, FasL, and Fas, on ex vivo or on activated PBMCs, was assessed by flow cytometry, by use of a FACScan flow cytometer (given above). PBMCs from patients or from healthy control subjects were washed in cold PBS and were incubated in PBS plus 3% FCS with normal rabbit serum (20 μL) for 30 min at 4°C, followed by the addition of 0.5 μg of PE-labeled anti–CTLA-4, 0.5 μg of PE-labeled anti-CD28, 0.5 μg of FITC-labeled anti-Fas, or 0.5 μg of PE-labeled anti-FasL. The background staining was determined by incubation of the cells, in 0.5 μg of FITC- or PE-labeled control isotype antibodies, for 30 min at 4°C in the dark. The samples were then washed twice with PBS-FCS and were analyzed by flow cytometry.

**Figure 1.** Proliferative T cell response and expression of CD28 and CD152 by peripheral blood mononuclear cells (PBMCs) from patients with paracoccidioidomycosis. PBMCs were cultured with fungal antigen (PbAg) (10 μg/mL), cell wall (CW) (30 μg/mL), phytohemagglutinin (PHA) (1 μg/mL), or lipopolysaccharide (LPS) (1 μg/mL), and T cell proliferation (A) and CD28 (B) and CD152 (C) expression were determined. Results are medians of 15 control subjects and 20 patients with paracoccidioidomycosis, tested individually. Inset in panel C shows expression of CD152 in T cells, ex vivo, from patients and control subjects. CTLA-4 expression (P < .05) in ex vivo PBMCs from patients and in PBMCs from patients cultured with CW and PHA was significantly higher than in control subjects.
Figure 2. Cytokine production by cells from patients with paracoccidioidomycosis. Peripheral blood mononuclear cells from control subjects and patients were cultured with fungal antigen (PbAg), cell wall (CW), heat-killed fungus (HKF), lipopolysaccharide (LPS), or phytohemagglutinin (PHA), and concentrations of interferon-γ (IFN-γ) (A), interleukin-4 (IL-4) (B), IL-10 (C), and transforming growth factor–β (TGF-β) (D) were evaluated by ELISA. Each column represents mean ± SD of values of 8 control subjects and 12 patients with paracoccidioidomycosis. *P < .05.

Cytokine assays. Cell-culture supernatants were harvested after 48 h of incubation in fungal antigens or in control stimuli, and the cytokines that had been secreted were assayed by commercial ELISA kits. Interferon (IFN)–γ, IL-4, and IL-10 were measured by the ELISA set (Genzyme). For TGF-β1 assays, samples were acidified by addition of HCl (20 mM) for 15 min and were neutralized with NaOH, and then TGF-β1 content was measured by the TGF-β1 Emax Immunoassay Kit (Promega). Optical densities were measured at 450 nm, by use of a microplate ELISA reader.

Statistical analysis. Results are expressed as mean ± SD or as medians. Statistical analysis was done by ordinary analysis of variance, which was followed by the Tukey’s multiple comparison test (INSTAT software; GraphPad), as indicated in the figure legends. All values were considered to be significantly different at P < .05.

RESULTS

CD-28 and CTLA-4 expression, by PBMCs from patients with paracoccidioidomycosis. Previous studies have determined that cell proliferative responses and IL-2 production, by T cells from patients, are depressed in response to fungal antigen and PHA [4, 19]. In fact, our results indicate that PBMCs from patients were unable to proliferate in response to fungal lysate, CW, or heat-inactivated yeast forms. The patients also exhibited a significantly lower T cell proliferative response to PHA than did control subjects (figure 1A).

To investigate the possible participation of costimulatory molecules in T cell hyporesponsiveness, we evaluated the kinetics of expression of CD28 and CD152 in PBMCs obtained from healthy subjects and from patients with paracoccidioidomycosis. We found that CD28 expression in cells from patients and control subjects was similar and did not change significantly after culture (24–96 h), in either the presence or absence of fungal antigens. Figure 1B shows the levels of expression of CD28 in cells incubated for 96 h with P. brasiliensis antigens, LPS, and PHA. In contrast, the expression of CTLA-4 (figure 1C, inset) in ex vivo PBMCs from patients was significantly higher than that in PBMCs from control subjects. In addition, the expression of CTLA-4 in PBMCs from patients was higher than that in PBMCs from control subjects, when the cells were cultured with CW (P < .05) or with PHA (P < .001), but not when the cells were cultured with either P. brasiliensis antigen or LPS. The expression of CTLA-4 in cells from healthy control subjects was almost undetectable at all times in the experiment (figure 1C). These results indicate that CTLA-4 expression could be involved in the T cell unresponsiveness found in PBMCs from patients.

Cytokine production by PBMCs from patients with paracoccidioidomycosis. To evaluate whether the decreased T cell proliferation could be due to a disturbance in the balance of cytokine production, we assayed IFN-γ, IL-4, IL-10, and TGF-β production, in supernatants from PBMCs that were obtained from 12 patients and 8 healthy control subjects and were cultured with P. brasiliensis antigen, CW, LPS, or PHA. As shown in figure 2, PHA induced similar levels of IFN-γ production by PBMCs from control subjects and from patients. However, CW and LPS, both of which induced considerable production of IFN-γ in cells from control subjects, failed to increase IFN-γ production in cells from patients with paracoccidioidomycosis (figure 2A). The levels of IL-4 (figure 2B) and IL-10 (figure 2C) produced by cells cultured with the 3 different antigens,
with LPS, and with PHA were similar in both groups. CW induced a significant production of IL-4, whereas LPS and PHA induced vigorous production of IL-10, regardless of the clinical status of the donor of PBMCs. By contrast, the release of TGF-β was consistently higher in supernatants of PBMCs from patients with paracoccidioidomycosis than it was in those from healthy control subjects, especially when cells were cultured in the presence of either LPS or PHA (Figure 2D).

Spontaneous and activation-induced cell death in PBMCs from patients with paracoccidioidomycosis. Next we examined the occurrence of apoptosis in ex vivo PBMCs from control subjects and from patients with paracoccidioidomycosis, by labeling the cells with FITC–Annexin V. The percentage of Annexin V–labeled cells from patients with paracoccidioidomycosis was significantly higher (mean ± SD, 14.48% ± 3.14%) than it was in cells from control subjects (1.73% ± 0.32%) (Figure 3A). This increased number of Annexin V+ cells in PBMCs from patients with paracoccidioidomycosis is suggestive of AICD occurrence in vivo. To study the possibility of AICD occurrence in vivo, we cultured PBMCs from patients or from control subjects, with fungal antigens, LPS, and PHA, and the number of apoptotic cells was determined. We found that, when PBMCs were cultured with antigens derived from P. brasiliensis, but not with LPS, a significant increase in the number of apoptotic cells in PBMCs from patients, but not in PBMCs from control subjects, was observed (Figure 3B). It is of importance that, although PBMCs cultured in the presence of PHA produced significantly increased levels of apoptosis in cells from patients and from healthy control subjects, the number of apoptotic cells was higher in PBMCs from patients. This increased induction of apoptosis observed in the cells stimulated with antigens seems to be biologically relevant, and it provides a possible explanation for the inability of T cells to proliferate on stimulation by antigens.

Expression of FasL in PBMCs from patients with paracoccidioidomycosis. Because there is a large body of evidence showing that AICD of peripheral lymphocytes can be triggered by the interaction of the Fas molecule with its ligand [20, 21], we next investigated the possible involvement of Fas-FasL interactions in this mechanism. We found that, although the ex vivo cells from patients showed a higher density of Fas expression (Figure 4B), the percentage of Fas-expressing cells was similar in both groups (Figure 4C). When Fas expression was evaluated after stimulation by fungal antigens, PHA, or LPS, no significant differences were detected between the groups (data not shown). By contrast, the expression of FasL was significantly higher in ex vivo cells from patients (Figure 4D and 4F). Although a mean of <5% of ex vivo cells from normal healthy persons (n = 9) were CD95L-positive, >10% of T cells

Figure 3. Increased levels of apoptosis in peripheral blood mononuclear cells from patients with paracoccidioidomycosis. A, Annexin V assay and flow cytometry were used to detect apoptotic cells. Comparison of 1 representative control subject (left) and 1 patient with paracoccidioidomycosis (middle) is shown. Right, Median percentages of apoptotic cells found in ex vivo cells from healthy controls (open circles) and patients with paracoccidioidomycosis (solid circles) (n = 20) are also shown. B, Cell death was assessed after 48 h of culture with fungal antigen (PbAg), cell wall (CW), heat-killed fungus (HKF), lipopolysaccharide (LPS), or phytohemagglutinin (PHA). Levels of apoptosis in cells from patients were higher (P < .05) than those in cells cultured in absence of either antigen or LPS.
Figure 4. Expression of CD95L in peripheral blood mononuclear cells (PBMCs) from patients with paracoccidioidomycosis. Surface expression of CD95 (A–C) and CD95L (D–F) was analyzed by flow cytometry in ex vivo cells. Figure shows intensity of fluorescence in cells from 1 representative control subject (A and D) and 1 patient with paracoccidioidomycosis (B and E). Thin lines, binding of isotype control antibody; thick lines, binding of CD95 (A and B) or CD95L (D and E) to cells from healthy control subjects or patients. Percentages of CD95+ T cells (C) found in PBMCs from patients (solid circles) (n = 20) and control subjects (open circles) (n = 13) and percentages of CD95L+ T cells (F) in PBMCs from patients with paracoccidioidomycosis (solid circles) (n = 13) and from healthy control subjects (open circles) (n = 9) are shown. P < .05, vs. expression of CD95L in cells from patients vs. that from control subjects.

from patients with paracoccidioidomycosis (n = 13) were CD95L-positive (figure 4F). We next investigated whether Fas-FasL interactions contribute to the AICD seen in T cells from patients. Therefore, cells were stimulated with P. brasiliensis antigen and with PHA, with or without an antibody to block Fas-FasL interactions [21], and the frequency of apoptotic cells was determined. The results showed that blockage of Fas-FasL interaction resulted in a significant reduction in antigen-induced apoptosis, in cells from both patients with paracoccidioidomycosis and healthy control subjects (figure 5). Similar results were obtained when we used CW as antigen (data not shown). These results suggest that the Fas-FasL pathway could be involved in the antigen-induced apoptosis of T cells from both patients with paracoccidioidomycosis and healthy control subjects and could be one of the mechanisms that modulate AICD in paracoccidioidomycosis infection.

Effect of CTLA-4 and CD95-CD95L interaction on the T cell proliferative response. We next investigated whether the apoptosis mediated by Fas-FasL interaction could contribute to the T cell unresponsiveness observed in patients with paracoccidioidomycosis. For these experiments, leukocytes were stimulated with antigens of P. brasiliensis and with PHA, with or without anti-FasL monoclonal antibody, and the cell-proliferation index was determined. We found that blockage of Fas-FasL interaction did not restore the T cell proliferative response to either P. brasiliensis antigens or PHA (figure 6A). Because we found increased CTLA-4 expression in PBMCs from patients with paracoccidioidomycosis, we investigated whether this molecule could be involved in the mechanism that mediates the T cell unresponsiveness. We therefore cultured leukocytes with PHA or P. brasiliensis antigens, in the presence or absence of anti–CTLA-4 monoclonal antibody, which blocks CTLA-4 engagement, and we quantified apoptosis and cell proliferation. Blockage of CTLA-4 engagement did not result in decreased apoptosis levels (data not shown) and did not restore the T cell proliferative response in cells from patients. Moreover, addition of anti–TGF-β did not significantly change the proliferation of T cells from control subjects or patients. However, simultaneous blocking of CD95-CD95L interaction and of CTLA-4 signaling, by the addition of both anti–CTLA-4 and anti-CD95L monoclonal antibodies, resulted in a significant increase (436%) in the T cell proliferation induced by PHA, but not in that induced by fungal antigens, in cells from patients with paracoccidioidomycosis (figure 6A).

To gain insight into the functional inactivation of T cells by either CTLA-4 or CD95L, we measured T cell production of IFN-γ in cultures with anti-FasL and anti–CTLA-4 monoclonal antibodies. The results show that blockage of CTLA-4 and CD95L did not change IFN-γ production when the cells from control subjects were cultured in the absence of antigen or in...
the presence of PHA. However, IFN-γ production was significantly increased when the cells from patients were cultured with anti-FasL or anti–CTLA-4 monoclonal antibody and with *P. brasiliensis* antigen. When we added CW to the cultures, similar results were found (data not shown). When the cells from patients were cultured with anti-CD95L and anti–CTLA-4 monoclonal antibodies that were either separately or simultaneously added to the cultures, the increased IFN-γ production was similar (figure 6B).

**DISCUSSION**

Here we have presented data that show, for the first time, that Fas- and FasL-mediated apoptosis and engagement of the down-regulatory molecule CTLA-4 are clearly involved in modulating T cell unresponsiveness in patients with paracoccidiomycosis. Our results confirm results published elsewhere that show that PBMCs from patients infected with *P. brasiliensis* do not proliferate in response to either fungal antigens or PHA. Because cell-mediated immunity is crucial for host defense of paracoccidiomycosis [4, 22–24], we investigated the mechanism underlying the T cell unresponsiveness seen in these patients. We did not find a significant imbalance in the levels of IFN-γ, IL-4, and IL-10 produced, after antigen and mitogen stimulation, by PBMCs, an imbalance that would have explained the T cell unresponsiveness. Moreover, the addition of a neutralizing antibody against IL-4 and IL-10 did not restore T cell proliferation (data not shown). Although *P. brasiliensis* infection has been associated with a Th2-type response, the data on cytokine production in patients with paracoccidiomycosis are controversial [6, 24–26]. Therefore, other mechanisms are possibly involved in the modulation of the T cell unresponsiveness seen in patients with paracoccidiomycosis.

Recently, evidence has emerged that shows that, because it provides an additional stimulus for both IL-2 production and IL-2 receptor expression, the costimulatory molecule CD28 is a crucial modulator of T cell activation [27]. Considering that cells from patients with paracoccidiomycosis show decreased IL-2 receptor expression [6], we hypothesized that CD28 expression could be down-regulated in T cells from patients with paracoccidiomycosis, as has been demonstrated to occur in T cells from persons infected with other parasites, such as *T. cruzi* [28]. However, we found normal levels of CD28 expression in the cells from patients, both ex vivo and after culture with fungal antigens, LPS, or PHA. Alternatively, the decreased ability of T cells to proliferate in response to specific or polyclonal stimulation could be due to increased expression of CTLA-4, a molecule that also binds to B7, promotes anergy, and negatively influences T cell effector function by inhibiting cell-cycle progression [14, 29]. In fact, CTLA-4 expression was significantly increased in cells from patients with paracoccidiomycosis but not from control subjects (figure 1C). Moreover, stimulation with fungal antigen or with PHA additionally increased CTLA-4 expression in leukocytes from patients with paracoccidiomycosis, a finding that suggests that specific stimulation prompts these cells to become unresponsive. Interestingly, increased expression of CTLA-4 has also been observed in cells from patients with malaria and from patients with human immunodeficiency virus (HIV) infection [30, 31]. In HIV-infected patients, the proliferation of T cells was inversely correlated with CTLA-4 expression, a correlation that suggests that CTLA-4 may be involved in the elimination and anergy of T cells in HIV-1 infection [31]. Also, increased CTLA-4 expression was correlated with decreased proliferative activity and IL-2 production, in T cells from patients with Hodgkin’s disease [32]. However, in contrast with what was observed in HIV-infected patients, addition of anti–CTLA-4 neutralizing monoclonal antibody to the cultures of PBMCs from patients with paracoccidiomycosis did not restore the T cell proliferative response (figure 6).

Other reports show that CTLA-4 engagement leads to TGF-β production and that CTLA-4 mediates its inhibitory effect on T cell activation via TGF-β [33]. TGF-β, another cytokine that is able to inhibit T cell proliferation [34] and macrophage activation [35], was persistently increased in supernatants of cells (figure 2) and in serum samples of patients, a finding that explains the higher levels of specific antibodies against fungal
antigen, as reported elsewhere [36]. However, the increased TGF-β production apparently does not also explain the decreased T cell proliferation, because the addition of neutralizing anti–TGF-β antibody did not change the pattern of cell proliferation. Moreover, inhibition of CTLA-4 engagement did not result in decreased production of TGF-β (data not shown). These results suggest that TGF-β production and CTLA-4 engagement are independent phenomena in the human immune response against *P. brasiliensis*. The possibility that they are independent is compatible with recent results that demonstrate that CTLA-4 and TGF-β represent distinct mechanisms for regulation of the T cell response [37].

An alternative explanation for the absence of a T cell response in these cultures is that CTLA-4 mediates antigen-specific apoptosis, as has been elsewhere reported to occur in activated human T cells [38]. In fact, we found high levels of Annexin V+ T cells in PBMCs from patients. However, the AICD found in T cells from patients seems to be not mediated by CTLA-4, because blockage of CTLA-4 did not change the apoptosis levels in the cells. The increased levels of Annexin V+ cells, after culture with fungal antigen or PHA, indicate that activation-induced cell death is occurring in vivo, as has been found to occur in patients with head and neck cancer [39]. Similarly, T cell hyporesponsiveness has been associated with induction
of apoptosis in other infectious diseases of chronic evolution, such as schistosomiasis, tuberculosis, malaria, and Chagas’ disease [12, 13, 40, 41].

We next examined the involvement of the CD95-CD95L system in the modulation of apoptosis in patients with paracoccidioidomycosis. The first indication that the Fas-FasL system could be involved in the modulation of apoptosis during paracoccidioidomycosis came from the observation that the expression of CD95L was greatly increased in cells from patients but not in cells from control subjects. The possibility that Fas-FasL is involved in the mediation of apoptosis was confirmed by the finding that neutralization of FasL leads to a significant reduction in levels of apoptosis in patients’ PBMCs cultured with antigens. However, considering that FasL blockage was unable to completely abrogate induction of apoptosis in these cultures, we cannot rule out the possibility that mechanisms other than Fas-FasL engagement are involved in the modulation of apoptosis in cells from patients with paracoccidioidomycosis. One possibility could be that the apoptosis observed is induced by tumor necrosis factor–α (TNF-α). However, recent reports have showed normal levels of TNF-α in serum samples [25] and in supernatants [26] of cells from patients with paracoccidioidomycosis. Alternatively, the T cell death could be mediated by perforin and IFN-γ feedback [42]. However, the possibility of perforin and IFN-γ involvement is remote, because the majority of apoptotic cells are CD4+ T lymphocytes (data not shown), and IFN-γ production by cells from patients with active disease is either very low (figure 2) or undetectable [7, 24–26].

Although our data point to a clear correlation between T cell unresponsiveness and Fas-FasL–mediated AICD, during P. brasiliensis infection, we observed that blockage of Fas-FasL interaction was unable to restore the proliferative response of T cells from patients with paracoccidioidomycosis. Although we do not fully understand this result, one possible explanation is that CTLA-4 expression remains increased in these cells (figure 1), a conclusion reinforced by the results showing that simultaneous inhibition of FasL and CTLA-4 resulted in a significant increase in the T cell proliferative response. Therefore, the blockage, via Fas-FasL interaction, of cell death is not sufficient to overcome the inhibitory antiproliferative effects of CTLA-4. Nevertheless, although the blockage of CTLA-4 and FasL led to an increased lymphoproliferation in response to PHA, it did not result in any improvement of fungal antigen-driven proliferative T cell response (figure 6). This could be because of the type of fungal antigens used in the cultures. However, similar results were obtained when we used purified gp43 as antigen, which is considered to be the main antigenic compound of P. brasiliensis [43]. Therefore, the antigen-specific T cells do not proliferate, either because of CTLA-4 expression or because they die from CD95-CD95L engagement. Similar mechanisms could explain the skin-test anergy to paracoccidioidin observed in patients with paracoccidioidomycosis [4].

Another important aspect of our study is the finding that blockage of CTLA-4 and FasL lead to an increased IFN-γ production by PBMCs from patients with paracoccidioidomycosis (figure 6). This is a very interesting finding: it indicates that, because of CTLA-4 expression on their surface, T cells do not produce IFN-γ after interaction with specific fungal antigens. This result is in accordance with the findings of low numbers of IFN-γ–producing cells in the skin and lymph node lesions of patients with paracoccidioidomycosis (A.P. Campanelli and J.S. Silva, unpublished data) and in their blood [7, 26, 27]. It also suggests that CTLA-4 impairs proliferation of T cells while it allows them to accumulate in the G0/G1 phase of the cell cycle and to produce IFN-γ, a suggestion in accordance with previously published results [44, 45].

In summary, the data we have presented in the present study show that Fas-FasL–mediated apoptosis and stimuli driven by CTLA-4 engagement are involved in the modulation of the immune response during P. brasiliensis infection in humans. Therefore, studies to understand the relationship between T cell activation, proliferation, and apoptosis may lead to significant improvements in the treatment of patients with fungal infections. Moreover, therapeutic protocols for CTLA-4 blockage may enhance T cell–mediated protection in patients infected with P. brasiliensis.

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References


