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Human Purified Protein Derivative-Specific CD4+ T Cells Use Both CD95-Dependent and CD95-Independent Cytolytic Mechanisms

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CTL, both CD4+ and CD8+, are essential in the eradication of intracellular pathogens. Data generated using murine T cells have suggested a critical role for CD95 (Fas, Apo-1) in CD4+ T cell-induced apoptosis of target cells. In contrast, CD8+ CTL predominantly use the perforin/granzyme lytic pathway. At present little is known about the mechanism of CD4+ CTL lytic function during intracellular infection in humans. We have used human CD4+ T cells specific for purified protein derivative (PPD) of Mycobacterium tuberculosis to explore whether CD95 is the dominant cytolytic mechanism. PPD-reactive CD4+ clones efficiently lyed Ag-pulsed autologous monocytes, adherent macrophages, and EBV-transformed B cells. Addition of an antagonistic CD95 Ab had a minimal effect on cytolysis, whereas addition of MgEGTA to block perforin/granzyme resulted in complete inhibition of killing. In contrast, lysis of activated peripheral blood B cells could be partially blocked with the antagonistic CD95 Ab. Supporting these observations, monocytes, macrophages, and EBV-transformed B cells were not lysed by an agonistic CD95 Ab. Activated B cells were readily lysed by the agonistic CD95 Ab. T cell clones triggered through the TCR with anti-CD3 were capable of lysing the CD95-sensitive Jurkat T cell line in a CD95-dependent manner, but were also able to release granzymes. We conclude that human CD4+ T cells are capable of lysing PPD-pulsed targets using both perforin/granzyme and CD95 pathways. The contribution of CD95 is strictly dependent on target cell susceptibility to CD95-mediated killing.

CA). Agonistic anti-human CD95 mAb (clone CH-11) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). CH-11 is an IgG Ab and is able to cross-link CD95 in solution and induce an apoptotic signal in susceptible cells. The agonistic CD95 Ab Fas M3 (Immunex Corp., Seattle, WA) is an IgG1 Ab that does not cross-link CD95 in solution and blocks CH-11- and CD95L-induced apoptosis (14). MgEGTA (0.1 M EGTA/0.2 M MgCl2 Sigma Chemical Co., St. Louis, MO; pH 7.4) was prepared by the method of MacLennan (20). Recombinant soluble human CD40 ligand (CD40L) was expressed and purified from the supernatant of Saccharomyces cerevisiae (21). Human IL-4 was produced at Immunex Corp.

**Cell lines**

The T lymphoma cell line Jurkat was obtained from American Type Culture Collection (Rockville, MD). EBV-transformed B cell lines were generated in our laboratory using supernatants from the cell line 9B5-8. Cell lines were maintained by continuous passage in RPMI culture medium supplemented with 10% FCS.

**Flow cytometry**

Cells to be analyzed for CD95 expression were first incubated at 4°C in a blocking solution of PBS containing 2% normal rabbit serum and 2% normal goat serum to prevent nonspecific binding of mouse Ig. Cells were washed in FACS buffer (PBS containing 0.5% FBS and 0.02% sodium azide) and incubated with either the anti-CD95 Fas M3 Ab (5 μg/ml) or an IgG1 control (Becton Dickinson Immunocytometry Systems, San Jose, CA; 5 μg/ml) for 30 min at 4°C in a total volume of 50 μl. Cells were then washed and incubated in 50 μl of a 1/100 dilution of goat anti-mouse IgG-phycocerythrin (Tago, Burlingame, CA) for 30 min at 4°C. Flow cytometry was performed using a FACS Calibur (Becton Dickinson), and data were collected on 10^4 viable cells.

**Separation of target cell subsets**

PBMC from healthy PPD-positive individuals (>10-mm Mantoux skin test) were isolated from heparinized blood by centrifugation over Ficoll-Hypaque (Sigma) and washed three times with culture medium. T cell-depleted targets were isolated from human PBMC by rosetting with 2-aminomethylisothiouronium bromide hydrobromide-treated sheep erythrocytes. These cells were then layered on Histopaque (Sigma), and the cell pellet was discarded. Following hypotonic lysis of sheep erythrocytes, PBMC from healthy PPD-positive individuals (Costar no. 3524) in 2 ml of culture medium. Cultures were incubated at 37°C in 5% CO2 for 5 to 7 days. At this time, FACS analysis revealed that these cells were >90% monocytes. Monocytes were maintained in polypropylene tubes to minimize adherence.

**Generation of PPD-specific T cell lines and clones**

PBMC from healthy PPD-positive individuals were isolated from heparinized blood by centrifugation over Ficoll-Hypaque and washed three times with culture medium. One million cells were cultured in the presence of PPD (Lederle, PA) at a concentration of 10 μg/ml in 16-mm wells (Costar no. 3524) in 2 ml of culture medium. Cultures were incubated at 37°C in 5% CO2 for 5 to 7 days. At this time, FACS analysis revealed that these cells were >85% CD4^+.

To generate a panel of CD4^+ T cell clones reactive to PPD, a cell line was incubated for 3 days in 5% CO2 as described above. T cells were then cloned by limiting dilution in the presence of 2 × 10^6 irradiated (3500 rad using a 137Cs source) heterologous PBMC in the presence of anti-CD3 (10 ng/ml) and rIL-2 (10 ng/ml). Cell culture media consisted of 200 μl of RPMI supplemented with 10% FBS. T cell clones were selected based upon their proliferative responses to PPD.

**Cytotoxicity assay**

A standard 4- to 5-h 51Cr release assay was used to assess cytolytic activity. Briefly, target cells were labeled with 50 μCi of 51Cr for 16 h at 37°C in 1 ml of culture medium in the presence of either PPD (25 μg/ml) or se-
Lysis of Jurkat targets by CD4⁺ redundant. lysis during the 4-h assay; and 3) the killing pathways were possibilities: 1) the T cell clones were deficient in their expression of CD95; 2) these targets were not susceptible to CD95-mediated lysis during the 4-h assay; and 3) the killing pathways were redundant.

Lysis of Jurkat targets by CD4⁺ T cell clones is CD95 dependent

To test whether these CD4⁺ T cell clones were able to express CD95L, clones were activated with immobilized (plate-bound) anti-CD3 and assessed for their ability to lyse the CD95-sensitive Jurkat cell line in a bystander assay. As shown in Figure 3A, the experiment suggested that lysis of these targets was probably occurring through the perforin/granzyme pathway. To date, a total of eight PPD-specific clones from two donors have been analyzed, and all are cytolytic. Of the four clones from two donors that have been evaluated, all lyse Ag-pulsed macrophages in a Ca²⁺-dependent manner (data not shown). We considered the following possibilities: 1) the T cell clones were deficient in their expression of CD95L; 2) these targets were not susceptible to CD95-mediated lysis during the 4-h assay; and 3) the killing pathways were redundant.

CD4⁺ CTL clones were capable of lysing Jurkat targets in a manner that was completely blocked by the addition of the anti-CD95 Fas M3 Ab. Supporting a primary role for CD95 in this assay, the removal of extracellular calcium with MgEGTA resulted in minimal inhibition of cytolytic activity (Fig. 3B). Thus, we concluded that these PPD-specific T cell clones were capable of using both the perforin-granzyme and CD95 cytolytic pathways. As a result, utilization of the CD95/CD95L pathway probably depends upon the susceptibility of the target cell to CD95-mediated apoptosis.

Expression of CD95 by a variety of targets

For a CD4⁺ CTL to kill its target via the CD95 apoptotic pathway, the target cell must both express CD95 and be susceptible to CD95-mediated apoptosis. We initially analyzed a variety of targets for CD95 expression. Flow cytometric analysis of purified cell subsets revealed the presence of CD95 on a variety of targets (Fig. 4). The Jurkat cell line, B cell blasts, elutriated monocytes, and EBV-transformed lymphoblastoid cells all exhibited high levels of CD95. Adherent macrophages expressed lower levels of CD95. The agonistic anti-CD95 Ab CH-11 was used to assess susceptibility to CD95-dependent lysis by 4-h ⁵¹Cr release or by uptake of trypan blue after overnight incubation with the CH-11 Ab (Table I). Jurkat cells and B cell blasts were both sensitive to CD95-mediated apoptosis, whereas monocytes and adherent macrophages were relatively refractory to cytolytic activity in these assays. Interestingly, the EBV-transformed lymphoblastoid cell line that expressed high levels of CD95 was relatively refractory to CH-11-mediated lysis. Similar data have been obtained using lymphoblastoid cell line (LCL) generated from four different individuals (data not shown). These data confirmed that CD95 expression was necessary but not sufficient for susceptibility to CD95-mediated lysis and predicted that among APC, only the B cell blast population would be lysed in a CD95-dependent manner.

Utilization of CD95 lytic pathway is correlated with CD95 susceptibility

To test the hypothesis that Ag-specific CD4⁺ T cell-mediated lysis could use both the perforin/granzyme and CD95 cytolytic pathways, PPD-specific T cell clones were tested for their ability to lyse a panel of target cells. As shown in Figure 5, these clones
were capable of efficient short term lysis of macrophages, elutriated monocytes, LCL, and B cell blast targets. However, significant inhibition with the CD95 Fas M3 Ab could only be demonstrated with the B cell blast targets (Table II). Addition of an isotype-matched control Ab had no effect. In addition, we assessed the ability of the PPD-specific T cell clones to lyse Mtb-infected monocytes. At an E:T cell ratio of 20:1, we observed 25% specific lysis, which was completely blocked by the addition of MgEGTA (data not shown).

CD4⁺ T cell clones release serine esterase when activated

While utilization of the perforin-granzyme pathway is inferred from the dependence of lytic activity on extracellular calcium, we sought to directly demonstrate the release of granule contents of the PPD-specific T cell clones upon activation. The CD4⁺ T cell clones were incubated in the presence of immobilized anti-CD3 for 4 h, and the supernatants were assessed for their ability to cleave the hexosaminidase substrate. As shown in Table III, PPD-specific

![Image](image1.png)

**Figure 4.** Expression of CD95 by various cell types. Adherent macrophages, elutriated monocytes, positively selected CD19⁺ B cells stimulated with CD40L (3 μg/ml) and IL-4 (30 ng/ml), an autologous lymphoblastoid cell line, and the Jurkat T cell line were stained with Fas M3 (5 μg/ml) or an isotype-matched control Ab followed by goat anti-mouse F(ab')₂ conjugated to phycoerythrin. Flow cytometry was performed with a FACSCalibur.

were capable of efficient short term lysis of macrophages, elutriated monocytes, LCL, and B cell blast targets. However, significant inhibition with the CD95 Fas M3 Ab could only be demonstrated with the B cell blast targets (Table II). Addition of an isotype-matched control Ab had no effect. In addition, we assessed the ability of the PPD-specific T cell clones to lyse Mtb-infected monocytes. At an E:T cell ratio of 20:1, we observed 25% specific lysis, which was completely blocked by the addition of MgEGTA (data not shown).

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![Image](image2.png)

**Figure 5.** A PPD-specific T cell clone efficiently lyses CD95-sensitive and CD95-insensitive targets. Clone 80-20 was assessed 10 days after anti-CD3 stimulation for its ability to lyse autologous adherent macrophages, elutriated monocytes, a lymphoblastoid cell line, and positively selected CD19⁺ cells treated for 3 days with CD40L (3 μg/ml) and IL-4 (30 ng/ml). Targets were incubated overnight with secreted proteins from Mtb (20 μg/ml; circles). Before the 5-h assay, Fas M3 (5 μg/ml; triangles), MgEGTA (2 mM; diamonds), or both (open squares) were added. Control targets are shown as squares. This experiment is representative of three such experiments.

### Table I. Susceptibility of various targets to CD95-mediated cell death

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Chromium Release⁺</th>
<th>Overnight Trypan⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH-11⁺</td>
<td>CH-11 + Fas M3⁺</td>
</tr>
<tr>
<td>Jurkat</td>
<td>31</td>
<td>1</td>
</tr>
<tr>
<td>Adherent macrophages</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Elutriated monocytes</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>E-</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>B cells</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td>LCL</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

⁺ A total of 5 x 10⁵ ⁵²Cr labeled targets were incubated in the presence of CH-11 (1 μg/ml) for 5 h. Percent specific lysis was calculated as described in Materials and Methods. Results represent the mean of triplicate determinations.

⁺⁺ Cells 1 x 10⁶ were incubated overnight in the presence of CH-11. Two hundred cells were counted, and cell viability assessed by trypan blue exclusion. Minimal (<5%) cell death was noted in the absence of CH-11.

⁺⁺⁺ CD95 agonist mAb CH-11 used at 1 μg/ml.

⁺⁺⁺⁺ CD95 agonist CH-11 along with CD95 antagonist mAb Fas M3 at 5 μg/ml.

⁺⁺⁺⁺⁺ Not done (ND).
confirm the potential for CD4⁺ CTL to use both the CD95 and perforin/granzyme cytolytic pathways.

Discussion

In vitro studies with murine CD4⁺ T cells have suggested that the predominant mechanism for their cytolytic capacity involves CD95L interaction with CD95 on the target cell (16–18). In this study, we have found that human PPD-specific CD4⁺ T cell clones are capable of efficiently lysing a variety of targets using both CD95-dependent and CD95-independent mechanisms. In short term Ag-dependent cytotoxicity assays, addition of the antagonistic CD95 Ab Fas M3 had no effect on the lysis of macrophage, monocyte, and LCL targets and had a partial effect on the lysis of B cell blasts. These data correlated well with the observed susceptibility to lysis by the agonistic CD95 Ab CH-11 on these targets. In our experiments, adherent macrophages and monocytes were relatively insensitive to CD95-mediated lysis. These data are consistent with the observation that human monocytes and macrophages are resistant to CD95-mediated apoptosis unless activated (25, 26). In contrast, B cell blasts were CD95 sensitive, a finding consistent with previous reports (27, 28).

Several experiments suggested that the short term cytosis we observed was due to activation of the perforin/granzyme system. First, we could detect the release of serine esterase following stimulation of the T cell clones and the presence of perforin mRNA by RT-PCR. Second, addition of MgEGTA resulted in complete inhibition of lysis of the CD95-insensitive targets and only partial inhibition of the B cell blast targets.

We show that the CD4⁺ T cell clones are capable of inducible CD95L expression by RT-PCR, and functionally in a bystander assay, as lysis of the CD95-negative Jurkat cell line was effectively inhibited by the antagonistic CD95 Ab, while addition of MgEGTA had little effect. Our experiments might underestimate the role of CD95 in two ways. First, chelation of extracellular calcium may inhibit T cell activation and thus the induction of CD95L expression, an obvious requirement for utilization of the CD95 lytic pathway. Second, if the kinetics of apoptosis induced by CD95 were slower than those of perforin-granzyme, our short term assays would favor detection of the former mechanism. Nonetheless, the efficient lysis of CD95-insensitive targets in a short term assay supports a dominant role for the perforin-granzyme mechanism of cytosis.

Our data also do not exclude the possibility of CD4⁺ T cell subsets that differentially use either the CD95 or perforin granzyme pathways. However, we have found that all our PPD-reactive T cell clones are capable of efficient short term cytosis of LCL targets. Additionally, it is possible that our CD4⁺ T cell clones are using an as yet undescribed, calcium-dependent mechanism that is independent of the perforin-granzyme pathway. However, experiments performed in the mouse model have suggested that the CD95 and perforin/granzyme mechanisms can account for all short term cytosis.

These observations may have important implications for the biologic role of CD4⁺ CTL. The ability of human CD4⁺ CTL clones to lyse a variety of targets using the perforin/granzyme mechanism would suggest a role for these cells in the recognition and the elimination of APC. Thus, CD4⁺ CTL may be important in host defense against intracellular pathogens, such as Mtb, whose host is the macrophage (29). Lysis of infected cells, particularly those in which containment has failed, might serve to release intracellular organisms into the extracellular space, allowing fresh macrophages to lyse the bacterium. Alternatively, cytotoxic T cells may play a unique role in host defense to Mtb through the release of granular constituents that promote the destruction of infected macrophages. The directed exocytosis of cytolytic granules by T cells induces apoptosis in the target cell. In this regard, it has been suggested that apoptosis will kill intracellular mycobacteria (30). However, mice deficient in the expression of perforin, granzyme, or CD95 (Fas) are still able to contain infection with Mtb (31, 32), suggesting that in the mouse model the secretion of macrophage-activating cytokines such as IFN-γ and TNF-α may be sufficient for protective immunity. Alternatively, other components of the cytotoxic granule may have a direct anti-mycobacterial effect (23, 33).

Since human CD4⁺ CTL are capable of using the CD95 lytic pathway, these cells may still have an important role in the modulation of the immune response. This role has been inferred from the development of a lymphoproliferative disorders both in the CD95-deficient lpr mouse (34, 35) and in patients with the Canale-Smith syndrome (36). An immunomodulatory role for CD95 is further supported by the observation that B cells become CD95

<table>
<thead>
<tr>
<th>Clone</th>
<th>CD95</th>
<th>Antagonista</th>
<th>MgEGTAa</th>
<th>Both</th>
</tr>
</thead>
<tbody>
<tr>
<td>80-16</td>
<td>35 ± 18d</td>
<td>66 ± 37</td>
<td>87 ± 29</td>
<td></td>
</tr>
<tr>
<td>80-20</td>
<td>42 ± 21</td>
<td>74 ± 35</td>
<td>95 ± 20</td>
<td></td>
</tr>
</tbody>
</table>

a CD19⁺ cells were positively selected from fresh, autologous PBMC and cultured in the presence of IL-4 and CD40L for 3 days.

b Antagonistic anti-CD95 antibody Fas M3 was added at 5 μg/ml.

c MgEGTA was added at 2 mM.

d Results are presented as the mean ± SD of three separate experiments.

Table II. Antagonistic CD95 antibody and MgEGTA inhibit CD4⁺ T cell lysis of B cell targets

<table>
<thead>
<tr>
<th>Clone</th>
<th>CD95</th>
<th>Optical Densityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>80-16</td>
<td>0.44 ± 0.04</td>
<td>0.66 ± 0.01</td>
</tr>
<tr>
<td>80-20</td>
<td>0.44 ± 0.01</td>
<td>0.75 ± 0.01</td>
</tr>
</tbody>
</table>

a CD4⁺ T cell clones were incubated in the presence of immobilized anti-CD3 for 2 h. Supernatants were assayed for the presence of serine esterase.

b Optical density read at 450 nm with 560 nm used as a reference.
sensitive when activated in the absence of Ag (28) and by the observation that activated CD4+ T cells lyse CD8+ T cells in a CD95-dependent manner (37). Recently, Vergelli et al. (38) have described autoreactive myelin basic protein-specific CD4+ T cell clones, a subset of which kill through CD95, a subset of which are presumed to kill through perforin, and a subset of which kill through both. Since we have not found evidence for PPD-reactive T cell clones that lyse exclusively through CD95, it is interesting to speculate that CD4+ T cell clones that are capable only of CD95-mediated lysis may play a unique immunoregulatory role.

In conclusion, we have found that human PPD-specific CD4+ CTL use both CD95-dependent and CD95-independent mechanisms. Collectively, the data suggest that CD4+ CTL may play an important role in the immune response to intracellular pathogens.

Acknowledgments

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References