Preferential Apoptosis of HIV-1-Specific CD4+ T Cells

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Preferential Apoptosis of HIV-1-Specific CD4\(^+\) T Cells\(^1\)

Feng Yun Yue,* Colin M. Kovacs,**† Rowena C. Dimayuga,‡ Xiao Xiao Jenny Gu,* Paul Parks,‡ Rupert Kaul,* and Mario A. Ostrowski\(^2\)\(^*\)†

In contrast to other viral infections such as CMV, circulating frequencies of HIV-1-specific CD4\(^+\) T cells in peripheral blood are quantitatively diminished in the majority of HIV-1-infected individuals. One mechanism for this quantitative defect is preferential infection of HIV-1-specific CD4\(^+\) T cells, although <10% of HIV-1-specific CD4\(^+\) T cells are infected. Apoptosis has been proposed as an important contributor to the pathogenesis of CD4\(^+\) T cell depletion in HIV/AIDS. We show here that, within HIV-1-infected individuals, a greater proportion of ex vivo HIV-1-specific CD4\(^+\) T cells undergo apoptosis compared with CMV-specific CD4\(^+\) T cells (45 vs 7.4%, respectively, \(p<0.05\), in chronic progressors). The degree of apoptosis within HIV-1-specific CD4\(^+\) T cells correlates with viral load and disease progression, and highly active antiretroviral therapy abrogates these differences. The data support a mechanism for apoptosis in these cells similar to that found in activation-induced apoptosis through the TCR, resulting in oxygen-free radical production, mitochondrial damage, and caspase-9 activation. That HIV-1 proteins can also directly enhance activation-induced apoptosis supports a mechanism for a preferential induction of apoptosis of HIV-1-specific CD4\(^+\) T cells, which contributes to a loss of immunological control of HIV-1 replication. *The Journal of Immunology, 2005, 174: 2196–2204.

CD4\(^+\) T cells are important as immune effectors against HIV-1, presumably, by providing optimal help for HIV-1-specific cytotoxic CD8\(^+\) T cells (1–5). Studies by Rosenberg et al. (6) have shown that individuals with strong HIV-1-specific CD4\(^+\) T cell proliferative responses to HIV-1 p24 Ag are able to better control their viremia than those with diminished or absent responses. Unfortunately, most infected individuals show poor or absent proliferative responses to HIV-1, even on successful antiretroviral therapy (7). In HIV-1-infected individuals, the frequency of IFN-\(\gamma\)-producing, HIV-1 gag-specific CD4\(^+\) T cells ranges from 0 to 1.3%, with average frequencies of 0.04–0.3%, depending on the assay methodology used (8–11). The frequency of IFN-\(\gamma\)-producing CD4\(^+\) T cells directed against the entire HIV-1 genome has been reported to range from 0 to 2.94% (9). These frequencies are remarkably low in contrast to those reported in CMV-seropositive individuals where CMV-specific CD4\(^+\) T cells range from 0.1 to 43% (median, 1.57%) of CD4\(^+\) T cells (12). The difference is surprising given that in HIV-1 infection the virus is readily detectable in plasma and is thus producing a strong antigenic stimulus. In addition, in an acute lymphocytic choriomeningitis virus (LCMV)\(^3\) murine model, >20% of CD4\(^+\) T cells have been shown to be LCMV specific at peak infection (13).

Such findings indicate a quantitative defect in the HIV-1-specific CD4\(^+\) T cell response. Recently, Douek et al. (14) showed that HIV-1 infects preferentially those CD4\(^+\) T cells that are HIV-1-specific, rather than CD4\(^+\) T cells specific for unrelated Ags. However, the HIV-1-infected cells represented <10.0% of HIV-1-specific CD4\(^+\) T cell population, suggesting that direct infection of these cells may not be entirely responsible for the observed quantitative defect.

Another potential mechanism for deletion of Ag-specific cells invokes apoptosis of these cells in the infected host. Increased spontaneous and activation-induced apoptosis of CD4\(^+\) and CD8\(^-\) T cells is clearly demonstrated in HIV-1 infection (15–17). However, it is unknown whether spontaneous or Ag-induced apoptosis occurs at a higher frequency in HIV-1-specific CD4\(^+\) T cells relative to CD4\(^+\) T cells of other specificities (e.g., CMV or EBV). In T cells, apoptosis can be triggered by signaling through cell surface death receptors, including the CD95 ligand/CD95 pathway (18). A second pathway of apoptosis induction involves mitochondrial damage and the opening of mitochondrial permeability transition pores, releasing apoptogenic proteins such as cytochrome \(c\), APAF-1, and caspase-9 (19). The latter pathway has been seen to operate in association with reactive oxygen species produced during T cell activation, leading to activation-induced cell death (20).

Recent studies have clearly shown that caspases, a family of cysteine proteases, play essential roles at various stages of apoptosis (19, 21–23). With triggering of cell death receptors such as CD95, caspase-8 is activated, whereas with mitochondrial damage, caspase-9 is activated. Both of these caspases have been shown to promote the cleavage of caspase-3 downstream (19, 22). Caspase-3, a major executor of apoptosis, when activated, results in cleavage of proteins related to cell structure, DNA repair, and mediators of protein synthesis, leading to dissolution of cell integrity and cell death (19, 21–23). Thus, activated caspase-3 has been identified as a marker for cellular apoptosis induced by various signaling pathways.

The aim of the current study was to evaluate the apoptotic potential of ex vivo HIV-1-specific and CMV-specific CD4\(^+\) T cells...
from a cohort of HIV-1-infected individuals displaying various rates of disease progression.

### Materials and Methods

#### Study participants

Clinical data from 20 HIV-1-infected individuals and 1 HIV-1-uninfected individual with primary CMV infection, who were recruited for this study, are listed in Table I. Three individuals were recently infected by HIV-1 within 6 mo of the study (diagnosed by recent seroreactivity to HIV-1) and were asymptomatic at time of blood draw. Thirteen individuals had chronic progressive HIV-1 infection defined as documented HIV-1 infection of at least 1 year, with evident CD4⁺ T cell decline and/or viral load >10,000 copies/ml (bDNA). All were asymptomatic. One HIV-1-infected individual (participant 17 (P17); see Table I) had slowly progressive HIV-1 infection, which we defined as having HIV-1 infection <7 years, less than a 50 CD4 T cell count decline/year, and a viral load <10,000 copies/ml (bDNA). Three individuals with long-term nonprogression were defined as having chronic infection for at least 7 years and no evidence of CD4⁺ T cell decline. One individual, P20, is a unique long-term nonprogressor who has high viral load set points despite stable CD4 count. All participants were antiretroviral naive at enrolment, and P1, P2, and P12 were studied before and after at least 6 mo of highly active antiretroviral therapy (HAART) who had viral loads <100 copies/ml at their subsequent study. All HIV-1-infected individuals in this study had positive CMV IgG Abs but negative IgM Abs (performed by EIA). We also had an opportunity to study a HIV-1-uninfected individual with primary CMV infection, P21. P21 is a 36-year-old woman who presented with acute mononucleosis. Investigations were negative for EBV (monospot negative) but were diagnostic of primary CMV infection. CMV IgG Abs were detected, CMV IgG was negative, and CMV antigenemia was positive at 1/100,000. Blood from P21 was sampled 20 wk after onset of symptoms. Informed consent was obtained from participants in accordance with the guidelines for conduct of clinical research at the University of Toronto and St. Michael’s Hospital. All investigational protocols were approved by the University of Toronto and St. Michael’s Hospital institutional review boards.

#### Source of Ags

We used the following Ags: yeast derived p55 of HIV-1 (Austral Biologicals) at 5 µg/ml, yeast cytochrome c protein (Sigma-Aldrich) control at 5 µg/ml was used as a negative control as previously done (10), and CMV lysate and control lysates were tested at various titrations and then used at a 1/200 final dilution (Virion).

#### Flow cytometry and intracellular staining

Intracellular staining of cytokines in fresh blood samples was performed according to BD Biosciences protocols (www.bdbiosciences.com). Briefly, fresh heparinized blood samples were incubated with Ag for 6 h in the presence of 10 µg/ml brefeldin A and 1 µg/ml anti-CD49d and CD28 Ab for costimulation (BD Biosciences) and then transferred to an 18°C water bath. The following day, the blood cells were lysed using FACS lysis solution (BD Biosciences) and then stained by a panel of conjugated Abs obtained from BD Biosciences (FITC, PE, PerCP, and allophycocyanin). The following Abs in various combinations were used: activated caspase-3-FITC, Bcl-2-PE, CD4-PerCP, IFN-γ-PE, or allophycocyanin, CD3-allophycocyanin, and respective isotype controls (BD Biosciences). For intracellular FLIP staining, a two-staining procedure involving initial staining with 1 µg/ml Rat IgG2a clone Dave-3 anti-FLIP Ab, followed by a FITC conjugated Ab to rat IgG at 1 µg/ml (Axkora). Cells were then washed and resuspended in 1% paraformaldehyde/BSA and then analyzed the following day on a FACSCalibur (BD Biosciences). Data were acquired by CellQuest software (BD Biosciences) and analyzed using Fio2 (Tree Star). A total of 100,000–200,000 events in the lymphocyte gate was acquired per sample.

#### TUNEL assay

Fresh heparinized blood samples were incubated with Ag for 6 h in the presence of 10 µg/ml brefeldin A and 1 µg/ml anti-CD49d and CD28 Ab for costimulation (BD Biosciences), and then lysed and fixed, as described above and then resuspended in 70% (v/v) ethanol overnight. The following day, cells were assayed by using the APO-BrdU TUNEL Assay kit (Molecular Probes) according to the manufacturer’s instructions. Gating was based on positive and negative control cells, which were supplied by Molecular Probes. In addition to Alexa Fluor 488 anti-BrdU Ab (FL1 gate), cells were also stained for intracellular IFN-γ-allophycocyanin and CD4-PerCP and then analyzed immediately by flow cytometry.

#### CD95 Ab studies

Freshly isolated PBMC from five HIV-1-infected individuals (four chronic progressors and one acute seroconverter) were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, and antibiotics (Invitrogen Life Technologies) overnight (16 h) at 37°C in a 5% CO₂ water bath. The following day, blood cells were lysed using FACS lysis and control lysates were tested at various titrations and then used at a 1/200 final dilution (Virion).

### Table I. Clinical, virologic, and immunologic characteristics of participants

<table>
<thead>
<tr>
<th>Participant</th>
<th>Clinical Diagnosis</th>
<th>CD4 Count (cells/mm³)</th>
<th>Viral Load (copies/ml)</th>
<th>HIVp55</th>
<th>CMV</th>
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</thead>
<tbody>
<tr>
<td>P1</td>
<td>Acute</td>
<td>280</td>
<td>278,936</td>
<td>0.52</td>
<td>1.21</td>
</tr>
<tr>
<td>P2</td>
<td>Acute</td>
<td>214</td>
<td>750,000</td>
<td>0.54</td>
<td>2.69</td>
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<td>P3</td>
<td>Acute</td>
<td>670</td>
<td>316</td>
<td>0.20</td>
<td>0.45</td>
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<tr>
<td>P4</td>
<td>Chronic progressor</td>
<td>230</td>
<td>142,561</td>
<td>0.94</td>
<td>2.63</td>
</tr>
<tr>
<td>P5</td>
<td>Chronic progressor</td>
<td>240</td>
<td>423,285</td>
<td>0.61</td>
<td>0.57</td>
</tr>
<tr>
<td>P6</td>
<td>Chronic progressor</td>
<td>220</td>
<td>250,000</td>
<td>0.09</td>
<td>3.41</td>
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<tr>
<td>P7</td>
<td>Chronic progressor</td>
<td>410</td>
<td>10,826</td>
<td>0.21</td>
<td>2.30</td>
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<tr>
<td>P8</td>
<td>Chronic progressor</td>
<td>250</td>
<td>125,000</td>
<td>0.24</td>
<td>0.46</td>
</tr>
<tr>
<td>P9</td>
<td>Chronic progressor</td>
<td>438</td>
<td>19,000</td>
<td>0.77</td>
<td>1.49</td>
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<td>P10</td>
<td>Chronic progressor</td>
<td>490</td>
<td>63,106</td>
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<td>1.70</td>
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<td>P11</td>
<td>Chronic progressor</td>
<td>400</td>
<td>47,271</td>
<td>0.37</td>
<td>2.90</td>
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<td>P12</td>
<td>Chronic progressor</td>
<td>560</td>
<td>23,104</td>
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<td>1.38</td>
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<tr>
<td>P13</td>
<td>Chronic progressor</td>
<td>320</td>
<td>17,498</td>
<td>0.11</td>
<td>0.62</td>
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<td>P14</td>
<td>Chronic progressor</td>
<td>720</td>
<td>40,800</td>
<td>0.30</td>
<td>1.80</td>
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<tr>
<td>P15</td>
<td>Chronic progressor</td>
<td>556</td>
<td>40,000</td>
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<td>0.62</td>
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<td>85,408</td>
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<td>P17</td>
<td>Slow progressor</td>
<td>840</td>
<td>359</td>
<td>0.85</td>
<td>1.94</td>
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<td>P18</td>
<td>LTNP</td>
<td>670</td>
<td>&lt;50</td>
<td>0.18</td>
<td>3.00</td>
</tr>
<tr>
<td>P19</td>
<td>LTNP</td>
<td>460</td>
<td>204</td>
<td>0.05</td>
<td>2.42</td>
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<td>P20</td>
<td>LTNP/high virus load</td>
<td>580</td>
<td>300,000</td>
<td>0.04</td>
<td>0.66</td>
</tr>
<tr>
<td>P21</td>
<td>Primary CMV/HIV negative</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
</tbody>
</table>

* Acute, recently acquired HIV infection within 6 mo; chronic progressor, HIV infected >1 year with CD4 decline or viral load >10,000; slow progressor, loss of <50 CD4/year or no progression, HIV infected ≤7 years, viral load <10,000; LTNP HIV-infected >7 years and no CD4 decline. n/d, not done.

† LTNP, long-term nonprogression.

‡ P20 has shown stable CD4 counts with high viral loads >10 years of HIV infection.

† LTNP, long-term nonprogression.
µg/ml monoclonal anti-CD95 Ab (IgM, Ch11; Immunootech) or isotype control (anti-TNP IgM; BD Pharmingen). The following day, specific and control Ags were added (p55 or CMV, same concentrations as above) to individual wells, as well as brefeldin A and 1 µg/ml anti-CD49d and CD28 Ab for costimulation (BD Biosciences), and the cells were harvested 6 h later for surface and intracellular IFN-γ and activated caspase-3 staining as described above.

Caspase inhibitor and manganese (III) tetraakis (5,10,15,20-benzoic acid) porphyrin (MnTBAP) studies

Freshly isolated PBMC from HIV-1-infected individuals were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, and antibiotics (Invitrogen Life Technologies) overnight (16 h) at 37°C in a 5% CO2 incubator at 1% humidity. The following day, cells were washed twice, resuspended in complete media, and stimulated for 6 h with HIV p55 Ag or control Ag in the presence of brefeldin A and anti-CD49d and CD28 Abs. The cells were then harvested and stained for IFN-γ and activated caspase-3 staining as described above.

Statistical analysis

Because the frequency of HIV-1-specific CD4+ T cells was often <0.5% of gated CD4+ T cells, to determine whether there was statistical significance between activated caspase-3 expression in HIV-1-specific CD4+ T cells compared with CMV-specific CD4+ T cells within an individual, we used the following formula that tests for significant differences between two proportions:

\[ p_{\text{HIV}} - p_{\text{CMV}} \sqrt{\frac{p_{\text{HIV}}(1 - p_{\text{HIV}}) + p_{\text{CMV}}(1 - p_{\text{CMV}})}{n_{\text{HIV}}n_{\text{CMV}}} z_{1 - a/2}^2} \]

where \( p_{\text{HIV}} \) is proportion of HIV-1-specific cells that express activated caspase-3; \( p_{\text{CMV}} \) is the proportion of CMV-specific cells that express activated caspase-3, \( n_{\text{HIV}} \) is the number HIV-1-specific CD4+ T cells, and \( n_{\text{CMV}} \) is the number of CMV-specific CD4+ T cells, and \( Z_{1 - a/2} \) is the quantile of standard normal and is 1.96, when \( a = 0.05 \).

Nonparametric statistical tests were used to avoid the assumption of normally distributed data sets. Paired groups were usually compared using Wilcoxon’s rank-sum test (two-tailed). Nonpaired or grouped with \( n < 5 \) were compared by the Student’s t test (two-tailed). For correlations of different variables within a group, we calculated Spearman’s correlation coefficient and tested whether it was statistically different from 0 by using an asymptotic normal approximation.

Results

Characterization of apoptotic CMV and HIV-1-specific memory CD4+ T cells in HIV-1-infected individuals

Because CMV-specific memory CD4+ T cells are easily detectable in CMV IgG Ab-positive individuals, we compared the apoptotic potential of HIV-1-specific memory CD4+ T cells to CMV-specific CD4+ T cells within the same individual in a cohort of HIV-1-infected individuals with varying rates of disease progression and HIV-1 plasma viral loads (see Table I). Using intracellular flow cytometry, we identified Ag-specific memory CD4+ T cells in whole blood samples by gating on CD4+ T cells, which express IFN-γ after brief exposure to the specific Ag in question. The number of Ag-specific cells were quantitated by calculating the frequency of cytokine-producing cells in Ag-stimulated conditions and subtracting the number of cytokine-producing cells in control Ag-stimulated conditions. Only samples that had background IFN-γ staining of <0.04% of total CD4+ T cells during control Ag-stimulated conditions were studied. As previously shown, for the cohort, we observed a greater frequency of ex vivo CMV-specific, IFN-γ-producing CD4+ T cells compared with those that were HIV-1 specific (median, 1.6 vs 0.23% of total CD4+ T cells, respectively, \( p < 0.05 \)). Caspase-3 is a key protease that is activated during the early stages of apoptosis and, as with other members of the caspase family, is synthesized as an inactive proenzyme (32 kDa) that is processed in cells undergoing apoptosis by self-proteolysis or cleavage by other caspases into a heterodimer of 17- and 12-kDa subunits. Ag-specific cells undergoing apoptosis were identified with a fluorescein-conjugated Ab that specifically recognizes these active subunits of caspase-3. A representative panel of three participants studied is illustrated in Fig. 1a, and summary data of all 20 participants from Table I are summarized in Fig. 1d. In the majority of participants (17 of 20), HIV-1-specific CD4+ T cells showed greater activated caspase-3 expression than those that were CMV specific, within the same blood sample. More specifically, activated caspase-3 expression was greater within HIV-1-specific CD4+ T cells in 16 of 17 with acute or chronic progressive HIV-1 (Table I; P1–P17), which was statistically significant in 16 of 17 individuals (see statistical analysis in Materials and Methods), whereas activated caspase-3 expression was not significantly greater within HIV-1-specific CD4+ T cells from the three long-term nonprogressors (P18–P20). For the entire cohort of HIV-1-infected individuals, a greater percentage of HIV-1-specific CD4+ T cells expressed activated caspase-3 compared with CMV-specific CD4+ T cells (mean, 28.7 vs 6.6%, respectively, \( p < 0.05 \)). In addition, ex vivo total CD4+ T cells that were unstimulated or stimulated with the mitogen, PHA showed 1.3 and 4.74% activated caspase-3 expression, respectively, on average (data not shown). Similarly, enhanced apoptosis of HIV-1-specific CD4+ T cells was also demonstrated using TUNEL staining (Fig. 1b and c) in three of three chronic progressors examined (mean, 33.7 vs 13.6%, for HIV-1 vs CMV, respectively, \( p < 0.05 \)). We were unable to assess apoptosis using annexin V staining because the fixation and permeabilization steps required to identify FIGURE 1. Characterization of apoptotic potential of virus-specific CD4+ T cells. Representative data from three HIV-1-infected individuals are shown in a. HIV-1- and CMV-specific cells were identified by culturing fresh whole blood samples in the presence of CMV lysate HIV-1 gag p55 Ag or control Ags (see Materials and Methods) for 6 h, with brefeldin A, and then assessed for IFN-γ expression by intracellular flow cytometry. The frequencies of Ag-specific cells seen in the second column are indicated as percentage of total CD4+ T cells and are calculated after subtraction from control Ag stimulation (always <0.04% of CD4+ T cells) in the first column. Cells are gated in the lymphocyte gate, and IFN-γCD4+ CD4+ expressing cells are then gated and examined for activated caspase-3 expression (third column). Positive caspase-3 expression is based on isotype-matched controls. Percentage of Ag-specific cells expressing activated caspase-3 are indicated above the gates for each sample in the third column. b, Apoptotic cells were examined using TUNEL staining. A representative experiment from one of three chronic progressors studied is shown. Gating for TUNEL positivity was based on positive and negative control cells supplied by Molecular Probes (data not shown). Summary data of TUNEL staining from three chronic progressors is shown in c. A value of \( p < 0.05 \) by Student’s t test. Summary data from all participants studied in Table I showing activated caspase-3 expression in Ag-specific cells are depicted in d. Each distinct symbol represents a particular participant. *p = 0.05 by Wilcoxon rank-sum test (two-tailed). ** Data from P20, a long-term nonprogressor with high HIV-1 viral loads. Correlations between viral load and CD4 count, and percentages of apoptosis of virus-specific CD4+ T cells in HIV-1-infected individuals are shown in e. Plasma viral load and CD4 count were determined in each participant from the same time point in which viral specific responses were measured. Values of \( p \) shown in each figure are determined by the Spearman rank correlation. The dashed line represents a regression line. f, Fresh blood from a HIV-1-uninfected individual (P21) with primary CMV infection was analyzed as in a.
Ag-specific cells abolished surface phosphatidylserine detection (data not shown).

Of note, we observed considerable variability of activated caspase-3 expression within HIV-1-specific CD4\(^+\) T cells between individuals (Fig. 1d). Thus, correlations were examined between HIV plasma viral load or CD4 count and activated caspase-3 expression within HIV-1- or CMV-specific CD4\(^+\) T cells (Fig. 1e). CD4 count negatively correlated with percentage of apoptotic HIV-1-specific CD4\(^+\) T cells but not with percentage of apoptotic CMV-specific CD4\(^+\) T cells (Fig. 1e). Similarly, activated caspase-3 expression within HIV-1-specific CD4\(^+\) T cells was significantly lower in slow or long-term nonprogressors (mean, 6%), than for recent seroconverters or chronic progressors (mean, 32 and 45%, respectively; p < 0.05 between all three groups). Activated caspase-3 expression within CMV-specific CD4\(^+\) T cells did not significantly differ between recent seroconverters, chronic progressors, and slow/long-term nonprogressors (means, 7 vs 7 vs 4%, respectively). In addition, activated caspase-3 expression did not differ between CMV- and HIV-1-specific CD4\(^+\) T cells in the slow/long-term nonprogressor group (6 vs 4%, respectively, p = 0.42). We studied one unusual individual (P20; Table I), a long-term nonprogressor, who was HIV-1 infected for at least 10 years, despite consistently having very high plasma viral loads >100,000 copies/ml. Ex vivo activated caspase-3 expression in both HIV-1- and CMV-specific CD4\(^+\) T cells was low in this individual (<5%; Fig. 1d), suggesting a viral phenotype that does not induce apoptosis.

It is possible that the CMV-specific CD4\(^+\) T cells from our HIV-1-infected cohort may not be responding to actively replicating CMV and thus have no antigenic stimulus in vivo to trigger apoptosis. We thus also studied a HIV-1-uninfected individual (Table I), a long-term nonprogressor who was HIV-1 infected for at least 10 years, despite consistently having very high plasma viral loads >100,000 copies/ml. Ex vivo activated caspase-3 expression in both HIV-1- and CMV-specific CD4\(^+\) T cells was low in this individual (<5%; Fig. 1d), suggesting a viral phenotype that does not induce apoptosis.

We next examined the effect of HAART on apoptotic potential of virus-specific CD4\(^+\) T cells. Six individuals who received HAART for at least 6 mo were studied (Fig. 2). P1, P2, and P12 (Table I) were studied before and after HAART (average of 8 mo; see Fig. 2, b and c). HAART was associated with a dramatic reduction in activated caspase-3 expression within HIV-1-specific CD4\(^+\) T cells (Fig. 2). Similarly, as previously demonstrated (8), we noted a decline in the absolute frequencies of HIV-1-specific CD4\(^+\) T cells post-HAART from 879/ml of blood to 330 cells/ml post-HAART (data not shown).

In summary, HIV-1-specific CD4\(^+\) T cells show greater apoptotic potential than CMV-specific CD4\(^+\) T cells, and the degree of apoptosis directly correlates with viral load (excluding P20, who represents an uncommon phenotype), with the greatest degrees of apoptosis being observed in chronic progressors with higher viral loads. The enhanced apoptosis of HIV-1-specific cells is reversible with HAART (Fig. 2).

Susceptibility to anti-CD95-induced apoptosis

Previous work has shown that HIV-1-specific CD8\(^+\) T cells have an increased propensity to anti-CD95-induced apoptosis (24). In contrast, susceptibility to anti-CD95 Ab-induced apoptosis of HIV-1-specific CD4\(^+\) T cells was not observed (Fig. 3) as only one of five individuals showed >50% enhanced activated caspase-3 expression within HIV-1-specific CD4\(^+\) T cells after CD95 stimulation. In contrast, in three of five individuals, CMV-specific CD4\(^+\) T cells had enhanced activated caspase-3 expression (>50%) with anti-CD95 Ab (Fig. 3), indicating that cytokine-producing, CMV-specific CD4\(^+\) T cells are more prone to CD95-induced apoptosis than those directed against HIV-1.

Bcl-2 and FLIP expression

Bcl-2 and FLIP are well-established inhibitors of apoptosis (19, 22). Bcl-2 blocks mitochondrial cytochrome c release, thus preventing activation of the caspase-9 pathway, and FLIP blocks...
CD95-mediated apoptosis by competing with pro-caspase-8 (19, 22). In HIV-1-infected individuals with chronic progressive infection, significantly lower levels of Bcl-2 were expressed within HIV-1-specific CD4+ T cells when compared with CMV-specific cells (Fig. 4, a and b; 32 vs 58%, respectively, p < 0.05). In individuals with slow or nonprogressive disease, differences in Bcl-2 expression between HIV-1- and CMV-specific CD4+ T cells were not observed (Fig. 4b; 46 vs 46%, p = 0.96). With regard to FLIP expression, although there was a trend to decreased FLIP within HIV-1- vs CMV-specific CD4+ T cells in chronic progressors, these differences were not statistically significant (Fig. 4c; 33 vs 38%, respectively, p = 0.58). Of note, however, was that FLIP expression was significantly increased in both CMV- and HIV-1-specific CD4+ T cells from slow/nonprogressors when compared with chronic progressors as a group (Fig. 4c; 80 and 86%, HIV-1 and CMV vs 33 and 38%, HIV-1 and CMV, respectively, p < 0.05).

Effect of caspase and free radical inhibitors on HIV-1-specific, IFN-γ-producing immunity

We postulated that if enhanced apoptotic potential of HIV-1-specific CD4+ T cells is partly responsible for the relatively low frequencies of IFN-γ-producing, HIV-1-specific CD4+ T cells in peripheral blood in HIV-1 infection, then treatment of PBMC with various apoptosis inhibitors may preserve HIV-1-specific CD4+ T cell immunity. We were interested in examining the effects of inhibition of two cell death pathways: death receptor-mediated (e.g., CD95) and mitochondrial pathways. Death receptor-mediated apoptosis predominantly induces caspase-8, whereas TCR-mediated activation induced apoptosis induces reactive oxygen species, resulting in mitochondrial damage and caspase-9 activation. Both pathways activate caspase-3 downstream. We thus studied the effects of pretreatment of ex vivo PBMC with the caspase-3 inhibitor, Boc-D-FMK; caspase-8 inhibitor, Z-IETD-FMK; caspase-9 inhibitor, Z-LEHD-FMK; and the free radical scavenger, MnTBAP, on rescuing HIV-1-specific CD4+ T cell responses. An example of a typical experiment is demonstrated in Fig. 5a. For this individual (Fig. 5a), pretreatment of ex vivo PBMC overnight with either a caspase-3 or caspase-9 inhibitor or MnTBAP markedly enhanced HIV-1-specific IFN-γ responses. Summary data from all participants studied are shown in Fig. 5b. Overall, inhibition of caspase-9 significantly enhanced HIV-1-specific CD4+ T cell responses. Inhibition of caspase-8 had no effect (zero of six individuals), and inhibition of caspase-3 showed a nonsignificant trend to enhanced responses (three of six individuals had >50% enhancement compared with control with caspase-3 inhibition). Pretreatment of ex vivo PBMC with MnTBAP enhanced IFN-γ responses to >50% of control in 7 of 14 individuals studied; however, these differences failed to reach statistical significance for the entire group.

Discussion

We have shown that ex vivo HIV-1-specific CD4+ T cells have greater apoptotic potential than those specific for CMV. That a large percentage of these HIV-1-specific CD4+ T cells are undergoing apoptosis (>30% of cells) indicates that HIV-1 Ag-specific apoptosis is likely a significant mechanism for loss of HIV-1-specific CD4+ T cell immune responses. In addition, the apoptotic potential of HIV-1-specific CD4+ T cells directly correlated with viral load and disease progression (i.e., CD4+ T cell count), suggesting a direct effect of viral replication on apoptosis. This also was shown clearly after HAART treatment, in which apoptosis of HIV-1-specific CD4+ T cells was dramatically reduced and approached to those that were CMV specific. It should also be noted that we had only used the p55 Ag of HIV gag to identify HIV-1-specific CD4+ T cells, and thus may be underestimating the degree of apoptosis in cells directed against other areas of the HIV-1 proteome.

A number of mechanisms have been postulated to play a role in the enhanced apoptosis of T cells in HIV-1-infected individuals. One could postulate that direct infection of HIV-1-specific CD4+ T cells could explain their preferential apoptosis. Our data do not support this as a sole mechanism for apoptosis, because on average only ~5% of HIV-1-specific CD4+ T cells have been previously shown to be infected (14), whereas, the percentage of apoptotic cells we have observed are at least 6-fold higher.

It has previously been shown that HIV-1-specific CD8+ T cells exhibit a 3-fold increase in sensitivity to CD95-induced apoptosis but not to activation induced apoptosis (24). However, we failed to demonstrate enhanced CD95-induced apoptosis in HIV-1-specific CD4+ T cells using a similar methodology. In addition, we were unable to rescue HIV-1-specific CD4+ T cell IFN-γ responses

**FIGURE 4.** Bcl-2 and FLIP expression within Ag-specific CD4+ T cells. Whole blood samples were stimulated with Ag as described in Fig. 1, and Ag-specific cells were assessed for intracellular Bcl-2 and FLIP. A representative experiment from a chronic progressor is shown in a; summary data of Bcl-2 expression from seven chronic progressors and four slow or long-term nonprogressors are shown in b; and summary data of FLIP expression in four chronic progressors and three slow or long-term nonprogressors in are shown in c. Each symbol represents an individual participant. HIV, HIV-1-specific CD4+ T cells; CMV, CMV-specific CD4+ T cells; CP, chronic progressor; SP, slow progressor. A value of p < 0.05 by the Wilcoxon rank-sum test (two-tailed).
with a caspase-8 inhibitor, which should block CD95-mediated apoptotic signaling pathways. We also observed no differences in intracellular FLIP expression, which counteracts caspase-8 activation, between HIV-1-specific and CMV-specific cells in chronic progressors. However, we did note significant decreases in FLIP expression in CD4\(^+\)/H11001 T cells of both specificities in chronic progressors when compared with slow or nonprogressors, suggesting a generalized enhanced sensitivity of CD4\(^+\)/H11001 T cells to CD95-mediated apoptosis in progressors.

Our findings support a mechanism of apoptosis of HIV-1-specific CD4\(^+\)/H11001 T cells that is consistent with mitochondrial damage and that specifically involves caspase-9 activation. Mitochondrial apoptosis is often observed with TCR-mediated activation-induced apoptosis (19, 22). This is supported by finding decreased Bcl-2 expression in HIV-1-specific CD4\(^+\)/H11001 T cells compared with those against CMV in chronic progressors and our ability to detect enhanced HIV-1-specific CD4\(^+\)/H11001 T cell IFN-γ frequencies after brief pretreatment of ex vivo cells with a caspase-9 inhibitor. Bcl-2 is an antiapoptotic protein that inserts into the outer mitochondrial membrane to maintain transmembrane integrity and is inhibited by other members of the Bcl-2 family (e.g., Bid, Bad, or Bax), which can then allow release of cytochrome c and caspase-9 activation and subsequent caspase-3 activation. Surprisingly, we obtained more consistent rescue of IFN-γ responses with caspase-9 inhibition compared with caspase-3 inhibition, supporting the observation that caspase-9 activation can also induce apoptosis independently of caspase-3 activation, probably by activating caspase-7 (19). Mitochondrial membrane integrity can also be damaged by an excess of free radical oxygen species after certain forms of T cell activation (20). We also observed that the free radical oxygen scavenger, MnTBAP, could enhance HIV-1-specific immune responses in 50% of individuals studied, again supporting a role of mitochondrial damage in apoptosis of HIV-1-specific CD4\(^+\)/H11001 T cells. Our findings also support those of Zaunders et al. (25, 26), who demonstrated in their study, high levels of spontaneous apoptosis observed in T cells obtained ex vivo during acute HIV-1 infection, which was associated with activation of caspases 3 and 9, and increased expression of the proapoptotic protein Bax and decreased expression of Bcl-2. In their cohort, high average HIV-1 viral loads of >500,000 copies/ml were reported, which suggests that high levels of circulating virus may also have generalized effects on T cells.

It is unclear whether the degree of apoptosis we observed is physiologic in response to the level of circulating Ag or pathologic due to direct effects of HIV-1. For example, the differences in apoptosis between CMV- and HIV-1-specific T cells may be related to functional differences of these cells in that most CMV-specific T cells in the individuals we studied are presumably resting memory T cells (as most individuals were not CMV viremic), whereas HIV-1-specific cells in chronic persistent infection are presumably active effector cells. Previous studies in the acute LCMV infection murine model show that older virus-specific memory cells, after Ag is
cleared, undergo less apoptosis than those found early in the infection (27, 28), with the enhanced apoptosis corresponding to the contraction phase of the expanded T cells before establishment of long-term memory. CMV-specific CD4+ T cells from our individual with primary CMV infection also did not show high levels of apoptosis, suggesting that the high levels of apoptosis may be unique to those cells specific for HIV-1. It should be noted, however, that if we had sampled P21 earlier (at the time of acute symptoms), we may have observed a greater degree of apoptosis because CMV viral loads would have been much higher at this time. In contrast to acute infections, however, chronic persistent viral infections are more complicated and likely are comprised of multiple expansions/contractions of virus-specific cells over time. It is also possible that the varying rates of apoptosis we observed between HIV-1- and CMV-specific cells may reflect the differentiation state of the Ag-specific cells. We have previously shown that, within the same individual, HIV-1-specific CD4+ T cells have an immature memory phenotype compared with CMV-specific cells, which tend to have a late effector phenotype (29). However, on the basis of previous studies, one would expect to see greater apoptosis within T cells with a late effector phenotype (30). Thus, the differences we observed cannot be explained by maturation phenotype of the cells. A comparison of other acute and chronic persistent virus infections in humans is clearly warranted to understand whether the high frequencies of apoptotic HIV-1-specific cells we have observed are appropriate or pathogenic.

There is considerable evidence showing that HIV-1 proteins can enhance the apoptotic potential of CD4+ T cells, above that usually observed with TCR-induced activation. HIV-1 gp120 expressed on APC has been shown to prime CD4+ T cells to apoptosis after signaling through their cognate Ag (TCR; Refs. 31 and 32). This latter mechanism also requires binding of the HIV-1 envelope to the CD4 receptor. Cicala et al. (33) demonstrated that the HIV-1 envelope induced activation of caspase-3 and caspase-6, both classical end effectors of apoptosis. Recent work exploring upstream mechanisms of envelope-mediated apoptosis showed that HIV-1 envelope induces activation of the proapoptotic proteins p53 and Puma, leading to Bax/Bak activation and inhibition of Bcl-2 (34, 35), indicating that HIV-1 envelope induces apoptosis through a mitochondrial damage mechanism. HIV-1 vpr has also been shown to induce loss of mitochondrial membrane integrity by competing with Bcl-2 and directly binding to the mitochondrial permeability transition pore complex (36). Also, the presence of the rare vpr R77Q mutation in virus isolates was associated with nonprogression and decreased apoptosis (36). Thus, it will be important to determine whether the one long-term nonprogressor in our cohort with high viral loads, but low levels of apoptosis in HIV-1-specific CD4+ T cells, carried this vpr mutant.

We postulate that HIV-1-specific memory CD4+ T cells, by virtue of their specificity, could be recruited to sites of HIV-1 replication and thereby be exposed to HIV-1 env and vpr proteins, thus priming them for apoptosis in contrast to CD4+ T cells of other specificities. Such a mechanism would be an efficient method for eliminating virus specific effector cells without having to directly infect them. Such high levels of apoptosis as observed here would explain the lack of detecting high frequencies of HIV-1-specific CD4+ T cells ex vivo, despite ongoing high levels of viral replication and Ag accumulation. This is in contrast to the relatively high levels of HIV-1-specific CD8+ T cells, which are observed at all stages of infection (9) because they should not be primed via HIV-1 envelope signaling through the CD4 receptor. It is also unclear from our data whether the generalized immune activation that is found in untreated HIV-1 infection is also driving apoptosis of Ag-specific cells, warranting future studies in this regard. For example, it would also be instructive to determine whether our one long-term nonprogressor with high viral load but little apoptosis within HIV-1-specific CD4+ T cells also had lower levels of T cell activation.

In summary, these results provide an additional mechanism to explain the progressive loss of HIV-1-specific CD4+ T cell response during acute and chronic infection, allowing uncontrolled viral replication. Our data also provide a rational basis for the clinical investigation of the recently described novel class of caspase inhibitors (37) in preserving and maintaining HIV-1-specific immune responses, particularly, in individuals who may have antiretroviral drug resistant virus.

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References