Involvement of the Fas/Fas Ligand Pathway in Activation-Induced Cell Death of Mycobacteria-Reactive Human \( \gamma\delta \) T Cells: A Mechanism for the Loss of \( \gamma\delta \) T Cells in Patients with Pulmonary Tuberculosis

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Involvement of the Fas/Fas Ligand Pathway in Activation-Induced Cell Death of Mycobacteria- Reactive Human \( \gamma \delta \) T Cells: A Mechanism for the Loss of \( \gamma \delta \) T Cells in Patients with Pulmonary Tuberculosis

Baiqing Li,\(^1\) Hamid Bassiri,\(^*\) Milton D. Rossman,\(^2\) Peter Kramer,\(^3\) A. Fusun-Oner Eyuboglu,\(^3\) Martha Torres,\(^4\) Eduardo Sada,\(^4\) Turgut Imir,\(^\¶\) and Simon R. Carding\(^4\)\(^*\)

Although the identity of T cells involved in the protection against *Mycobacterium tuberculosis* (Mtb) in humans remain unknown, patients with pulmonary tuberculosis (TB) have reduced numbers of Mtb-reactive, V\(\gamma\delta^+/V\delta^+\) T cells in their blood and lungs. Here we have determined whether this \( \gamma \delta \) T loss is a consequence of Mtb Ag-mediated activation-induced cell death (AICD). Using a DNA polymerase-mediated dUTP nick translation labeling assay, 5% or less of freshly isolated CD4\(^+\)\(\alpha\beta\) or \( \gamma \delta \) T cells from normal healthy individuals and TB patients were apoptotic. However, during culture Mtb Ags induced apoptosis in a large proportion of V\(\gamma^+/V\delta^+\) peripheral blood T cells from healthy subjects (30–45%) and TB patients (55–68%); this was increased further in the presence of IL-2. By contrast, anti-CD3 did not induce any significant level of apoptosis in \( \gamma \delta \) T cells from healthy subjects or TB patients. Mtb Ag stimulation rapidly induced Fas and Fas ligand (FasL) expression by \( \gamma \delta \) T cells, and in the presence of metalloproteinase-inhibitors >70% of \( \gamma \delta \) T cells were FasL\(^+\). Blockade of Fas-FasL interactions reduced the level of Mtb-mediated \( \gamma \delta \) T cell apoptosis by 75 to 80%. Collectively, these findings demonstrate that Mtb-reactive \( \gamma \delta \) T cells are more susceptible to AICD and that the Fas-FasL pathways of apoptosis is involved. AICD of \( \gamma \delta \) T cells, therefore, provides an explanation for the loss of Mtb-reactive T cells during mycobacterial infection. *The Journal of Immunology*, 1998, 161: 1558–1567.

Tuberculosis (TB) remains the leading cause of mortality among human infectious diseases today in the world. Although the importance of CD4\(^+\)\(\alpha\beta\) T cells in mycobacterial immunity has been clearly demonstrated (1, 2), several lines of evidence suggest that \( \gamma \delta \) T cells may also be important. Among T cells in normal healthy individuals, more \( \gamma \delta \) than \( \alpha \beta \) are *Mycobacterium tuberculosis* (Mtb)-reactive (3, 4). Mtb-reactive \( \gamma \delta \) T cells express a TCR encoded by a single V\(\gamma\) (V\(\gamma\)) and V\(\delta\) (V\(\delta\)) gene (5–7). \( \gamma \delta \) T cells from both normal and mycobacterium-sensitized subjects proliferate vigorously and release cytokines in vitro in response to a variety of Mtb-derived protein (3, 6, 8–12) and nonprotein (13–16) Ags. Increases in \( \gamma \delta \) T cell numbers in mice (17, 18) and humans (19, 20) infected with Mtb have also been reported.

Recently, we have shown that in patients with active pulmonary TB, the numbers of V\(\gamma^+/V\delta^+\) Mtb-reactive T cells in the blood and bronchoalveolar lavage were significantly reduced compared with those in healthy PPD\(^+\) individuals and in patients with unrelated pulmonary granulomatous diseases (21). This correlation between the changes in the \( \gamma \delta \) T cell repertoire and manifestations of disease in patients with pulmonary TB is consistent with a direct role for these cells in the immunopathogenesis of TB.

In the present study we have investigated the mechanisms that could account for the loss or the absence of V\(\gamma^+/V\delta^+\) T cells in TB patients. Possible causes include functional inactivation (energy), inactivation or dysfunction of other immune cell populations necessary for \( \gamma \delta \) T cell activation and growth (11, 22, 23), and elimination as a consequence of activation-induced cell death (AICD). Of these, AICD is an attractive possibility, since programmed cell death and apoptosis of Ag-activated T cells are known to be important mechanisms for controlling T cell responses and maintaining homeostasis (24). The finding that \( \gamma \delta \) T cells from normal PPD\(^+\) individuals (6, 25) and patients with advanced clinical forms of TB (26) can be induced to undergo apoptosis after activation with either anti-CD3 and IL-2 (6, 25) or mycobacterial Ags (26) suggest that the loss of \( \gamma \delta \) T cells may be a consequence of the elimination (clonal deletion) of Mtb-specific \( \gamma \delta \) T cells. However, it is not known whether this occurs in vivo, what pathways of AICD are involved, and whether AICD can explain the loss of V\(\gamma^+/V\delta^+\) T cells in patients with active TB.

Consequently, we have investigated whether AICD is an outcome of the exposure of \( \gamma \delta \) T cells to Mtb both in vivo and during culture. We have analyzed and compared \( \gamma \delta \) T cell populations from normal healthy individuals and TB patients for evidence of
apoptotic death directly ex vivo and after in vitro culture with different types of Mtb-derived Ags. Our results show that compared with CD4⁺ αβ T cells, γδ T cells are more susceptible to Mtb Ag-mediated AICD and that this susceptibility is increased further in the presence of IL-2. We also show that Fas-Fas ligand (FasL) interactions are involved in this Mtb-mediated AICD of γδ T cells.

Materials and Methods

Study population

The patient and control subject populations are described in Table I. Peripheral blood samples were obtained from a total of 32 patients diagnosed with pulmonary TB who were recruited from the Hospital of University of Pennsylvania (n = 2), the Bengbu Medical College Affiliated Hospital (people’s Republic of China; n = 13), and Gazi University Hospital (Ankara, Turkey; n = 17). The diagnosis of active pulmonary TB was established in all patients (21 men, 38 ± 6.4 yr of age, range from 21–66 yr; 11 women, 33 ± 11 yr of age, range from 18–52 yr) by the presence of recent clinical symptoms of TB, positive culture of Mtb and smear test for acid-fast bacilli (AFB) from sputum or broncho-brush samples, and abnormal chest radiograph. All TB patients were HIV⁻. Eighteen patients at the time of sampling were receiving anti-mycobacterial therapy (ethambutol, pyrazinamide, morfanizamide and/or streptomycin) for at least 3 mo before analysis. To evaluate the contribution that endogenously produced TNF-α added to cultures of Mtb Ag-expanded lymphocytes for 72 h before analysis. In some experiments analyzing FasL expression, cells were cultured for 48 h. In some experiments analyzing FasL expression, cells were cultured in the presence of 5 mM EDTA to inhibit metalloprotease-mediated cleavage of cell surface FasL (28). EDTA was only present for the last 4 h of culture before analysis. For the derivation of γδ T cells, 20 to 20 ng/ml of recombinant TNF-α (PeproTech, Rocky Hill, NJ) was added to cultures of Mtb Ag-expanded lymphocytes for 72 h before analysis. To evaluate the contribution that endogenously produced TNF-α makes to apoptotic death of T cell during in vitro culture, neutralizing

<table>
<thead>
<tr>
<th>Group and Source</th>
<th>No. of Patients</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>I. PPD⁺ normal</td>
<td>34</td>
<td>Healthy, normal, PPD⁺</td>
</tr>
<tr>
<td>II. Inactive TB</td>
<td>12</td>
<td>Patients with history of TB, PPD⁺, abnormal chest x-ray, negative AFB</td>
</tr>
<tr>
<td>III. Active TB</td>
<td>32</td>
<td>Patients with history of TB, PPD⁺, abnormal chest x-ray, positive AFB</td>
</tr>
<tr>
<td>Nontreated</td>
<td>14</td>
<td>No anti-Mtb drug therapy at time of sampling</td>
</tr>
<tr>
<td>Treated</td>
<td>18</td>
<td>Ethambutol, pyrazinamide, morfanizamide and/or streptomycin for 2 mo or less (n = 15) or &gt;2 mo (n = 3)</td>
</tr>
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</table>

Flow cytometry

Fifty-microliter aliquots of freshly isolated or cultured PBMC (containing 2–10 × 10⁶ cells) in PBS with 5% FCS and 0.1% NaN₃ (staining buffer) were added to individual wells of V-bottom 96-well plates. All Ab incubations were conducted on ice for 30 min each. Cell samples were first incubated with biotin-conjugated Abs, washed, and incubated with fluoro-chrome-conjugated streptavidin (SA-Red 670, Life Technologies, Gaithersburg, MD) and fluorochrome-conjugated-Ab Abs (Dako Corp., Carpinteria, CA) were used as compensation control reagents for two- and three-color flow cytometric analyses. Fluorochrome-conjugated mouse IgG isotype control Abs were purchased from Becton Dickinson. Phorbol ester, ionomycin, and murine IgM were obtained from Sigma (St. Louis, MO).

Lymphocyte culture

PBMC isolated from TB patients and normal subjects were cultured in RPMI/10% human serum supplemented with 25 mM HEPES at 37°C in 5% CO₂. Cells were cultured at 5 × 10⁶/ml (1 ml/well) in 24-well tissue culture plates (Life Technologies) in the presence of 1) 1 to 100 U/ml recombinant human IL-2 (Boehringer Mannheim, Indianapolis, IN), 2) 2.5 µg/ml anti-CD3 Ab (OKT3), 3) 1 µg/ml of culture filtrate from H37Rv (provided by Dr. Michael E. Ellis, Colorado State University, Fort Collins, CO), 4) 2 µg/ml of supernatant from heat-treated H37Rv (SPT) provided by Dr. Henry Boom (Case Western Reserve University, Cleveland, OH), or 5) medium alone. For the majority of experiments, cells were cultured for 48 h. In some experiments analyzing FasL expression, cells were cultured in the presence of 5 mM EDTA to inhibit metalloprotease-mediated cleavage of cell surface FasL (28). EDTA was only present for the last 4 h of culture before analysis. For the derivation of γδ and Vγ9/Vδ2⁺ T cell lines, PBMC were cultured for up to 14 days by stimulating PBMC with anti-CD3 or Mtb-SHT, adding 1 U/ml of IL-2 after 5 and 10 days of culture.

Apoptosis assay

Apoptotic cells were identified among suspensions of freshly isolated or cultured PBMC using a DNA polymerase-mediated dUTP nick translation labeling (DUNTL) assay provided by Dr. J. Ashwell (National Institutes of Health, Bethesda, MD). For evaluating TNF-induced apoptosis, 2 to 20 ng/ml of recombinant TNF-α (PeproTech, Rocky Hill, NJ) was added to cultures of Mtb Ag-expanded lymphocytes for 72 h before analysis.
rabbit anti-human TNF-α antiserum (1/5 to 1/100 dilution) was included in some cultures. Nonimmune rabbit serum was used as a control. As a positive control for Fas-mediated apoptosis, cells were incubated with 200 ng of the anti-Fas IgM Ab, CH-11, or control Ab (purified murine IgM) for 20 h before analysis. In some experiments Fab fragments of the anti-Fas Ab, APO-1 (0.01–10 μg/ml), were included to block Fas-mediated apoptosis. Cells were stained with Abs for cell surface Ags, fixed with 1% paraformaldehyde/PBS, then washed in staining buffer (PBS, 2% FCS, and 0.1% NaN₃) followed by permeabilization buffer (0.1% Triton-X 100 and 0.1% sodium citrate). Cells were resuspended in labeling buffer (50 mM Tris (pH 7.4), 10 mM MgSO₄·7H₂O, 0.1 mM DTT, 1 nmol/ml dATP/CTP/GTP, and 0.7 nmol/ml dTTP) containing 40 pmol of FITC-12-dUTP (Boehringer Mannheim, Indianapolis, IN) and either active (2 U) or denatured DNA polymerase (Promega, Madison, WI) and incubated at 37°C for 90 min before analysis by flow cytometry. Stained cells were run on a FACScan and analyzed as described above. The frequency of apoptotic (dUTP⁺) cells was determined by subtracting the frequency of cells incorporating dUTP in the presence of denatured (control) DNA polymerase from the frequency of cells incorporating dUTP in the presence of active polymerase. The frequency of dUTP⁺ cells was used to determine the number of viable cells.

Statistical analysis

For comparing the proportions of γδ T cells freshly isolated from PB and bronchoalveolar lavage in different patient and control groups, Student’s t test was used. For comparison of γδ T cells and subsets, considered non-parametric, Mann-Whitney U test or Wilcoxon’s signed rank sum test was used. Values of p < 0.05 were chosen for rejection of the null hypothesis.

Results

Drug therapy is not responsible for alterations in the population size of Mtb-reactive Vγ9/Vδ2 T cells in TB patients

Previously we have demonstrated a strong correlation between the absence or loss of the major Mtb-reactive subset of γδ T cells, Vγ9+/Vδ2+ cells, and manifestations of active pulmonary TB (21). To determine whether chemotherapy adversely affected Vγ9+/Vδ2+ T cells and if drug treatment had any beneficial effect on this population of T cells, the number of peripheral blood Vγ9+/Vδ2+ T cells was analyzed in patients during and after drug therapy (Fig. 1). Nontreated patients were those that had not received any antimycobacterial drugs before analysis. Treated patients received drug therapy before analysis and were divided into two groups depending upon the duration of treatment: 2 mo or less (average, 1.2 mo for active TB patients; n = 15) and >2 mo (average, 8 mo for disease-free subjects (n = 5) and 16 mo for TB patients with active disease (n = 3)). Of the patients treated for >2 mo, the average period of treatment for three patients with active TB was longer due to treatment failure. For five of the treated patients we were able to obtain blood samples both before and 1–2 mo after beginning drug treatment. The active disease group of patients consisted of those whose sputum at the time of analysis was AFB smear test and Mtb culture positive. Inactive disease patients were those originally diagnosed with pulmonary TB who, after a period of drug treatment and at the time of sampling, were AFB smear test and Mtb culture negative.

Using two- and three-color flow cytometry, the frequency of Vγ9+/Vδ2+ T cells in TB patients with active disease (39 ± 7% of all γδ T cells; Fig. 1) was, as reported previously (21), significantly (p < 0.0001) reduced compared with healthy individuals (84 ± 8% of all γδ T cells). This reduced frequency of Vγ9+/Vδ2+ T cells in TB patients reflected a decrease in absolute numbers of these cells, since as a group, the number of peripheral blood γδ T cells in patients with active TB (82 ± 43 cell/μl) and nonactive TB (83 ± 40 cells/μl) was comparable to that in normal PPD+ control subjects (94 ± 42 cells/μl). A similar decrease in Vγ9+/Vδ2+ cells was seen in the lungs of TB patients (data not shown) (21). As we have described previously (21), as a result of the loss of Vγ9+/Vδ2+ T cells, the γδ T cell repertoire in TB patients is dominated by Vγ9−/Vδ2+ T cells, which in the majority of normal, healthy, PPD+ subjects represent only a small proportion of the peripheral blood γδ T cell repertoire (21). What accounts for the increase in number of these unusual γδ T cell subsets in TB patients and the nature of the Ag(s) that drives their expansion are not known. However, since the Mtb reactivity of γδ T cells is contained entirely within the Vγ9+/Vδ2+ subset (29), it is unlikely to be mediated by Mtb Ags.

In drug-treated TB patients decreased numbers of Vγ9+/Vδ2+ cells were associated with patients that had persistent positive cultures for Mtb (i.e., active disease) regardless of the period of drug treatment. Reduced numbers of Vγ9+/Vδ2+ T cells were seen in a group of 15 treated patients with active disease for >2 mo or less (Fig. 1) and in three patients with positive sputum cultures for Mtb after 8, 16, and 18 mo (active disease patient group treated for 2 mo in Fig. 1). Persistently reduced numbers of Vγ9+/Vδ2+ T cells were seen in five patients analyzed before and 30 (n = 3) or 60 (n = 2) days post-treatment (data not shown). By comparison, the frequency of Vγ9+/Vδ2+ cells in treated patients who were sputum culture negative for Mtb (inactive disease group in Fig. 1) was significantly higher (p < 0.001) than that in patients with active TB, although still slightly lower than that in normal subjects. Importantly, this recovery of Vγ9+/Vδ2+ cell numbers in patients successfully treated with antimycobacterial drugs makes it unlikely that the loss of Mtb-reactive γδ T cells seen in TB patients is due to the adverse affect of antimycobacterial drugs on the survival or growth of Vγ9+/Vδ2+ cells. Together, these findings...
To investigate the underlying basis of the loss of Mtb-reactive and AICD in vitro among 5 million PBMC before (freshly isolated) and after 48 h of culture. The data obtained and shown in Figure 2 represent the cytometric determination of the frequencies of CD4\(^+\) T cells exhibiting spontaneous and AICD in vitro culture. Five million PBMC from PPD\(^+\) normal individuals (n = 7) or from patients with active pulmonary TB (n = 6) were analyzed before (Fresh) or after culture for 48 h in complete medium alone (Media) or in medium containing IL-2 (10 U/ml), anti-CD3 Ab (2 \(\mu\)g/ml), supernatant from heat-treated Mtb (Mtb-SHT; 2 \(\mu\)g/ml), or culture filtrate from Mtb (Mtb-Filtrate; 1 \(\mu\)g/ml). The DUNTL assay and flow cytometry were used to distinguish between and quantitate viable (\(dUTP^+\)) and apoptotic (\(dUTP^-\)) cells as described in Materials and Methods. The results shown represent the mean (\(\pm SD\)) number of \(dUTP^+\) and \(dUTP^-\) T cells present per 5 \(\times\) 10\(^6\) PBMC analyzed. The p values shown are for comparing the number of \(dUTP^+\) cells cultured in medium alone vs medium containing different stimuli; * indicates \(p < 0.02\); ** indicates \(p < 0.01\); *** indicates \(p < 0.005\).

**FIGURE 2.** \(\gamma\delta\) T cells display increased susceptibility to undergo spontaneous and AICD during in vitro culture. Five million PBMC from PPD\(^+\) normal individuals (n = 7) or from patients with active pulmonary TB (n = 6) were analyzed before (Fresh) or after culture for 48 h in complete medium alone (Media) or in medium containing IL-2 (10 U/ml), anti-CD3 Ab (2 \(\mu\)g/ml), supernatant from heat-treated Mtb (Mtb-SHT; 2 \(\mu\)g/ml), or culture filtrate from Mtb (Mtb-Filtrate; 1 \(\mu\)g/ml). The DUNTL assay and flow cytometry were used to distinguish between and quantitate viable (\(dUTP^+\)) and apoptotic (\(dUTP^-\)) cells as described in Materials and Methods. The results shown represent the mean (\(\pm SD\)) number of \(dUTP^+\) and \(dUTP^-\) T cells present per 5 \(\times\) 10\(^6\) PBMC analyzed. The p values shown are for comparing the number of \(dUTP^+\) cells cultured in medium alone vs medium containing different stimuli; * indicates \(p < 0.02\); ** indicates \(p < 0.01\); *** indicates \(p < 0.005\).

\(\gamma\delta\) T cells exhibit increased susceptibility to spontaneous death and AICD in vitro

To investigate the underlying basis of the loss of Mtb-reactive \(\gamma\delta\) T cells, we determined whether Mtb Ag stimulation resulted in AICD and could account for the reduced number of V\(\gamma\delta^+/V\beta^+\) T cells in TB patients. Apoptotic cells were identified and quantitated using a flow cytometry-based assay in which DNA strand breaks, a hallmark feature of apoptosis, were detected in cells using an in vitro DNA polymerase-mediated DUNTL method. Freshly isolated or cultured cells were first stained with anti-TCR Abs, fixed, permeabilized, and incubated with FITC-dUTP in the presence of DNA polymerase. As a control, duplicate aliquots of cells were incubated with FITC-dUTP in the presence of DNA polymerase. A similar proportion of CD4\(^+\) \(\alpha\beta\) (3.1 \(\pm\) 2.2%) and \(\gamma\delta\) (3.0 \(\pm\) 0.9%) T cells from patients with active TB was also \(dUTP^+\) (Fig. 2). By contrast, it was possible to detect differences in the susceptibility of CD4\(^+\) \(\alpha\beta\) and \(\gamma\delta\) T cells from normal subjects and TB patients during short term (48 h) in vitro culture (Fig. 2). Five different conditions were used: complete medium alone, anti-CD3, IL-2, and two different preparations of Mtb Ags representative of secreted (culture filtrate; Mtb-Filtrate) and cell-associated (supernatant from heat-treated bacteria; Mtb-SHT) Ags. Both of these Mtb Ags are capable of stimulating \(\alpha\beta\) T cells, whereas the Mtb-SHT antigenic preparation is very effective at promoting \(\gamma\delta\) T cell growth in normal individuals (12, 21).

In the absence of any exogenous Ag (medium alone) the level of apoptosis (spontaneous death) in \(\gamma\delta\) (17–24%) and V\(\gamma\delta^+/V\beta^+\) (16–27%) T cells, from PPD\(^+\) normal subjects was slightly higher than that in CD4\(^+\) \(\alpha\beta\) (10–15%) T cells (Fig. 2). Mtb Ags induced levels of apoptosis in \(\gamma\delta\) T cells and V\(\gamma\delta^+/V\beta^+\) T cells from the same individuals that were significantly higher (\(p < 0.01\) for Mtb-SHT and \(p < 0.02\) for Mtb-Filtrate) than the level of spontaneous death. The results obtained from the analysis of T cells from patients with non-active TB were very similar to those described for PPD\(^+\) normal subjects (data not shown). By contrast, none of the stimuli used induced levels of apoptosis in CD4\(^+\) \(\alpha\beta\) T cells that were significantly higher than those in cultures containing medium alone.

The profile of Ag-mediated apoptosis in cultured T cells from patients with active TB was similar to that in T cells from normal

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subjects. However, the overall level of T cell apoptosis was slightly higher in PBMC from TB patients. Whereas 30 to 45% of γδ T cells from normal subjects were induced to undergo apoptosis by Mtb-SHT or Mtb-filtrate, these Ags induced apoptosis in >50% (55–68%) of γδ T cells from TB patients (Fig. 2). Although this increase in apoptotic Vγ9+/Vδ2+ T cells is small, it is significant, since there are now more dead (apoptotic) than viable (dUTP−) cells present in these cultures, providing an explanation for the lack of growth of these γδ T cells when cultured with Mtb Ags (21). Also of note was the finding that IL-2 induced levels of apoptosis in γδ and Vγ9+/Vδ2+ cells from TB patients that were significantly higher (p < 0.01 and p < 0.02, respectively) than the level of spontaneous death, perhaps reflecting prior activation in vivo. Expression of the activation-associated Ags, CD25 and CD69, by a large proportion (20–35%) of freshly isolated peripheral blood Vγ9+/Vδ2+ T cells from TB patients (B. Li and S. R. Carding, unpublished observations) is consistent with this interpretation and suggests that IL-2-induced AICD in vitro is a consequence of activation in vivo as a result of exposure to Mtb Ags.

As seen from the analysis of PPD+ subjects, CD4+ αβ T cells from TB patients were, compared with γδ T cells, markedly less susceptible to AICD during 48 h of culture with the various stimuli. In these cultures only anti-CD3 was able to induce levels of apoptosis that were significantly higher (p < 0.01) than those occurring in medium alone (Fig. 2).

Collectively, the results of our analysis of apoptotic T cells suggest that γδ T cells, and in particular the Mtb-reactive Vγ9+/Vδ2− subset, are more susceptible to Mtb Ag-mediated AICD than are CD4+ αβ T cells. To investigate this phenomena further, the pathways involved in AICD of γδ T cells were investigated.

**Blockade of Fas-FasL interactions abrogates Mtb-mediated AICD of γδ T cells**

A large amount of evidence suggests that AICD of peripheral lymphocytes can be triggered by the interaction of the Fas molecule with its ligand (27, 30–32). A previous study of murine T cells has shown that γδ T cells express higher levels of FasL mRNA than αβ T cell clones (33). However, to date the involvement of Fas and FasL interactions in AICD of bulk polyclonal populations of human γδ T cells in mycobacterial disease has not been investigated.

To determine whether or how much the Fas-FasL pathway contributed to γδ T cell apoptosis induced by Mtb Ags, we investigated what effect Ab-mediated blockade of Fas-FasL interactions had on Mtb Ag-mediated apoptosis of Vγ9+/Vδ2+ T cells. For this experiment two T cell lines (>98% CD3+) derived by culturing PBMC from a PPD+ donor with the Mtb-SHT Ag preparation for 10 to 14 days were used. The first line (Expt. 1 in Fig. 3) comprised 45% CD4+ TCRγδT cells and 55% TCRγδT (>90% Vγ9+/Vδ2+) T cells, and the second (Expt. 2, Fig. 3) consisted of 95% TCRγδT (>95% Vγ9+/Vδ2+) T cells. These lines were stimulated a second time with anti-CD3 or Mtb-SHT in the presence or the absence of Fab fragments of the APO-1 Ab that blocks Fas-FasL interactions (27). Forty-eight hours later the frequency of apoptotic cells was determined using the DUNTL assay. The efficacy of the APO-1 Ab was demonstrated by its ability to almost completely inhibit CH-11 Ab-induced apoptosis of αβ CD4+ (90%) and γδ (85–95%) T cell lines (Fig. 3). A final concentration of 2 μg/ml of APO-1 Ab was shown to be optimal for inhibition of CH-11-induced apoptosis (data not shown) and was used for inhibiting Mtb-mediated AICD of αβ and γδ T cells.

In the presence of the APO-1 Ab, the levels of Mtb-SHT-induced γδ T cell apoptosis and anti-CD3-induced apoptosis of CD4+ αβ T cells were reduced by 70 to 85% and 65 to 75%, respectively (Fig. 3). By contrast, the frequency of apoptotic γδ and αβ T cells in cultures containing medium alone was not significantly reduced in the presence of the APO-1 Ab. These results demonstrate that the Fas-FasL pathway of AICD mediates Mtb Ag-induced apoptosis of γδ T cells. By contrast, the Fas-FasL pathway does not appear to be involved in the spontaneous death of cultured γδ or αβ T cells. Finally, the ability of Mtb-SHT to induce apoptosis in the T cell line that comprised almost entirely (95%) γδ T cells suggests that their death is a result of “fratricide”
expression by Mtb-stimulated gd we decided to investigate the kinetics of induction of Fas and FasL TCR, CD4, or Fas-specific Abs and flow cytometry. TCR directly ex vivo by staining with FITC-, PE-, and Tricolor-conjugated anti-
expression of these molecules is regulated differently in such as EDTA, can increase the level of expression by preventing their cleavage from the surface. To determine whether our analysis had underestimated the amount of FasL expressed by αβ and γδ T cells, PBMC from PPD + normal subjects or TB patients. The frequency of FasL + γδ T cells did not change significantly when analyzed at 13 days poststimulation, although FasL expression did appear to be up-regulated more slowly by αβ T cells, since the highest frequency of positive cells was seen 13 days after stimulation (data not shown).

Cell surface expression of FasL has been shown to be regulated by metalloproteinas (28, 35), and inhibitors of these enzymes, such as EDTA, can increase the level of expression by preventing their cleavage from the surface. To determine whether our analysis had underestimated the amount of FasL expressed by αβ and γδ T cells, PBMC from PPD + subjects were cultured with Mtb Ags in the presence of 5 mM EDTA before analysis of FasL expression. EDTA was added during the last 4 h of the 48-h culture period. During this 4-h incubation period, cell viability did not change significantly (data not shown). A striking increase in FasL expression by γδ T cells was seen in the presence of EDTA. Approximately 70% of γδ T cells activated in the presence of EDTA were FasL + compared with 10% or less in the absence of EDTA (Fig. 6). However, there was no evidence of stimulus (Ag)-specific modulation of FasL expression for γδ T cells, since the majority of γδ T cells cultured in medium alone were FasL +, and there was no

**Fas expression by Mtb-stimulated αβ and γδ T cells**

Since our analysis of T cell apoptosis demonstrated that αβ and γδ T cells have different susceptibilities to anti-CD3- and Mtb Ag-mediated AICD (Fig. 2), these stimuli were used to compare Fas expression. Cell surface Fas expression by freshly isolated and cultured peripheral blood T cells from PPD + subjects and patients with active pulmonary TB was detected by Ab staining and flow cytometry. Analysis of freshly isolated cells showed that in PPD + healthy individuals a higher proportion of γδ T cells (38 ± 9%; n = 10) than of CD4 + αβ T cells (12 ± 8%; n = 10) was Fas + (Fig. 4A). The level of Fas expression by both αβ and γδ T cells was variable, with the level of expression low in the majority of positive cells (Fig. 4A). In patients with active TB, the proportions of Fas-expressing αβ T cells and γδ T cells were similar to those in healthy subjects (Fig. 5).

Activation in vitro resulted in increased numbers of Fas + T cells, and the kinetics of Fas expression by αβ CD4 and γδ T cells induced by anti-CD3 and Mtb-SHT were distinct (summarized in Fig. 5). In cultures of Mtb-SHT-stimulated PBMC, Fas was up-regulated more rapidly by γδ T cells than by CD4 + αβ T cells. For example, at 48 h poststimulation the frequency of Fas + γδ T cells was significantly (p < 0.006) higher than that for CD4 + αβ T cells (Figs. 4B and 5). In fact, the frequency of CD4 + αβ T cells that were Fas + at this time in culture was lower than that among freshly isolated cells (Figs. 4, A and B). Beyond 48 h of culture the proportions of γδ and CD4 + αβ T cells that were Fas + were similar, with maximal levels of positive cells seen 10 days after activation (Fig. 5). In contrast to Mtb-SHT activation, anti-CD3 stimulation of PBMC from PPD + subjects resulted in the rapid (within 24 h) up-regulation of Fas by αβ CD4 T cells, which was sustained over the 14-day culture period. By contrast, up-regulation of Fas by γδ T cells occurred more slowly, with the highest frequency of positive cells occurring 10 days after stimulation.

In cultures of T cells from TB patients a similar profile of Fas expression by αβ and γδ T cells was seen (Figs. 4B and 5). Of note, the delayed up-regulation of Fas by γδ T cells from PPD + subjects was more pronounced in TB patient samples. Thus, differences in the temporal expression of Fas distinguish the responses of αβ and γδ T cells to different antigenic stimuli and Mtb-derived Ags.

**FasL expression by Mtb-stimulated αβ and γδ T cells**

Consistent with previous studies (28), cell surface-associated FasL was expressed by very few (1.4% or less) freshly isolated αβ and γδ T cells (Fig. 6). There were also no obvious differences in the frequency of FasL + T cells in normal or TB PBMC samples. In contrast, it was possible to detect differences in the kinetics and level of FasL expression by αβ and γδ T cells during in vitro culture (Fig. 6). An increase in the frequency of FasL + γδ T cells was seen within 48 h of culture, representing a three- to fivefold increase. The stimuli used were equivalent in their ability to induce FasL expression by γδ T cells, and there were no significant differences in the level of FasL expression by γδ T cells from PPD + normal subjects or TB patients. The frequency of FasL + γδ T cells did not change significantly when analyzed at 13 days poststimulation, although FasL expression did appear to be up-regulated more slowly by αβ T cells, since the highest frequency of positive cells was seen 13 days after stimulation (data not shown).
significant increase in the frequency of positive cells after culture with any of the stimuli. Although the frequency of FasL-expressing αβ CD4 T cells was also increased in the presence of EDTA, it was restricted to a small (11–23%) subset of cells. There was, however, some evidence of Ag-specific modulation of its expression in the presence of EDTA (Fig. 6). Whereas stimulation with anti-CD3 or Mtb-SHT did not significantly change the frequency of FasL\(^{1}\)ab T cells, there was a twofold increase in FasL\(^{1}\)ab T cells in response to Mtb-filtrate compared with that in cells cultured in medium alone.

Alternative mechanisms of γδ T cell apoptosis; TNF pathway

Since TNF is an apoptosis-associated molecule that shares amino acid homology with FasL and has been shown to induce apoptosis of activated lymphocytes (36, 37), we investigated whether this cytokine might contribute to Mtb-mediated γδ T cell apoptosis. Mtb Ag-specific T cells lines were established by culturing PBL from PPD\(^{1}\) normal subjects or TB patients in the presence of Mtb-filtrate or Mtb-SHT antigenic preparations and were restimulated with Ag in the presence of TNF-α for 3 days before DUNTL analysis.

Mtb-stimulated CD4\(^{+}\) αβ T cells from both PPD\(^{+}\) healthy subjects and TB patients were refractory to TNF-α-induced apoptosis, with 10% or less dUTP\(^{1}\) cells detected. Similarly, it was not possible to detect any significant difference in the level of γδ or V\(\gamma\delta\)\(^{+}\)/V\(\gamma\delta\)\(^{+}\) T cell apoptosis after stimulation with Mtb-SHT or -filtrate in the absence or the presence of TNF-α (Fig. 7). Also, the inability of neutralizing anti-human TNF-α antiserum (1/5 to 1/100) to significantly reduce the level of spontaneous apoptosis of CD4\(^{+}\) αβ, γδ, or V\(\gamma\delta\)\(^{+}\)/V\(\gamma\delta\)\(^{+}\) T cells from TB patients (data not shown) suggests that any TNF-α produced by these cells as a result of prior activation in vivo is not responsible for the death of these cells. We interpret these results as evidence of TNF-α not being involved in Mtb Ag-mediated AICD of γδ T cells.
**IL-2 influences the outcome of Mtb Ag stimulation of Vγ9/Vδ2+ T cells**

Whereas we and others have previously shown that the Mtb-SHT Ag preparation can promote the growth of Mtb-reactive, Vγ9+/Vδ2+ T cells, the data presented here demonstrate that this Ag preparation can also promote γδ T cell apoptosis. To attempt to explain this apparent paradox we investigated the possibility that the presence of additional growth factors, such as IL-2, might influence the outcome of γδ T cell activation by Mtb-SHT. The rationale for this experiment was based upon the observation by Kabelitz and colleagues that whereas anti-CD3 Abs alone are mitogenic for Vγ9+/Vδ2+ T cells, when combined with IL-2 they induce γδ T cell death by apoptosis (6, 25).

γδ T cells from a PPD+ healthy subject were expanded in vitro by stimulation with Mtb-SHT. After approximately 12 days these cultures contained >95% Vγ9+/Vδ2+ cells. The cells were then stimulated a second time with anti-CD3 or Mtb-SHT in the absence or the presence of exogenous IL-2 (1–100U/ml) or with IL-2 alone, and 48 h later the proportion of apoptotic cells was determined using the DUNTL assay. At concentrations of 10 U/ml and higher, IL-2 alone was able to induce a small but significant (p < 0.02) increase in γδ T cell apoptosis above that due to spontaneous death in culture (Media, in Fig. 8), reflecting, perhaps, the death of residual activated cells from the initial in vitro stimulation. By contrast, IL-2 had potent synergistic apoptotic activity when combined with Mtb-SHT (Fig. 8). In the presence of IL-2 the level of apoptosis induced by Mtb-SHT was increased by approximately twofold (26 ± 4 and 55 ± 5% dUTP+ cells in the absence and the presence of IL-2, respectively). This synergistic activity of Mtb-SHT and IL-2 was seen at IL-2 concentrations >1 U/ml, with the maximal effect obtained between 10 and 100 U/ml. By contrast, other cytokines, including TNF-α (Fig. 7), IL-4, and IFN-γ (data not shown), alone or in combination with Mtb-SHT, did not increase the level of γδ T cell apoptosis.

In agreement with the results of previous studies (6, 25), a similar, although less potent, synergistic effect of IL-2 on γδ T cell apoptosis was seen when it was combined with anti-CD3 (Fig. 8). In the presence of 10 and 100 U/ml of IL-2, the frequency of apoptotic γδ T cells was significantly greater (p < 0.01) than that after stimulation with anti-CD3 alone. Not surprisingly, the presence of IL-2 severely reduced the ability of Mtb-SHT to promote the growth of Vγ9+/Vδ2+ T cells. Whereas the Mtb-SHT Ag preparation expanded Vγ9+/Vδ2+ T cells, on the average, >50-fold after 10 to 12 days of culture (21) (data not shown), a <5-fold increase was seen in the presence of IL-2 (data not shown). The ability of the anti-Fas Ab, APO-1, to almost completely inhibit γδ T cell apoptosis induced by IL-2 and Mtb-SHT or anti-CD3 (Fig. 8) demonstrates that the synergistic effect of IL-2 on Mtb-mediated AICD of Vγ9+/Vδ2+ T cells is mediated by Fas-FasL interactions. Together, these findings demonstrate that IL-2 influences the outcome of Mtb-SHT Ag stimulation of γδ T cells and, in combination with the Mtb-SHT Ag preparation, promotes apoptotic cell death rather than growth.

**Discussion**

Collectively, the observations we have made show that compared with αβ T cells, Mtb-reactive, Vγ9+/Vδ2+, γδ T cells are more susceptible to Mtb Ag-mediated AICD, and that the Fas-FasL pathway is involved in γδ T cell apoptosis. These findings provide an explanation for the loss or the absence of Vγ9+/Vδ2+ T cells in patients with active TB and may also explain the reduced response of γδ T cells to Mtb infection in the absence of IL-2. However, the precise mechanisms that underlie the increased susceptibility of γδ T cells to be induced by Mtb Ag in the absence of IL-2 require further investigation.
in patients with active TB. The demonstration of Ag (Mtb)-mediated AICD is both consistent with and now extends the observations of previous studies demonstrating that γδ T cells readily undergo apoptosis in culture with anti-CD3 and IL-2 (6, 24, 25). Interestingly, our in vitro studies show that Mtb Ags are more effective than anti-CD3 at inducing AICD of (Vγ9/Vδ2) γδ T cells. This difference may be related to differences in the outcome of signaling via the TCR vs the CD3 complex alone. For example, since we have shown that the presence of IL-2 potentiates anti-CD3 and Mtb-SHT-mediated Vγ9/Vδ2 T cell apoptosis, it may be due to qualitative or quantitative differences in the ability of these stimuli to induce IL-2 production by γδ T cells or other (αβ) T cells present in these cultures.

By identifying the pathways involved in Mtb-mediated γδ T cell apoptosis, our findings extend those made recently by Duarte and colleagues describing Mtb (H37Ra)-mediated γδ T cell apoptosis (26) in patients with advanced forms of TB. Our demonstration of the rapid and sustained expression of FasL, by activated polyclonal populations of γδ T cells in both healthy subjects and TB patients is in agreement with a recent study showing that Vδ1-expressing γδ T cell clones obtained from synovial fluid of patients with Lyme arthritis (38) express high levels of FasL for a longer period after in vitro activation than αβ T cells. Up-regulation of FasL expression by γδ T cell clones from Lyme disease patients was also associated with broad spectrum cytotoxicity. However, using conventional 51Cr release assays or DNA fragmentation of [3H]Tdr-labeled cells (JAM assay) to detect cell-mediated cytotoxic activity, we were unable to demonstrate any autologous cell-directed cytotoxicity by Mtb-stimulated FasL+ Vδ2+ γδ T cells (B. Li and S. R. Carding, unpublished observations). Indeed, our finding that Mtb-mediated AICD of γδ T cells is abrogated by blocking Fas-FasL interactions suggests that activation by Mtb Ags results in expression of both Fas and FasL, making them targets for and effectors of Fas-mediated cytotoxicity. The more rapid up-regulation and persistent expression of Fas that occur in γδ T cells compared with CD4+ αβ T cells after Mtb-SHT stimulation is also consistent with this interpretation and may explain in part the increased susceptibility of γδ T cells to AICD.

Although the in vitro culture conditions used in this study may not necessarily reflect those found in vivo during mycobacterial infection, the results we have obtained suggest that the conditions under which γδ T cells are exposed to Mtb Ags is an important factor in determining their response and fate. For example, patients with clinically advanced forms of pulmonary TB will have high levels of bacteria due to the inability to contain and prevent their spread. This would presumably result in chronic T cell activation, high local concentrations of IL-2, and sustained expression and production of Fas and FasL. These are conditions that we have shown to result in AICD of Mtb-reactive Vγ9/Vδ2 T cells. Conversely, conditions under which bacterial growth is contained and restricted to the lung would result in acute exposure of T cells to relatively low amounts of Ag, resulting in transient or lower levels of T cell activation and IL-2 production. This would produce conditions that in the majority of individuals exposed to Mtb would favor the survival and expansion of Vγ9/Vδ2+ T cells. Our findings that IL-2 influences the outcome of Mtb Ag stimulation of γδ T cells (Fig. 8) and that the magnitude, kinetics, and duration of γδ T cell responses to intracellular bacteria in vivo are directly related to the dose of bacteria and the sites at which infection is established (39) are consistent with this hypothesis.

Although Mtb Ags could directly mediate AICD of γδ T cells, they could also be indirectly involved. For example, by the ability of the bacteria to modulate the activity of macrophages (22, 40) and CD4+ αβ T cells (21, 23, 41) that normally interact with and regulate the response of γδ T cells to Mtb. Whatever the mechanism, the persistence of high levels of bacteria and chronic exposure of Vγ9+Vδ2+ T cells to Mtb Ags provide an explanation for why in patients with active disease this population of γδ T cells remains low but recovers in patients that respond to drug therapy. The lack of response of residual γδ T cells from patients with advanced clinical forms of TB to in vitro challenge with Mtb-SHT (21) and other Ags (42) could reflect the elimination, rather than hyporesponsiveness (anergy), of Mtb-reactive cells in vivo as a result of chronic stimulation and AICD. Studies to analyze the Vγ9/Vδ2-TCR repertoire in TB patients should provide additional insights into γδ T cell function in mycobacterial disease.

The current findings may also bear on observations that in various animal models of infectious and autoimmune disease, inflammation and disease pathology are more rapid in onset and more severe in the absence of γδ T cells. For example, the inflammatory response is accelerated in a model of orchitis in which γδ T cells are depleted (43). Unusual lesions and pathologies develop in tissues of bacteria-infected (44–46) or parasite-infected (47) γδ-deficient or -deleted mice. Also, inflammation is more aggressive in collagen- (48) and adjuvant-induced (49) arthritis in the absence of γδ T cells. Together with the finding that γδ T cells can modulate the activity of other immune cell populations (50–55), it seems likely, therefore, that they perform an immunoregulatory function, being able to initiate as well as regulate the immune response to a variety of pathogens. Based upon our previous analyses of influenza virus-induced pneumonia in mice, we have proposed that γδ T cells are part of an inflammatory immune cell circuit made up of macrophages and αβ T cells as well as defined subsets of γδ T cells (reviewed in Refs. 50 and 51). Through the production of specific cytokines, these γδ T cells are able to modulate the activity of the other cellular components of the inflammatory immune cell circuit and to resolve the immune response. Thus, in individuals infected with Mtb, the absence of such an immunoregulatory (Vγ9/Vδ2) γδ T cell population and the inability to effectively down-modulate inflammatory immune responses could exacerbate cell and tissue necrosis and promote disease progression rather than protective immunity. A role for γδ T cells in the prevention or amelioration of immunopathology is attractive in light of the exaggerated pathology in Mtb-infected mice lacking γδ T cells and in TB patients in whom Mtb-reactive γδ T cells are reduced in number (21).

Although our studies cannot establish whether the γδ T cell phenotype seen in patients with active TB is causally related to disease, they do suggest that Vγ9/Vδ2+ T cells are an important component of the cell-mediated immune response to Mtb. Studies are in progress to determine how Mtb-reactive Vγ9/Vδ2+ T cells can interact with and if they can modulate the function of other immune cell populations. Overall, the current findings are consistent with the concept that γδ T cells, particularly the Vγ9+γδ T cells, provide important insights into the role of regulatory (Vγ9/Vδ2) γδ T cell populations in regulating the response of other immune cells to Mtb. Whatever the mechanism, the persistence of high levels of bacteria and chronic exposure of Vγ9+/Vδ2+ T cells to Mtb Ags provide an explanation for why in patients with active disease this population of γδ T cells remains low but recovers in patients that respond to drug therapy. The lack of response of residual γδ T cells from patients with advanced clinical forms of TB to in vitro challenge with Mtb-SHT (21) and other Ags (42) could reflect the elimination, rather than hyporesponsiveness (anergy), of Mtb-reactive cells in vivo as a result of chronic stimulation and AICD. Studies to analyze the Vγ9/Vδ2-TCR repertoire in TB patients should provide additional insights into γδ T cell function in mycobacterial disease.

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References


