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# HIV-Specific CD8$^{+}$ T Cells Exhibit Markedly Reduced Levels of Bcl-2 and Bcl-$x_L$[1]

**Constantinos Petrovas,* Yvonne M. Mueller,* Ioannis D. Dimitriou,* Paul M. Bojczuk,* Karam C. Mounzer,[‡] James Witek,[†] John D. Altman,[§] and Peter D. Katsikis[2]***

**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.**

Human immunodeficiency virus-specific CD8$^{+}$ T cell responses play a central role in controlling HIV infection (1–4). We have recently described an increased sensitivity of HIV-specific CD8$^{+}$ T cells to CD95/Fas-mediated apoptosis (5), which may impair their function as serial killers. Thus, understanding the molecular defects of these cells may lead to strategies that improve the survival and effector function of HIV-specific CD8$^{+}$ T cells.

In HIV infection, CD4$^{+}$ and CD8$^{+}$ T cells exhibit increased spontaneous, activation-induced, and CD95/Fas-induced apoptosis (6–9). Why T cells in HIV infection are prone to undergo apoptosis is, however, largely unknown. A number of studies have indicated that the expression of antiapoptotic molecules such as Bcl-2 and Bcl-$x_L$ may be impaired in peripheral blood T cells from HIV-infected individuals. Sensitivity to apoptosis of CD8$^{+}$ T cells from HIV-infected individuals was found to be related to decreased ex vivo levels of the antiapoptotic molecule Bcl-2 (10, 11). Additionally, induction of Bcl-$x_L$, another member of the Bcl-2 antiapoptotic family (12), is greatly impaired in PBMC from asymptomatic HIV-infected individuals (13). In contrast, spontaneous and CD95/Fas-induced apoptosis is minimal in peripheral blood T cells from healthy donors, and this correlates with high levels of Bcl-2 (14, 15), suggesting that this apoptosis may be regulated, at least in part, by a mechanism that involves Bcl-2 family members.

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-15R$\alpha$ 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

Departments of *Microbiology and Immunology and [†]Medicine, Drexel University College of Medicine, Philadelphia, PA 19129; [‡]Philadelphia FIGHT, Philadelphia, PA 19107; and [§]Department of Microbiology and Immunology, Emory University, Atlanta, GA 30329

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[2] Address correspondence and reprint requests to Dr. Peter D. Katsikis, Department of Microbiology and Immunology, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA 19129. E-mail address: katsikis@drexel.edu

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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-$x_L$ 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-$x_L$ 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-$x_L$ 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/$\mu$l (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 (NLVPMVATV) peptide 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-$x_L$ levels, anti-CD4 FITC/anti-CD8 PE-Cy5/HIV- or CMV-specific tetramer allophycocyanin and anti-CD45RA FITC/anti-CD62L allophycocyanin/anti-CD8 PE-Cy5 combinations were stained along with Bcl-2 and Bcl-$x_L$ Abs; 2) for apoptosis and survival measurement, annexin V FITC/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, Seatle, WA), while annexin V PE was purchased from eBioscience (San Diego, CA). All mAbs above were purchased from eBioscience.

Briefly, $1 \times 10^6$ cells were stained with tetramers and Abs in HBSS (Cellgro, Herndon, VA), 3% FBS (Life Technologies, Carlsbad, CA), and 0.02% $NaN_3$ for 30 min on ice; washed twice with HBSS, 3% FBS, and 0.02% $NaN_3$; and fixed with 1% paraformaldehyde. When annexin V staining was performed, 2.5 mM $CaCl_2$ 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 Calibrite beads (BD Biosciences).

For standardization of the Bcl-2 and Bcl-$x_L$ stains, we used the same frozen PBMC sample from a healthy control in every experiment as a standard. Flow cytometry was set up so that the mean fluorescence intensity (MFI)[3] of this sample was always within 10% of the first experiment performed. This standard was used to make sure the stains and the same experimental conditions for MFI determination were identical from experiment to experiment. In addition, healthy individuals were always run together with patients' PBMC with at least two healthy donors included in each experiment staining HIV patients for Bcl-2 and Bcl-$x_L$.

### *Apoptosis studies*

For spontaneous apoptosis measurement, freshly isolated PBMCs were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin sulfate (Cellgro) for 14 h at 37°C, 5% $CO_2$, at a density of $1 \times 10^6$ cells/ml/well in 24-well plates (Costar, Corning, NY) in the presence or absence of 5 ng/ml human rIL-15 (PeproTech, Rocky Hill, NJ).

For CD95/Fas-induced apoptosis, PBMC were cultured in plates coated with 5 $\mu$g/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% $CO_2$. 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 $1 \times 10^6$ cells/ml/well in the presence or absence of 5 ng/ml IL-15 for 7 days at 37°C, 5% $CO_2$ 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-$x_L$ staining*

Intracellular levels of Bcl-2 and Bcl-$x_L$ 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 cytotofix/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-$x_L$ staining, an anti-Bcl-$x_L$ 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-LETD-fluoromethylketone (FMK)) and caspase-3 (FAM-DEVD-FMK) (Serologicals, Norcross, GA). Freshly isolated PBMC were cultured at $1 \times 10^6$ cells/ml/well in plates coated with 5 $\mu$g/ml anti-Fas mAb in the absence or presence of 5 ng/ml IL-15. Following overnight incubation, 300 $\mu$l of cell suspension were transferred to a new tube and further incubated for 1 h at 37°C, 5% $C0_2$ in the presence of either FAM-LETD-FMK or FAM-DEVD-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 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 $\mu$g/ml aprotinin, 10 $\mu$g/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 $\beta$-actin staining, a second probing was performed on the same membrane using an anti-$\beta$-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).

[3] Abbreviations used in this paper: MFI, mean fluorescence intensity; FMK, fluoromethylketone.

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 $CD8^{high}$ 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. 1*C*). 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 ($CD8^{low}$ + $CD8^{high}$) 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 $CD8^{low}$ + $CD8^{high}$ 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 $CD8^{high}$ 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. 1*A*).

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-$x_L$ levels compared with CMV-specific $CD8^+$ T cells in HIV-infected individuals*

Bcl-$x_L$ is another potent antiapoptotic factor that belongs to the Bcl-2 family of antiapoptotic molecules (12). In contrast to Bcl-2, Bcl-$x_L$ 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 $CD8^{high}$ gate was used (Fig. 2, *A* and *B*). Surprisingly, Bcl-$x_L$ 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



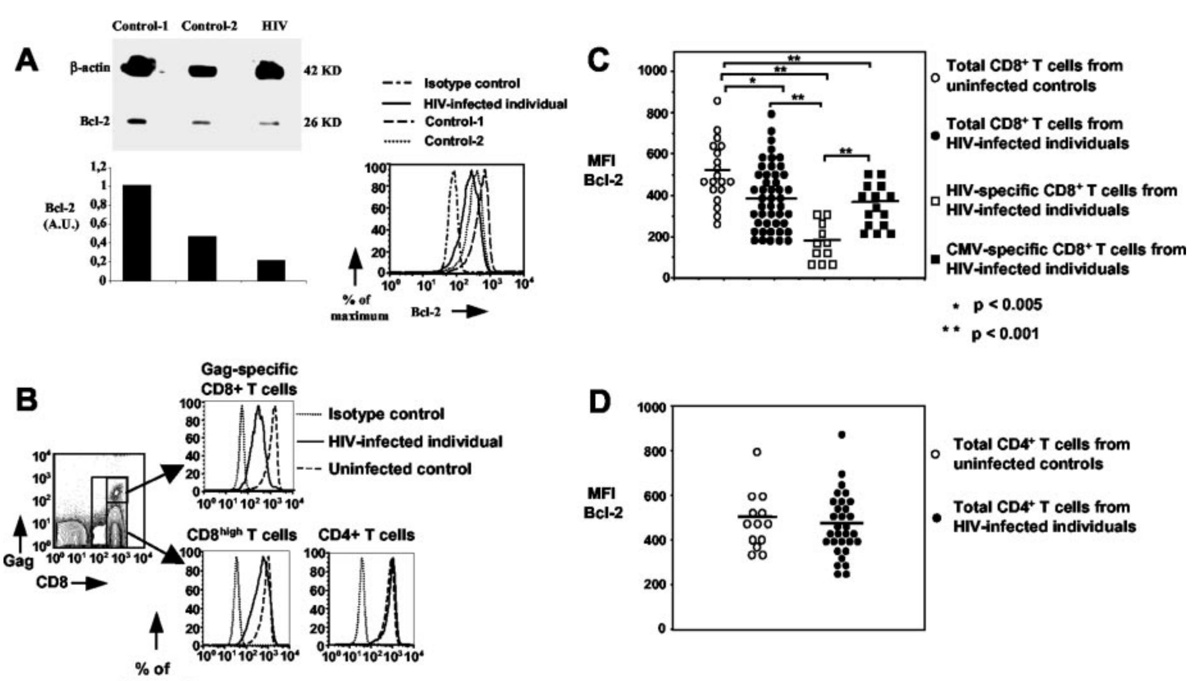


**FIGURE 1.** HIV-specific $CD8^+$ T cells express reduced Bcl-2 protein levels ex vivo. *A*, Western blot showing the Bcl-2 levels in purified total $CD8^+$ T cells from two healthy controls and one HIV-infected individual. Bar graphs (*left*) depicting Bcl-2 levels normalized against β-actin (in arbitrary 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 $CD8^{high}$ and $CD8^{high}$ + CD8 $^{low}$ gate (large CD8 gate). Histograms depict ex vivo Bcl-2 expression in HIV-specific, total $CD8^{high}$ 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 $CD8^{high}$ 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 $CD8^{high}$ T cells from HIV-infected individuals ($n = 46$), and total $CD8^{high}$ 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.

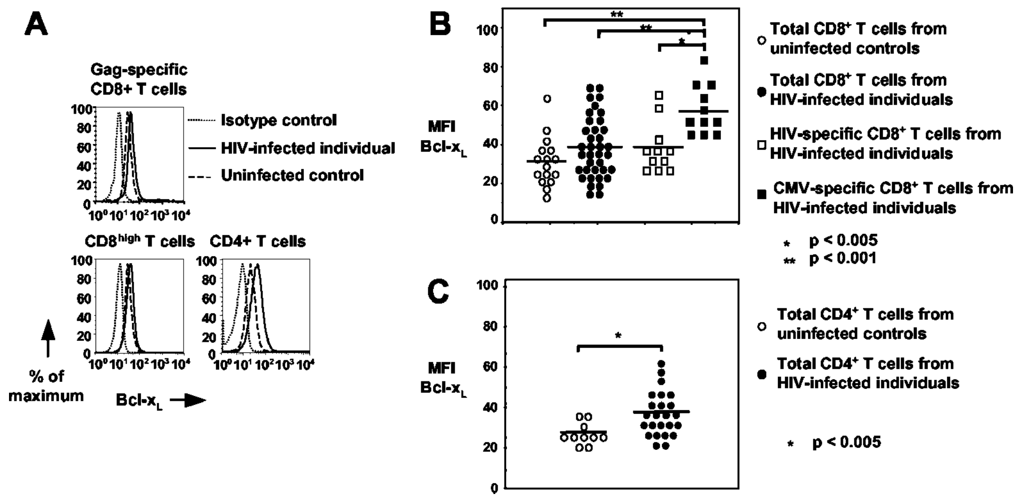


**FIGURE 2.** Impaired ex vivo expression of Bcl-$x_L$ in HIV-specific CD8$^+$ T cells compared with CMV-specific CD8$^+$ T cells. *A*, Representative histograms showing ex vivo Bcl-$x_L$ levels in HIV-specific CD8$^+$ T cells, total CD8$^{high}$ T cells, and CD4$^+$ T cells from one HIV-infected individual and an uninfected control. Cells were gated as in Fig. 1. Uninfected controls in Gag-specific CD8$^+$ T cell plot depict histogram for total CD8$^{high}$ T cells. *B*, Pooled data showing MFI of Bcl-$x_L$ staining for HIV-specific CD8$^+$ T cells ($n = 11$), CMV-specific CD8$^+$ T cells ($n = 11$), and total CD8$^{high}$ T cells from HIV-infected individuals ($n = 37$), and total CD8$^{high}$ T cells from uninfected controls ($n = 16$). *C*, Pooled data showing MFI of Bcl-$x_L$ 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. 2*B*). Again, comparable data were obtained when gating on CD8$^{low}$ + CD8$^{high}$ 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-$x_L$ 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-$x_L$ in CD45RA$^+$CD62L$^-$ (MFI = 446 ± 80 and 45 ± 6, respectively, for Bcl-2 and Bcl-$x_L$) compared with CD45RA$^-$CD62L$^-$ (MFI = 346 ± 52 and 35 ± 6, respectively, for Bcl-2 and Bcl-$x_L$) CD8$^+$ T cells from HIV-infected individuals ($n = 8$), although this difference was not statistically significant. By comparing the Bcl-$x_L$ 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-$x_L$ 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-$x_L$ (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-$x_L$ 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-$x_L$ 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-$x_L$ 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-$x_L$ in the effector memory phenotypes of these virus-specific populations are needed to conclusively answer this question.

Finally, Bcl-$x_L$ 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 ($p < 0.0001$, $r^2 = 0.4$) (Fig. 3*A*). Furthermore, an even stronger inverse correlation was found between ex vivo Bcl-2 levels and CD95/Fas-induced apoptosis of CD8$^+$ T cells ($p < 0.0001$, $r^2 = 0.63$) (Fig. 3*A*). Finally, treatment-specific CD95/Fas-induced apoptosis of CD8$^+$ T cells also correlated inversely with Bcl-2 levels in these cells ($p < 0.0001$, $r^2 = 0.52$) (Fig. 3*A*). No correlation was found

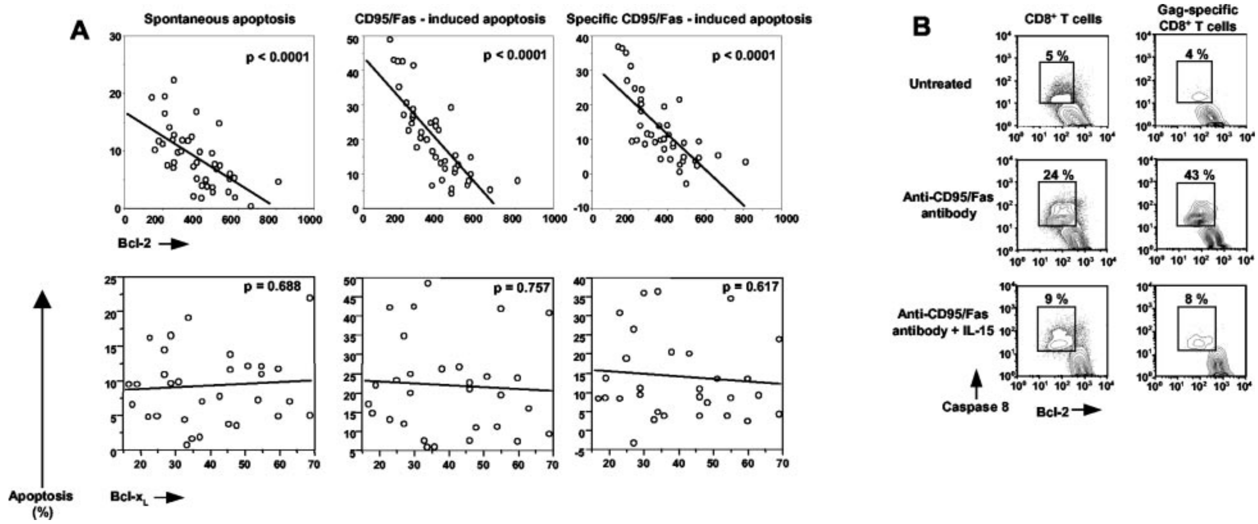


**FIGURE 3.** Low Bcl-2 expression correlates with high sensitivity to apoptosis of CD8$^+$ T cells from HIV-infected individuals. *A*, The correlation between the percentage of spontaneous or anti-CD95/Fas-induced CD8$^+$ T cell apoptosis and ex vivo Bcl-2 (*upper left, middle, and right panels*, $n = 42$ and $n = 41$, respectively) or Bcl-$x_L$ (*lower left, middle, and right panels*, $n = 31$ and $n = 30$, respectively) levels in HIV-infected individuals is shown. Apoptosis was measured by annexin V staining of total CD8$^+$ T cells in untreated or anti-CD95/Fas-treated cultures. For treatment-specific apoptosis calculations, see *Materials and Methods*. *B*, Representative flow cytometry showing in vitro caspase 8 activity vs Bcl-2 levels in HIV-specific and total CD8$^+$ T cells from one HIV-infected individual. Spontaneous apoptosis and apoptosis after culturing PBMCs with anti-CD95/Fas Ab ± IL-15 for 14 h are shown. Cells were gated first for lymphocytes by forward and side light scatter, and then for HIV-specific CD8$^+$ T cells by tetramer and CD8 staining. The percentage of caspase 8-positive cells is shown. One representative experiment of three performed is presented.

between Bcl-$x_L$ levels and CD8$^+$ T cell apoptosis (Fig. 3*A*) as well as between Bcl-2 levels and CD4$^+$ T cell counts or HIV viral load (data not shown).

### *Apoptotic HIV-specific CD8$^+$ T cells have reduced Bcl-2 and Bcl-$x_L$ levels*

To directly demonstrate that sensitivity to apoptosis was linked to Bcl-2 expression levels in CD8$^+$ T cells, we stained cells for both caspase 8 activity and Bcl-2 levels. Spontaneous apoptosis occurred in cells expressing low Bcl-2 levels for both HIV-specific CD8$^+$ T cells (MFI = 114 ± 16 vs 435 ± 137 for apoptotic and nonapoptotic cells, respectively; $n = 3$) and total CD8$^+$ T cells (MFI = 97 ± 4 vs 433 ± 83 for apoptotic and nonapoptotic cells, respectively; $n = 3$) from HIV-infected individuals (Fig. 3*B*). HIV-specific CD8$^+$ T cells undergoing CD95/Fas-induced apoptosis clearly had reduced levels of Bcl-2 expression (MFI = 99 ± 14, $n = 3$), while cells with high levels of Bcl-2 were resistant to apoptosis (MFI = 375 ± 28, $n = 3$) (Fig. 3*B*). 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. 3*B*). 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-$x_L$ levels in total CD8$^+$ T cells (Fig. 3*A*), HIV-specific CD8$^+$ T cells that undergo CD95/Fas-induced apoptosis have decreased Bcl-$x_L$ 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 cells, respectively). Lower Bcl-$x_L$ levels were also detected in HIV-specific CD8$^+$ T cells (MFI = 16 ± 3 vs 40 ± 8 for apoptotic and nonapoptotic cells, 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-$x_L$, Bcl-$x_L$ is lower in apoptotic cells, suggesting a role in apoptosis sensitivity for this molecule also.

### *IL-15 augments Bcl-2 and Bcl-$x_L$ 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-$x_L$ levels were simultaneously assessed in IL-15-treated cultures, IL-15 was able to eliminate the percentage of caspase 8$^+$ Bcl-2$^{low}$ 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-2$^{low}$ 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. 3*B*). A similar effect of IL-15 was observed with caspase 8$^+$ Bcl-$x_L$$^{low}$ HIV-specific CD8$^+$ T cells (24 ± 9% vs 7 ± 1% in the absence and presence of IL-15, respectively) or caspase 8$^+$ Bcl-$x_L$$^{low}$ 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$^+$

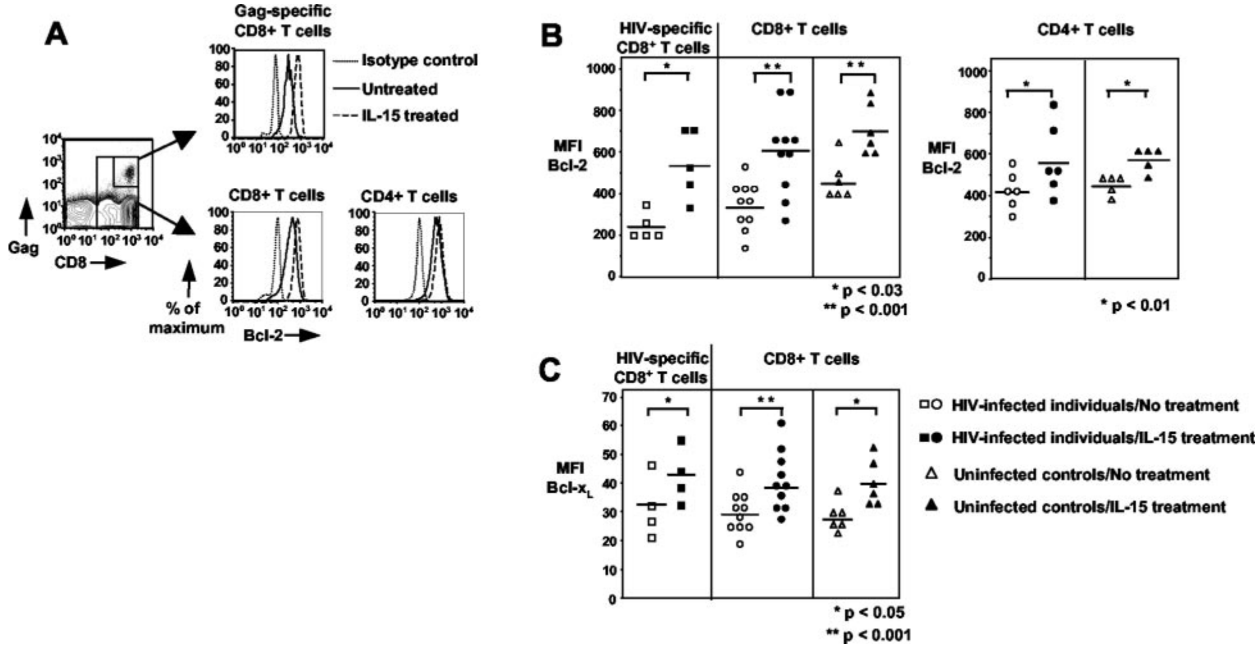


**FIGURE 4.** IL-15 enhances the expression of Bcl-2 and Bcl-$x_L$ in vitro. *A*, Representative flow cytometry showing HIV-specific $CD8^+$ T cells, total $CD8^+$ T cells, and $CD4^+$ T cells from an HIV-infected individual after culturing PBMC for 14 h in the presence or absence of IL-15. Histograms represent Bcl-2 levels in HIV-specific $CD8^+$ T cells, total $CD8^+$, and $CD4^+$ T cells. *B*, Pooled data showing MFI of Bcl-2 staining for HIV-specific $CD8^+$ T cells ($n = 5$), total $CD8^+$ T cells ($n = 10$), and $CD4^+$ T cells ($n = 6$) from HIV-infected individuals cultured in the absence (open shapes) or presence (filled shapes) of IL-15. Bcl-2 levels in $CD8^+$ T cells ($n = 6$) and $CD4^+$ T cells ($n = 5$) from uninfected controls are also shown. *C*, Pooled data showing MFI of Bcl-$x_L$ staining for HIV-specific $CD8^+$ T cells ($n = 4$) and total $CD8^+$ T cells ($n = 10$) from HIV-infected individuals, and total $CD8^+$ T cells ($n = 6$) from uninfected controls after culture ± IL-15 for 14 h. Horizontal lines depict mean values. The $p$ values were calculated using the paired Student $t$ test.

T cells, as IL-15 treatment did not affect CD95/Fas expression (data not shown).

Intriguingly, IL-15 significantly increased Bcl-$x_L$ 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-$x_L$ MFI levels from 32 ± 5 (untreated cells) to 41 ± 4 (IL-15-treated cells) (Fig. 4*C*). Bcl-$x_L$ 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. 4*C*). IL-15 also increased the levels of Bcl-$x_L$ 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. 4*C*). Our findings suggest that the antiapoptotic function of IL-15 is mediated, at least in part, by up-regulation of Bcl-2 and Bcl-$x_L$ 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-$x_L$*

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-$x_L$. 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 ($5 \times 10^3 \pm 2 \times 10^3$ vs $23 \times 10^3 \pm 5 \times 10^3$ for untreated and IL-15-treated cells, respectively) (Fig. 5*A*). 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. 5*A*).

In parallel, we examined the effect of IL-15 on the expression of Bcl-2 and Bcl-$x_L$. 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. 5*B*). Similarly, IL-15 enhances Bcl-$x_L$ expression in Gag-specific $CD8^+$ T cells (MFI = 103 ± 40, $n = 3$) compared with untreated cells (MFI = 36 ± 14.) (Fig. 5*B*). A 2- to 3-fold induction of Bcl-2 and Bcl-$x_L$ expression by IL-15 was observed, when total $CD8^+$ T cells from HIV-infected persons and healthy donors were examined (Fig. 5*B*). 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-$x_L$.

## 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-$x_L$ than CMV-specific $CD8^+$ T cells, which are not as sensitive to apoptosis (5). Reduced Bcl-2 and Bcl-$x_L$ 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-$x_L$ 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,

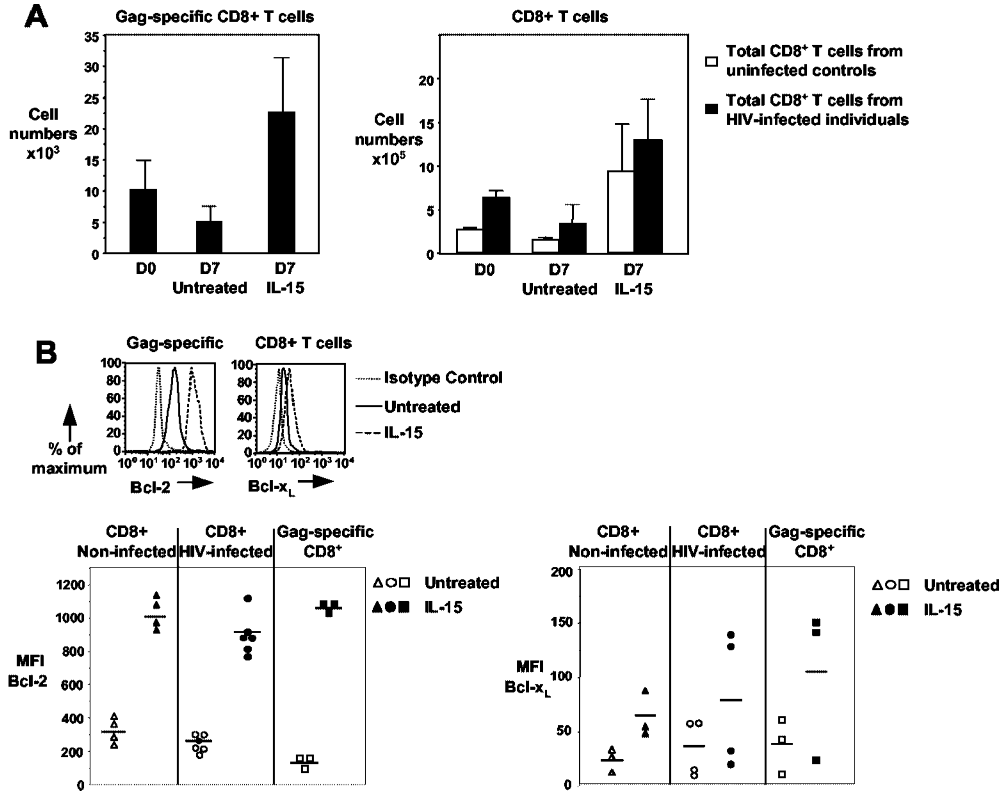


**FIGURE 5.** Increased levels of Bcl-2 and Bcl-$x_L$ correlate with enhanced survival of Gag-specific CD8$^+$ T cells in long-term cultures. *A*, Pooled data showing absolute numbers of live (annexin V$^-$) Gag-specific CD8$^+$ T cells ($n$ = 3) and total CD8$^+$ T cells from HIV-infected individuals ($n$ = 6), and total CD8$^+$ T cells from healthy donors ($n$ = 4) after 7-day culture in the absence or presence of IL-15. Bars depict means ± SE. *B*, Representative histograms showing Bcl-2 and Bcl-$x_L$ levels in Gag-specific CD8$^+$ T cells from HIV-infected individual after 7-day culture ± IL-15. Cells gated first on annexin V$^-$ cells, and then for HIV-specific and total CD8$^+$ T cells by tetramer and CD8 staining. Pooled data presenting MFI of Bcl-2 and Bcl-$x_L$ in Gag-specific CD8$^+$ T cells ($n$ = 3), total CD8$^+$ T cells from HIV-infected individuals ($n$ = 6 and $n$ = 4 for Bcl-2 and Bcl-$x_L$, respectively), and total CD8$^+$ T cells from healthy donors ($n$ = 4 and $n$ = 3 for Bcl-2 and Bcl-$x_L$, respectively). Horizontal lines depict mean values. The $p$ values were calculated using the paired Student $t$ test.

which inhibits the apoptosis of HIV-specific CD8$^+$ T cells (26), induces both Bcl-2 and Bcl-$x_L$ 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-$\beta$ (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 3, 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. Antiapoptotic 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-$\alpha$-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-$\alpha$ 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-$x_L$ 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-15R$\alpha$ 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-$x_L$ 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 antiretroviral therapy may provide a novel way to restore and enhance the immune response against HIV.

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