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HIV-Specific CD8\(^+\) T Cells Exhibit Markedly Reduced Levels of Bcl-2 and Bcl-x\(_L\)\(^1\)

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Human immunodeficiency virus-specific CD8\(^+\) T cells are highly sensitive to spontaneous and CD95/Fas-induced apoptosis, and this sensitivity may impair their ability to control HIV infection. To elucidate the mechanism behind this sensitivity, in this study we examined the levels of antiapoptotic molecules Bcl-2 and Bcl-x\(_L\) in HIV-specific CD8\(^+\) T cells from HIV-infected individuals. Bcl-2 expression was markedly decreased in HIV-specific CD8\(^+\) T cells compared with CMV-specific and total CD8\(^+\) T cells from HIV-infected individuals as well as total CD8\(^+\) T cells from healthy donors. CD8\(^+\) T cell Bcl-2 levels inversely correlated with spontaneous and CD95/Fas-induced apoptosis of CD8\(^+\) T cells from HIV-infected individuals. HIV-specific CD8\(^+\) T cells also had significantly lower levels of Bcl-x\(_L\) compared with CMV-specific CD8\(^+\) T cells. Finally, IL-15 induces both Bcl-2 and Bcl-x\(_L\) expression in HIV-specific and total CD8\(^+\) T cells, and this correlated with apoptosis inhibition and increased survival in both short- and long-term cultures. Our data indicate that reduced Bcl-2 and Bcl-x\(_L\) may play an important role in the increased sensitivity to apoptosis of HIV-specific CD8\(^+\) T cells and suggest a possible mechanism by which IL-15 increases their survival. The Journal of Immunology, 2004, 172: 4444–4453.

Various stimuli can up-regulate Bcl-2 and Bcl-x\(_L\) expression in T lymphocytes. IL-2-mediated survival of CD4\(^+\) T cells is dependent on IL-2R\(\beta\) chain that induces Bcl-2 expression (16). Similarly, IL-7 can enhance the survival of naive T cells by up-regulating Bcl-2 expression (17). The costimulatory molecule OX40 regulates CD4\(^+\) T survival by promoting the expression of Bcl-2 and Bcl-x\(_L\) (18). In contrast, activation of CD28, 4-1BB, and CD40 costimulatory pathways promotes the expression of Bcl-x\(_L\) rather than Bcl-2 (19–21).

IL-15 is a pleiotropic cytokine that is involved in the generation and maintenance of NK and memory CD8\(^+\) T cells (22, 23). In HIV-infected individuals, IL-15 can enhance activation and proliferation of CD8\(^+\) T cells (24, 25). Most importantly, we have recently shown that IL-15 increases survival and effector function (IFN-\(\gamma\)-production and direct ex vivo cytotoxicity) of HIV-specific CD8\(^+\) T cells (26) as well as that of total CD8\(^+\) T cells (27) from HIV-infected individuals. IL-15 may mediate this effect by up-regulating antiapoptotic molecules because IL-15 has been shown to be a potent up-regulator of Bcl-2 in T cells (28), while IL-15Ra deficiency leads to reduced expression of Bcl-2 in CD8\(^+\) T cells (29). Furthermore, IL-15 has been shown to up-regulate Bcl-2 in CD8\(^+\) T cells from HIV-infected individuals (11, 30). Whether this is the mechanism of action on HIV-specific CD8\(^+\) T cells, however, is yet unknown.

In an attempt to elucidate the mechanism responsible for the increased apoptosis of HIV-specific CD8\(^+\) T cells we previously described (26), we examined directly ex vivo the protein levels of the Bcl-2 and Bcl-x\(_L\) antiapoptotic molecules in HIV-specific and total CD8\(^+\) T cells from HIV-infected individuals. We report in this study that Bcl-2 levels are greatly reduced in HIV-specific CD8\(^+\) T cells compared with CMV-specific and total CD8\(^+\) T cells from HIV-infected individuals as well as CD8\(^+\) T cells from healthy subjects. Reduced Bcl-2 levels inversely correlated with both spontaneous and CD95/Fas-mediated apoptosis of CD8\(^+\) T cells from HIV-infected individuals, indicating that reduced Bcl-2 levels may contribute to apoptosis sensitivity. Bcl-x\(_L\) levels were also significantly reduced in HIV-specific CD8\(^+\) T cells compared...
with CMV-specific CD8+ T cells from HIV-infected individuals. Finally, IL-15 that inhibits apoptosis of HIV-specific CD8+ T cells (26) increases both Bcl-2 and Bcl-xL levels in both HIV-specific and total CD8+ T cells. This effect of IL-15 is accompanied by apoptosis inhibition and increased survival in both short- and long-term cultures. These findings suggest that reduced Bcl-2 and Bcl-xL levels may be responsible for the increased apoptosis sensitivity of HIV-specific CD8+ T cells and indicate that IL-15 increases the survival of HIV-specific CD8+ T cells by augmenting Bcl-2 and Bcl-xL levels.

Materials and Methods

Patients

Peripheral blood was collected from HIV-infected individuals (n = 48) following Drexel University Institutional Review Board approval and obtaining informed consent. All individuals were HIV positive for at least 1 year (range 1–22); the median CD4 count was 380 cells/μl (range 4–703); the median viral load was 1.286 RNA copies/ml blood (range <50–287,077); n = 45 were asymptomatic and n = 30 patients were on antiretroviral treatment. Control samples were obtained from 19 HIV-negative age-matched healthy individuals. All assays were performed on freshly isolated PBMC from HIV+ and HIV− individuals.

Flow cytometry

Flow cytometry was performed on freshly isolated PBMC, as previously described (5, 26). Briefly, PBMC were isolated by density centrifugation using Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) from heparinized venous blood of HIV-infected individuals and HIV-seronegative controls. HIV-specific and CMV-specific CD8+ T cells were identified using tetramers of HLA class I A*0201 loaded with either HIV-Gag p17 77–85 (SLYNTVATL), HIV-Pol 476–484 (ILKEPVHGV), or CMV p65 495–503 (NLVPVVATP) tetramer and tetramers of HLA class I A3 loaded with HIV-Nef 71–80 (QVPLRPMTYK) peptide, as previously described (31). The following Ab combinations were used: 1) for Bcl-2 and Bcl-xL levels, anti-CD4 FITC/anti-CD8 PE-Cy5/HIV− or CMV-specific tetramer allophycocyanin and anti-CD45RA FITC/anti-CD62L allophycocyanin and annexin V PE/anti-CD8 PE-Cy5/HIV− or CMV-specific tetramer allophycocyanin and annexin V PE/anti-CD4 FITC/anti-CD8 PE-Cy5/HIV− or CMV-specific tetramer allophycocyanin. Annexin V FITC was a kind gift from J. Tait (University of Washington, Seattle, WA), while annexin V PE was purchased from eBioscience (San Diego, CA). All Abs above were purchased from eBioscience.

Brieﬂy, 1 × 10^6 cells were stained with tetramers and Abs in HBSS (Cellgro, Herndon, VA), 3% FBS (Life Technologies, Carlsbad, CA), and 0.02% NaNO3 for 30 min on ice; washed twice with HBSS, 3% FBS, and 0.02% NaNO3, and fixed with 1% paraformaldehyde. When annexin V staining was performed, 2.5 mM CaCl2 was included in all steps. Samples were collected on a FACSCalibur (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo software (Treestar, San Carlos, CA). Flow cytometry was calibrated every day using Calibrate beads (BD Biosciences).

For standardization of the Bcl-2 and Bcl-xL stains, we used the same frozen PBMC sample from a healthy control in every experiment as a standard. Flow cytometry was calibrated every day using Calibrite beads (BD Biosciences). Flow cytometry was calibrated every day using Calibrite beads (BD Biosciences). Flow cytometry was calibrated every day using Calibrite beads (BD Biosciences). Flow cytometry was calibrated every day using Calibrite beads (BD Biosciences).

Apoposis studies

For spontaneous apoptosis measurement, freshly isolated PBMCs were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine, 105 U/ml penicillin, and 100 μg/ml streptomycin sulfate (Cellgro) for 14 h at 37°C, 5% CO2, at a density of 1 × 10^6 cells/ml in 24-well plates (Costar, Corning, NY) in the presence or absence of 5 ng/ml human rIL-15 (PeproTech, Rocky Hill, NJ).

3 Abbreviations used in this paper: MFI, mean fluorescence intensity; FMK, fluoro-methylketone.

For CD95/Fas-induced apoptosis, PBMC were cultured in plates coated with 5 μg/ml anti-Fas mAb (IgM, CH11; Immunotech, Brea, CA) in the presence or absence of 5 ng/ml IL-15 for 14 h at 37°C, 5% CO2. Cells were harvested and stained for apoptosis. Apoptotic cells were determined by annexin V positivity using flow cytometry.

Specific apoptosis was calculated using the formula: (percentage of induced apoptosis − percentage of spontaneous apoptosis) × 100/(100 − percentage of spontaneous apoptosis).

For long-term survival studies, PBMCs were cultured at 1 × 10^6 cells/ml in the presence or absence of 5 ng/ml IL-15 for 7 days at 37°C, 5% CO2 before cells were harvested, counted using 0.1% trypan blue solution (Cellgro), and stained with anti-CD4 FITC/anti-CD8 PE-Cy5/annexin V PE/HIV-specific tetramer allophycocyanin.

Intracellular Bcl-2 and Bcl-xL staining

Intracellular levels of Bcl-2 and Bcl-xL proteins were measured directly ex vivo in freshly isolated PBMCs or after culture for 14 h or 7 days in the presence or absence of 5 ng/ml IL-15. After surface staining with anti-CD4 FITC/anti-CD8 PE-Cy5/HIV− or CMV-specific tetramer allophycocyanin or anti-CD45RA FITC/anti-CD62L allophycocyanin/anti-CD8 PE-Cy5, cells were permeabilized with cytofix/cytoperm buffer (BD Biosciences) for 20 min on ice, and intracellular staining was performed for 1 h on ice with either an anti-Bcl-2 PE hamster mAb or a hamster isotype control PE mAb (BD Biosciences). For Bcl-xL staining, an anti-Bcl-xL PE and an IgG3-PE isotype control mAb were used (Southern Biotechnology Associates, Birmingham, AL). Cells were analyzed using a FACSCalibur and FlowJo software, as above.

In vitro caspase activity

In vitro caspase activity was detected using the caspa-Tag kit for caspase-8 (FAM-LET-D fluoromethylketone (FMK)) and caspase-3 (FAM-DEVDFMK) (Serologicals, Norcross, GA). Freshly isolated PBMC were cultured at 1 × 10^6 cells/ml/well in plates coated with 5 μg/ml anti-Fas mAb in the absence or presence of 5 ng/ml IL-15. Following overnight incubation, 300 μl of cell suspension were transferred to a new tube and further incubated for 1 h at 37°C, 5% CO2 in the presence of either FAM-LET-FMK or FAM-DEV-FMK peptide, according to the manufacturer’s instructions. Cells were then washed and stained with annexin V PE/anti-CD8 PE-Cy5/ HIV− or CMV-specific tetramer allophycocyanin. Alternatively, cells were stained with anti-CD8 PE-Cy5/HIV− or CMV-specific tetramer allophycocyanin, and subsequently, intracellular staining for Bcl-2 levels was performed, as described above. Data were collected using a FACSCalibur and analyzed using FlowJo software, as above.

CD8+ T cell purification: Western blot analysis

Total CD8+ T cells were purified from freshly isolated PBMC using the CD8 Positive Isolation Kit (Dynal Biotech, Great Neck, NY), according to the manufacturer’s instructions. Whole cell extracts were prepared from 10^6 purified CD8+ T cells in Tris-glycine-2× SDS sample buffer (Invitrogen, San Diego, CA) supplemented with 50 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM benzamidine, sonicated, and debris were removed by centrifugation. Boiled samples were resolved in 4–20% gradient gel (Invitrogen), and proteins were transferred to nitrocellulose membrane (Invitrogen). Following blocking with PBS-Tween 20 (0.05%)-5% skim milk, membranes were probed with a mouse anti-human Bcl-2 mAb (BD Biosciences) for 16 h at 4°C. After washing with PBS-Tween 20 (0.05%), membranes were incubated with secondary anti-mouse HRP-conjugated Ab for 2 h at room temperature. For β-actin staining, a second probing was performed on the same membrane using an anti-β-actin mAb (Sigma-Aldrich, St. Louis, MO). Protein bands were visualized by the Chemiluminescence Reagent Plus kit (PerkinElmer, Wellesley, MA). Quantitation was performed using a Bio-Rad computing imaging system (Gel Doc 2000; Bio-Rad, Hercules, CA).

Statistical analysis

Statistical analysis was performed using Student’s t test, paired Student’s t test, and regression analysis. Values of p < 0.05 were considered significant. The JMP statistical analysis program was used (SAS Institute, Cary, NC).

Results

Ex vivo Bcl-2 levels are greatly reduced in HIV-specific CD8+ T cells

We recently reported that HIV-specific CD8+ T cells are susceptible to spontaneous as well as CD95/Fas-induced apoptosis (5).
Because Bcl-2 is a potent inhibitor of apoptosis (12, 32) and Bcl-2 protein expression has been reported to be down-regulated in CD8+ T cells from HIV-infected individuals (10), we sought to investigate the ex vivo levels of the antiapoptotic molecule Bcl-2 in HIV-specific CD8+ T cells. For this purpose, freshly isolated PBMCs from HIV-positive individuals, as well as healthy controls, were stained with specific Abs and tetramers, and subsequently, the endogenous Bcl-2 protein levels were determined by intracellular staining.

Gating on the CD8high T cells, Bcl-2 expression was found to be markedly reduced in HIV-specific CD8+ T cells (MFI = 186 ± 26, n = 11) compared with total CD8+ T cells from either HIV-infected individuals (MFI = 370 ± 24, n = 46, p < 0.005) or healthy donors (MFI = 514 ± 35, n = 19, p < 0.005) (Fig. 1, B and C). This was true for all HIV-specific epitopes examined (A2-gag, 202 ± 34, n = 6; A2-pol, 146 ± 43, n = 2; and A3-nef, 178 ± 69, n = 3 specific CD8+ T cells). Bcl-2 levels were also reduced in total CD8+ T cells from HIV-infected individuals compared with healthy controls, as previously described (10) (Fig. 1, B and C). Furthermore, Bcl-2 levels in HIV-specific CD8+ T cells were reduced compared with CMV-specific CD8+ T cells (MFI = 336 ± 22, n = 15) from HIV-infected individuals (p < 0.001) (Fig. 1C). This correlates with our previous data showing that CMV-specific CD8+ T cells from HIV-infected individuals are not as sensitive to apoptosis as HIV-specific CD8+ T cells (5). Similar data were obtained when the total CD8 gate (CD8low + CD8high) was used (MFI = 361 ± 20, 496 ± 30, 186 ± 26, and 336 ± 22 for total CD8+ T cells from HIV-infected individuals, healthy donors, and HIV- and CMV-specific CD8+ T cells, respectively). This CD8low + CD8high gate, however, it should be pointed out, contains a small NK cell population (2.4 ± 1%). It should also be noted that all virus-specific CD8+ T cells fall within the CD8high gate.

Reduced Bcl-2 levels in purified CD8+ T cells of HIV+ patients were also confirmed by Western blot, thus validating our intracytoplasmic stains (Fig. 1A).

In contrast to total CD8+ T cells, no difference between Bcl-2 levels in CD4+ T cells from HIV-infected individuals (MFI = 476 ± 24, n = 31) and those from healthy controls was observed (MFI = 493 ± 36, n = 13) (Fig. 1, B and D). Taken together, these findings clearly demonstrate that ex vivo Bcl-2 protein levels in HIV-specific CD8+ T cells are greatly reduced.

**HIV-specific CD8+ T cells express lower Bcl-xL levels compared with CMV-specific CD8+ T cells in HIV-infected individuals**

Bcl-xL is another potent antiapoptotic factor that belongs to the Bcl-2 family of antiapoptotic molecules (12). In contrast to Bcl-2, Bcl-xL levels in HIV-specific CD8+ T cells (MFI = 38 ± 4, n = 11) and total CD8+ T cells from HIV-infected individuals (MFI = 39 ± 2, n = 37) were moderately increased compared with total CD8+ T cells from healthy controls (MFI = 31 ± 3, n = 16) when the CD8high gate was used (Fig. 2, A and B). Surprisingly, Bcl-xL expression was significantly higher in CMV-specific CD8+ T cells from HIV-infected individuals (MFI = 56 ± 4, n = 11) compared with HIV-specific CD8+ T cells (MFI = 38 ± 4, p < 0.005) and

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**FIGURE 1.** HIV-specific CD8+ T cells express reduced Bcl-2 protein levels ex vivo. A, Western blot showing the Bcl-2 levels in purified total CD8+ T cells from two healthy controls and one HIV-infected individual. Bar graphs (left) depicting Bcl-2 levels normalized against β-actin (in arbitrary units, A.U.), and the corresponding flow cytometry histograms (right) are shown in the lower panel. B, Representative flow cytometry from one HIV-infected individual showing HIV-specific CD8+ T cells, total CD8+ T cells, and CD4+ T cells. Cells were gated first for lymphocytes by forward and side light scatter and then for HIV-specific, total CD8+ and CD4+ T cells by tetramer and CD8 or CD4 staining. Analysis of CD8+ T cells was done using both CD8low and CD8high gate (large CD8 gate). Histograms depict ex vivo Bcl-2 expression in HIV-specific, total CD8high T cells and CD4+ T cells from a representative HIV-infected individual and an uninfected control. Uninfected controls in Gag-specific CD8+ T cell histogram depict total CD8high T cells. C, Pooled data showing MFI of Bcl-2 staining for HIV-specific CD8+ T cells (n = 11), CMV-specific CD8+ T cells (n = 15), and total CD8high T cells from HIV-infected individuals (n = 46), and total CD8high T cells from uninfected controls (n = 19). D, Pooled data showing MFI of Bcl-2 staining for CD4+ T cells from HIV-infected individuals (n = 31) and uninfected controls (n = 13). Horizontal lines depict mean values. The p values were calculated using Student’s t test.
A. Representative histograms showing ex vivo Bcl-xL levels in HIV-specific CD8+ T cells, total CD8high T cells, and CD4+ T cells from one HIV-infected individual and an uninfected control. Cells were gated as in Fig. 1. Uninfected controls in Gag-specific CD8+ T cell plot depict histogram for total CD8high T cells. B, Pooled data showing MFI of Bcl-xL staining for HIV-specific CD8+ T cells (n = 11), CMV-specific CD8+ T cells (n = 11), and total CD8high T cells from HIV-infected individuals (n = 37), and total CD8high T cells from uninfected controls (n = 16). C, Pooled data showing MFI of Bcl-xL staining for CD4+ T cells from HIV-infected individuals (n = 24) and CD4+ T cells from healthy donors (n = 10). Horizontal lines depict mean values. The p values were calculated using Student’s t test.

FIGURE 2. Impaired ex vivo expression of Bcl-xL in HIV-specific CD8+ T cells compared with CMV-specific CD8+ T cells. A, Representative histograms showing ex vivo Bcl-xL levels in HIV-specific CD8+ T cells, total CD8high T cells, and CD4+ T cells from one HIV-infected individual and an uninfected control. Cells were gated as in Fig. 1. Uninfected controls in Gag-specific CD8+ T cell plot depict histogram for total CD8high T cells. B, Pooled data showing MFI of Bcl-xL staining for HIV-specific CD8+ T cells (n = 11), CMV-specific CD8+ T cells (n = 11), and total CD8high T cells from HIV-infected individuals (n = 37), and total CD8high T cells from uninfected controls (n = 16). C, Pooled data showing MFI of Bcl-xL staining for CD4+ T cells from HIV-infected individuals (n = 24) and CD4+ T cells from healthy donors (n = 10). Horizontal lines depict mean values. The p values were calculated using Student’s t test.

total CD8+ T cells (MFI = 39 ± 2, p < 0.001) (Fig. 2B). Again, comparable data were obtained when gating on CD8low + CD8high cells (MFI = 37 ± 2, 33 ± 3, 38 ± 4, and 56 ± 4 for total CD8+ T cells from HIV-infected individuals, healthy donors, and HIV- and CMV-specific CD8+ T cells, respectively).

Because the differentiation state of CMV-specific CD8+ T cells differs from that of HIV-specific CD8+ T cells (5, 33), it is possible that the observed differences between them in Bcl-2 and Bcl-xL levels were due to their different effector phenotypes. To indirectly address this, we compared the expression level of these molecules in effector memory CD8+ T cells. First, we found higher levels of Bcl-2 and Bcl-xL in CD45RA+CD62L− (MFI = 446 ± 80 and 45 ± 6, respectively, for Bcl-2 and Bcl-xL) compared with CD45RA−CD62L− (MFI = 346 ± 52 and 35 ± 6, respectively, for Bcl-2 and Bcl-xL) CD8+ T cells from HIV-infected individuals (n = 8), although this difference was not statistically significant. By comparing the Bcl-xL levels in the CMV-specific CD8+ T cell population and the two effector memory phenotypes of total CD8+ T cells in HIV-infected individuals, we can start to address the possibility that higher expression of Bcl-xL in CMV- compared with HIV-specific CD8+ T cells is due to preferential differentiation of CMV-specific CD8+ T cells to the CD45RA+CD62L− phenotype. CMV-specific CD8+ T cells that are composed of both CD45RA+CD62L− and CD45RA−CD62L− are significantly higher for Bcl-xL (MFI = 56 ± 4) compared with the CD45RA−CD62L− CD8+ T population (MFI = 35 ± 6, p < 0.002), but not the CD45RA+CD62L− (MFI = 45 ± 6). HIV-specific CD8+ T cells that are predominantly CD45RA−CD62L− had similar Bcl-xL levels with CD45RA+CD62L− CD8+ T cells (MFI = 38 ± 4 vs 35 ± 6). Thus, these comparisons suggest that maturation may determine the levels of Bcl-xL with CD45RA+CD62L− CD8+ T cells expressing more, and this could explain the difference seen between HIV- and CMV-specific CD8+ T cells. In contrast, however, when we compared Bcl-2 levels, we find that HIV-specific CD8+ T cells have much lower levels of Bcl-2 compared with CD45RA+CD62L− CD8+ T cells (186 ± 26 vs 346 ± 52, respectively, p < 0.006). The CMV-specific CD8+ T cells also have lower levels of Bcl-2 compared with CD45RA−CD62L− CD8+ T cells (336 ± 22 vs 446 ± 80, respectively, p < 0.04), but did not differ from the CD45RA+CD62L− CD8+ T cells (336 ± 22 vs 346 ± 52, respectively). Thus, our data indicate that although higher expression of Bcl-xL in CMV-specific CD8+ T cells compared with HIV-specific cells may be due to their different maturation status, this, however, does not seem to be the case for Bcl-2 expression. Future studies directly comparing the levels of Bcl-2 and Bcl-xL in the effector memory phenotypes of these virus-specific populations are needed to conclusively answer this question.

Finally, Bcl-xL was also found up-regulated in CD4+ T cells from HIV-infected individuals (n = 24) compared with noninfected donors (n = 10) (MFI = 36 ± 2 vs MFI = 26 ± 2, p < 0.01) (Fig. 2, A and C). Because the balance between proapoptotic and antiapoptotic factors determines whether a lymphocyte will live or die (32), our data reveal a functional deficiency of antiapoptotic molecules in HIV-specific CD8+ T cells compared with other virus-specific CD8+ T cells from HIV-infected individuals that potentially makes them more susceptible to apoptosis.

Spontaneous and CD95/Fas-induced apoptosis inversely correlates with ex vivo levels of Bcl-2 in CD8+ T cells from HIV-infected individuals

To determine whether the ex vivo levels of Bcl-2 were predictive of apoptosis sensitivity and thus indirectly establish a potential link between Bcl-2 levels and apoptosis, we investigated whether reduced ex vivo levels of Bcl-2 correlated with increased sensitivity to spontaneous and CD95/Fas-induced apoptosis of CD8+ T cells from HIV-infected individuals. Measurement of CD8+ T cell apoptosis by annexin V staining after overnight cultures revealed a significant inverse correlation between spontaneous CD8+ T cell apoptosis and ex vivo Bcl-2 protein levels in these cells (p < 0.0001, r2 = 0.4) (Fig. 3A). Furthermore, an even stronger inverse correlation was found between ex vivo Bcl-2 levels and CD95/Fas-induced apoptosis of CD8+ T cells (p < 0.0001, r2 = 0.63) (Fig. 3A). Finally, treatment-specific CD95/Fas-induced apoptosis of CD8+ T cells also correlated inversely with Bcl-2 levels in these cells (p < 0.0001, r2 = 0.52) (Fig. 3A). No correlation was found...
respectively; compared with nonapoptotic HIV-specific CD8+ T cells, respectively). Lower Bcl-xL levels were also detected in HIV-infected individuals (MFI 375 ± 28, n = 3) (Fig. 3B). Similar findings were found when total CD8+ T cells from HIV-infected individuals were examined (MFI = 94 ± 17 vs 586 ± 45 for apoptotic and nonapoptotic, respectively) (Fig. 3B). Similar data were obtained when cells were stained for both caspase 3 activity and Bcl-2 (data not shown). Although apoptosis sensitivity does not correlate with ex vivo Bcl-xL levels in total CD8+ T cells (Fig. 3A), HIV-specific CD8+ T cells that undergo CD95/Fas-induced apoptosis have decreased Bcl-xL levels (MFI = 15 ± 2, n = 3) compared with nonapoptotic HIV-specific CD8+ T cells (MFI = 40 ± 3). Comparable data were obtained for total CD8+ T cells from HIV-infected individuals (MFI = 16 ± 1 vs 42 ± 2 for apoptotic and nonapoptotic, respectively). Lower Bcl-xL levels were also detected in HIV-specific CD8+ T cells (MFI = 16 ± 3 vs 40 ± 8 for apoptotic and nonapoptotic, respectively) and total CD8+ T cells (MFI = 19 ± 3 vs 42 ± 4 for apoptotic and nonapoptotic cells, respectively) from HIV-infected individuals undergoing spontaneous apoptosis. Thus, our data strongly suggest a relation between ex vivo Bcl-2 protein levels and sensitivity to spontaneous or CD95/Fas-induced apoptosis of HIV-specific CD8+ T cells and indicate that reduced Bcl-2 levels may be responsible for this apoptosis sensitivity. Although such a correlation could not be shown for Bcl-xL, Bcl-xL is lower in apoptotic cells, suggesting a role in apoptosis sensitivity for this molecule also.

**IL-15 augments Bcl-2 and Bcl-xL levels in CD8+ T cells from HIV-infected individuals**

We recently described that IL-15 is a survival factor for both HIV-specific and total CD8+ T cells from HIV-infected individuals and potently inhibits spontaneous and CD95/Fas-induced apoptosis (26, 27). Consequently, we examined the possible involvement of Bcl-2 molecule in the antiapoptotic effect of IL-15 by determining Bcl-2 protein levels in HIV-specific CD8+ T cells treated with IL-15. IL-15 overnight treatment at a concentration of 5 ng/ml increased Bcl-2 levels by 2-fold in HIV-specific CD8+ T cells (MFI = 238 ± 23 in the absence vs 506 ± 73 in the presence of IL-15, n = 5) (Fig. 4, A and B). Similarly, IL-15 enhances the Bcl-2 levels in total CD8+ T cells from HIV-infected individual (MFI = 343 ± 35 vs 594 ± 63 in the presence or absence of IL-15, respectively, n = 10), while Bcl-2 levels in CD4+ T cells were also increased, albeit to a lower extent (MFI = 413 ± 37 vs 561 ± 70, n = 6) (Fig. 4, A and B).

When caspase 8 activity and Bcl-2 or Bcl-xL levels were simultaneously assessed in IL-15-treated cultures, IL-15 was able to eliminate the percentage of caspase 8+ Bcl-2low HIV-specific CD8+ T cells (32.3 ± 6% vs 11 ± 2% in the absence or presence of IL-15, respectively) as well as caspase 8+ Bcl-2low total CD8+ T cells (26 ± 2% vs 13 ± 1% in the absence or presence of IL-15, respectively) when cells were treated with anti-CD95/Fas mAb (Fig. 3B). A similar effect of IL-15 was observed with caspase 8+ Bcl-xLlow HIV-specific CD8+ T cells (24 ± 9% vs 7 ± 1% in the absence and presence of IL-15, respectively) or caspase 8+ Bcl-xLlow total CD8+ T cells (19 ± 1% vs 8 ± 2% in the absence or presence of IL-15, respectively) when cells were treated with anti-CD95. This was not due to reduced levels of CD95/Fas on CD8+ T cells from HIV-infected individuals.
T cells, as IL-15 treatment did not affect CD95/Fas expression (data not shown).

Intriguingly, IL-15 significantly increased Bcl-xL protein levels in HIV-specific and total CD8+ T cells from HIV-infected individuals and healthy donors. In HIV-specific CD8+ T cells, IL-15 increased Bcl-xL MFI levels from 32 ± 5 (untreated cells) to 41 ± 4 (IL-15-treated cells) (Fig. 4C). Bcl-xL levels from total CD8+ T cells from HIV-infected individuals also increased with IL-15 treatment (MFI = 29 ± 2 vs 39 ± 3 for untreated and IL-15-treated cells, respectively) (Fig. 4C). IL-15 also increased the levels of Bcl-xL in total CD8+ T cells from healthy donors from an MFI of 28 ± 2 for untreated cells to 40 ± 3 in IL-15-treated cells (Fig. 4C). Our findings suggest that the antiapoptotic function of IL-15 is mediated, at least in part, by up-regulation of Bcl-2 and Bcl-xL, antiapoptotic molecules.

Enhanced long-term survival of Gag-specific CD8+ T cells in the presence of IL-15 is accompanied by increased levels of Bcl-2 and Bcl-xL.

We recently reported that IL-15 enhances the long-term survival of HIV-specific CD8+ T cells as well as that of total CD8+ T cells from HIV-infected individuals (26). In this study, we examined whether this effect of IL-15 is associated with increased expression of the antiapoptotic molecules Bcl-2 and Bcl-xL. Purified PBMCs were cultured for 7 days in the absence or presence of IL-15 (5 ng/ml). Consistent with our previous findings (26), IL-15 increased by 5-fold the absolute numbers of live Gag-specific CD8+ T cells in 7-day cultures (5 × 10^3 ± 2 ± 10^3 vs 23 ± 10^3 ± 5 × 10^3 for untreated and IL-15-treated cells, respectively) (Fig. 5A). Additionally, IL-15 treatment increased the absolute number of Gag-specific CD8+ T cells by 2-fold compared with the number of cells placed in culture, indicating that IL-15 also induced a slow proliferation of these cells. Similar data were obtained with total CD8+ T cells from HIV-infected individuals as well as healthy donors (Fig. 5A).

In parallel, we examined the effect of IL-15 on the expression of Bcl-2 and Bcl-xL. A marked 7-fold induction of Bcl-2 levels in Gag-specific CD8+ T cells (MFI = 1046 ± 7, n = 3) compared with untreated cells (MFI = 143 ± 19) was observed in these 7-day cultures (Fig. 5B). Similarly, IL-15 enhances Bcl-xL expression in Gag-specific CD8+ T cells (MFI = 103 ± 40, n = 3) compared with untreated cells (MFI = 36 ± 14.) (Fig. 5B). A 2- to 3-fold induction of Bcl-2 and Bcl-xL expression by IL-15 was observed, when total CD8+ T cells from HIV-infected persons and healthy donors were examined (Fig. 5B). Taken together, our data clearly show that enhanced long-term survival of Gag-specific CD8+ T cells by IL-15 is accompanied by increased levels of the antiapoptotic molecules Bcl-2 and Bcl-xL.

Discussion

We previously described that HIV-specific CD8+ T cells are highly sensitive to spontaneous as well as CD95/Fas-induced apoptosis, and that HIV-infected macrophages can kill these cells by a CD95/Fas-mediated mechanism (5). In this study, we report that HIV-specific CD8+ T cells have greatly reduced ex vivo levels of the antiapoptotic molecule Bcl-2. Furthermore, HIV-specific CD8+ T cells have lower levels of Bcl-xL than CMV-specific CD8+ T cells, which are not as sensitive to apoptosis (5). Reduced Bcl-2 and Bcl-xL levels were found in CTL against all HIV epitopes examined. To the best of our knowledge, this is the first report investigating the direct ex vivo levels of Bcl-2 and Bcl-xL in HIV-specific CD8+ T cells. Such a decrease in antiapoptotic molecules may affect the survival of these cells and impair their ability to control HIV infection. We also show in this study that IL-15,
which inhibits the apoptosis of HIV-specific CD8⁺ T cells (26), induces both Bcl-2 and Bcl-x₁ levels in these cells, suggesting the up-regulation of these antiapoptotic molecules as a potential mechanism by which IL-15 is acting to inhibit apoptosis of these cells.

It has been reported previously that a subset of CD8⁺ T cells from HIV-infected individuals that is sensitive to spontaneous and CD95/Fas-induced apoptosis is characterized by reduced ex vivo levels of Bcl-2 (10). In agreement with this, we observed decreased ex vivo levels of Bcl-2 in total CD8⁺ T cells from HIV-infected subjects compared with CD8⁺ T cells from healthy controls, and this correlated with both spontaneous and CD95/Fas-induced apoptosis. Most importantly, however, we show that Bcl-2 levels are dramatically reduced in HIV-specific CD8⁺ T⁺ cells, which are much more prone to spontaneous and CD95/Fas-induced apoptosis compared with CMV-specific and total CD8⁺ T cells, as we have previously described (5). Finally, we directly demonstrate an association between Bcl-2 levels and sensitivity to apoptosis of HIV-specific CD8⁺ T cells, as we show that only Bcl-2 low HIV-specific CD8⁺ T cells undergo spontaneous and CD95/Fas-induced apoptosis, whereas those that are Bcl-2 high are resistant to apoptosis.

A number of mechanisms may be responsible for the Bcl-2 down-regulation in HIV-specific CD8⁺ T cells, which we describe in this work. The deficiency of growth factors or cytokines, such as IL-2, which has been reported in HIV-infected individuals (7, 34), may influence Bcl-2 levels. Because CD4⁺ T cell help is required to sustain CD8⁺ T cell responses (35) and HIV-specific CD4⁺ T cells are lost early during HIV infection (36), the lack of HIV-specific CD4⁺ T cell help during the generation and maturation of HIV-specific CD8⁺ T cells could also be, at least in part, responsible for the low expression of Bcl-2 in HIV-specific CD8⁺ T cells. HIV gp120 protein binding to the chemokine receptor CCR5 can induce caspase-dependent death of CD4⁺ T and CD8⁺ T cells (37, 38), and such a mechanism could lead to down-modulation of Bcl-2 in HIV-specific CD8⁺ T cells in vivo, particularly in the presence of TGF-β (39). In support of this, CD8⁺ T cell sensitivity to spontaneous apoptosis and low Bcl-2 levels were recently shown to correlate with elevated levels of the chemokine receptor CCR5 (11).

Enhanced generation of reactive oxygen species has been found in circulating T cells from patients infected with HIV (40). It is possible that reactive oxygen species generation could potentially lead to Bcl-2 down-regulation, as it has been proposed elsewhere (41). Alternatively, chronic activation of CD8⁺ T cells, which is observed in HIV infection, could lead to Bcl-2 reduction (11, 42). In accordance with this, we have found that Bcl-2 is decreased more in CD8⁺ T cells expressing the activation marker CD38 compared with nonactivated CD8⁺ T cells (C. Petrovas and P. Katsikis, unpublished observations).

Comparable Bcl-2 levels in CD4⁺ T cells from HIV-infected and healthy individuals were found. It has been proposed that CD4⁺ T cells with low Bcl-2 are rapidly eliminated in vivo (10), and consequently, they cannot be detected ex vivo. Alternatively, Bcl-2 may play a different role in apoptosis of CD4⁺ compared...
with CD8\(^+\) T cells. The fact that CD4\(^+\) T cells from HIV-infected individuals are very sensitive to spontaneous and CD95/Fas-induced apoptosis (8), while having normal levels of Bcl-2, whereas Bcl-2 levels correlate with this apoptosis in CD8\(^+\) T cells, supports this hypothesis. Our findings showing normal Bcl-2 levels in CD4\(^+\) T cells differ from a previous study that showed reduced levels of Bcl-2 in CD4\(^+\) T cells from HIV-infected patients (43). This discrepancy, however, may be due to differences in patient populations, as this later study focused on patients that were low responders to highly active antiretroviral therapy.

The Bcl-x\(_{L}\) antiapoptotic factor has been implicated in CD4\(^+\) (18, 44) as well as CD8\(^+\) (20, 45) T cell survival. In HIV infection, the induction of Bcl-x\(_{L}\) is markedly impaired in activated PBMC from asymptomatic HIV-infected patients (13). In our current study, we found lower ex vivo Bcl-x\(_{L}\) expression in HIV-specific compared with CMV-specific CD8\(^+\) T cells from HIV-infected individuals. This could be attributed to impaired costimulatory signals such as CD40 and CD28, which are well-known inducers of Bcl-x\(_{L}\) (19, 46). This lack of costimulatory signals could be more pronounced for HIV-specific CD8\(^+\) T cells due to the absence of HIV-specific CD4\(^+\) T cell help, as these later cells are lost early during HIV infection (36, 47). Direct regulation of Bcl-x\(_{L}\) gene by members of STAT family has been recently described (48, 49). A deregulation of STATs that has been seen in peripheral T cells during HIV infection (50) may also contribute to low levels of Bcl-x\(_{L}\) gene expression in HIV infection.

It has been previously proposed that there is a reciprocal feedback regulatory pathway for Bcl-2 and Bcl-x\(_{L}\) expression (12, 51). Our data indicate that such a reciprocal regulatory mechanism may be impaired in HIV-specific CD8\(^+\) T cells given that they cannot express higher levels of Bcl-x\(_{L}\) compared with total CD8\(^+\) T cells, despite their significantly lower Bcl-2 levels. Given that the relative ratio between proapoptotic and antiapoptotic factors is important for cell survival (32), we hypothesize that the high levels of Bcl-x\(_{L}\) in CMV-specific CD8\(^+\) T cells compensate for the reduced Bcl-2 levels in these cells and protect them from apoptosis. In contrast, HIV-specific CD8\(^+\) T cells, which have low levels of both antiapoptotic molecules, are prone to apoptosis.

Because CMV- and HIV-specific CD8\(^+\) T cells differ in their effector memory phenotype (5, 33), it is possible that the differing levels of Bcl-2 and Bcl-x\(_{L}\) in these cells could be attributed to their different maturation state. We find that CD45RA\(^-\)CD62L\(^-\)CD8\(^+\) T cells do express more Bcl-x\(_{L}\) and Bcl-2 levels compared with CD45RA\(^+\)CD62L\(^-\) cells. Although the increased Bcl-x\(_{L}\) levels in CMV-specific CD8\(^+\) T cells may be due to these cells containing more CD45RA\(^+\)CD62L\(^-\) cells compared with HIV-specific CD8\(^+\) T cells, the reduced Bcl-2 levels in HIV-specific CD8\(^+\) T cells, which are nearly exclusively CD45RA\(^+\)CD62L\(^-\), were significantly lower than CD45RA\(^-\)CD62L\(^-\) CD8\(^+\) T cells. Thus, the differences between CMV- and HIV-specific CD8\(^+\) T cells cannot simply be attributed to differences in their effector memory phenotypes. Future studies are needed to directly address these questions.

We found a strong inverse correlation between the ex vivo levels of Bcl-2 in CD8\(^+\) T cells from HIV-infected individuals and the susceptibility to spontaneous as well as CD95/Fas-induced apoptosis. However, we did not find this correlation in CD4\(^+\) T cells from HIV\(^+\) patients. Furthermore, we directly demonstrate that HIV-specific CD8\(^+\) T cells low for Bcl-2 and Bcl-x\(_{L}\) are those undergoing spontaneous and CD95/Fas-induced apoptosis. Our findings therefore strongly suggest that reduced Bcl-2 levels are, at least in part, responsible for the enhanced apoptosis sensitivity and reduced long-term survival of HIV-specific CD8\(^+\) T cells. CD95/ Fas apoptosis takes place through the activation of caspase 8, either directly by caspase 8 (type I cells) or indirectly through mitochondrial pathway (type II cells) (52). Whether primary CD4\(^+\) and CD8\(^+\) T cells belong to one or the other type is not clear. Our data indicate that different intracellular pathways are involved in CD95/Fas-induced apoptosis of CD4\(^+\) compared with those of CD8\(^+\) T cells. Supportive of this is our previous finding that IL-15 can significantly inhibit the CD95/Fas-induced apoptosis of CD8\(^+\) T cells, while it has little or no effect on the apoptosis of CD4\(^+\) T cells, despite the fact that it induces the Bcl-2 levels in these cells (26).

Bcl-2 has been shown to inhibit CD95/Fas-induced apoptosis under certain experimental conditions (53–55). In addition, several studies have revealed the involvement of ceramide in Fas-induced apoptosis (56, 57). The ganglioside GD3, which is synthesized from accumulated ceramide upon apoptosis induction, targets mitochondria in a Bcl-2-controlled manner (58). Such a connection between the CD95/Fas apoptotic pathway and Bcl-2 could be important for the induction of cell death in certain types of cells. The involvement of ceramide in the apoptosis of CD8\(^+\) T cells from HIV-infected individuals is an attractive hypothesis because elevated ceramide levels have been found in HIV-infected patients (59). Furthermore, numerous studies have revealed a mitochondrial dysfunction in T lymphocytes from HIV-infected patients, implicating these organelles in the progression of lymphocyte apoptosis in HIV infection (40, 60, 61).

Our data indicate that Bcl-2 family molecules may play a critical role in regulation of HIV-specific CD8\(^+\) T cell apoptosis. It is important, however, to point out that other regulatory molecules may also be involved and determine the apoptotic fate of these cells. Antia apoptotic factors such as c-FLIP can act at early stages of death receptor-mediated apoptosis (62), while others such as the second mitochondria-derived activator of caspase and members of the inhibitor of apoptosis protein family regulate the apoptosis at downstream levels (63). Today, there is little information on the molecular mechanisms of primary CD8\(^+\) T cell apoptosis, particularly in HIV infection. Similar expression of c-FLIP protein in peripheral blood lymphocytes from HIV-infected individuals and healthy donors was previously found (64), while the altered expression of antiapoptotic factors such as inhibitor of apoptosis protein family molecules under in vitro treatment with HIV or HIV proteins has also been described (39, 65). However, the expression of these molecules in specific CD8\(^+\) subpopulations is not known. Obviously, further studies are needed to elucidate the contribution of these other antiapoptotic factors in the apoptosis of HIV-specific CD8\(^+\) T cells. The development of reagents that allow for intracytoplasmic measurement of these molecules will facilitate these studies.

In this study, we examined the relation between Bcl-2/Bcl-x\(_{L}\) levels and the sensitivity of HIV-specific and total CD8\(^+\) T cells to spontaneous and CD95/Fas-induced apoptosis. Increased TNF-α-induced apoptosis of total CD8\(^+\) T cells from HIV-infected patients was described in a recent study (66), while we have previously shown that TRAIL may be involved in the apoptosis of these cells also (67). However, the sensitivity of HIV-specific CD8\(^+\) T cells to TNF family members such as TRAIL and TNF-α is currently not known. Future studies on the role of these molecules in HIV-specific CD8\(^+\) T cell apoptosis would add significant insight to our knowledge of CTL depletion in HIV infection.

IL-15 is a multipotent cytokine with functional activity in immune and nonimmune cells (22, 23). It plays an important role for both innate immune response (68) and homeostasis of memory CD8\(^+\) T cells (69–71). We recently described that IL-15 can inhibit the apoptosis of HIV-specific and total CD8\(^+\) T cells from HIV-infected individuals (26, 27). Furthermore, IL-15 augments
the effector function (IFN-γ production and direct ex vivo killing) of these cells (26, 27). In this study, we report that this inhibition of apoptosis is associated with induction of both Bcl-2 and Bcl-xL molecules. This is in disagreement with a previous work (30) that described no effect of IL-15 on CD95/Fas-induced apoptosis of total CD8+ T cells from HIV-infected individuals, although IL-15 could induce Bcl-2 expression. Differences in apoptosis assays used could explain this disagreement. Because Bcl-2 levels and survival of CD8+ T cells are reduced in IL-15-treated knockout mice (29), our findings raise the question as to whether IL-15 production is impaired in HIV-infected individuals, and this contributes to the apoptosis sensitivity of HIV-specific CD8+ T cells. Indeed, IL-15 production by PBMC has been reported to be compromised in HIV-positive individuals (72, 73).

In long-term cultures, up-regulation of both Bcl-2 and Bcl-xL in HIV-specific CD8+ T cells was observed in the presence of IL-15. As previously described, IL-15 was able to induce both proliferation and survival of these cells (26). These two signals by IL-15 appear to be separate, as inhibition of phosphatidylinositol 3-kinase by a specific inhibitor (LY294002) can inhibit the proliferative effect of IL-15 on HIV-specific CD8+ T cells without affecting its antiapoptotic effect (C. Petrovas and P. Katsikis, unpublished observations). Thus, it appears that the signaling pathways responsible for the survival and proliferative effect of IL-15 are distinct. Elucidating the signaling responsible for the antiapoptotic effect of IL-15 may reveal targets for therapeutic intervention.

Our studies showing that IL-15 can inhibit apoptosis and up-regulate antiapoptotic molecules such as Bcl-2 in HIV-specific CD8+ T cells further support the use of IL-15 as a means to increase the survival and function of the HIV-specific CD8+ T cells in HIV-infected individuals. Immunotherapy such as IL-15 treatment that increases antiapoptotic factors in combination with anti-tiretroviral therapy may provide a novel way to restore and enhance the immune response targeted against HIV.

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References


