Modulation of Bcl-2 Protein by CD4 Cross-Linking: A Possible Mechanism for Lymphocyte Apoptosis in Human Immunodeficiency Virus Infection and for Rescue of Apoptosis by Interleukin-2

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We have previously demonstrated that CD4 cross-linking (CD4XL) results in apoptosis of CD4$^+$ T cells and augmentation of Fas antigen (CD95, APO-1) expression in CD4$^+$ and CD8$^+$ T cells. Here we demonstrate that CD4XL mediated by both, anti-CD4 monoclonal antibody (MoAb) or human immunodeficiency virus (HIV) envelope protein gp120 reduces the expression of the proto-oncogene Bcl-2 in CD4$^+$ T cells, but not in CD8$^+$ T cells, concurrently with the induction of CD4$^+$ T cell-apoptosis. Additionally, the Bcl-2^{dim} population expressed high levels of Fas antigen. Bax, an antagonist of Bcl-2, was brightly expressed even in the Bcl-2^{dim} population. Addition of interleukin (IL)-2 rescued CD4$^+$ T cells from CD4XL-induced Bcl-2 downmodulation and apoptosis induction. These results support the hypothesis that CD4 ligation by HIV-1 envelope protein in vivo in HIV-infected patients selectively reduces Bcl-2 protein in CD4$^+$ T lymphocytes, thereby facilitating Fas/Fas-ligand triggered apoptosis; furthermore the findings reported expand the rationale for use of IL-2 in HIV disease.

ACCELERATED SPONTANEOUS and activated-induced T-cell apoptosis has been well documented in peripheral blood lymphocytes,[1-3] as well as in the lymph nodes of human immunodeficiency virus (HIV)-infected individuals.[4,5] Although the mechanisms involved in this accelerated apoptosis have not yet been fully elucidated, HIV-1 envelope protein-mediated CD4 cross-linking (CD4XL) is an attractive explanation for the depletion of CD4$^+$ T cells. We previously demonstrated that: (1) CD4XL performed in peripheral blood mononuclear cells (PBMC) results in apoptosis of CD4$^+$ T cells[3], (2) CD4XL-induced apoptosis of CD4$^+$ T cells requires the presence of accessory cells such as monocytes[3], (3) CD4XL augments Fas antigen (CD95, APO-1) expression in CD4$^+$ and CD8$^+$ T cells[6] and, (4) CD4XL induces tumor necrosis factor (TNF)-α and interferon (IFN)-γ, but not interleukin (IL)-2 secretion.[6] Moreover, we recently discovered that the Fas-ligand (FasL) expression was augmented in monocytes after CD4XL.[7] These results suggest that CD4XL is a potent mechanism for upregulating the proapoptotic molecules Fas and FasL, which are pivotal for initiating the apoptosis signaling pathway, and which are known to be upregulated in HIV infection.[8-13]

The balance between life and death by apoptosis, however, is controlled by opposing forces of proapoptotic and antiapoptotic factors. The proto-oncogene Bcl-2 is known to protect cells against many forms of programmed cell death and induced cell death, including growth factor deprivation (reviewed in Wyllie,[14] Henkart,[15] and Nunez[16]) and was originally cloned from the breakpoint of a t(14;18) translocation present in many human B cell lymphomas.[17,18] Additionally, many investigators have reported an inverse relationship of Fas antigen and Bcl-2 protein in peripheral lymphocytes and tumor cell lines.[19-22] We demonstrate here that CD4XL in PBMC, which results in apoptosis induction, reduces the Bcl-2 expression in CD4$^+$ T cells, but not in CD8$^+$ T cells. Also, our results show that the Bcl-2^{dim} population clearly expresses higher levels of Fas antigen than the Bcl-2^{bright} population. Furthermore, IL-2, a known inducer of Bcl-2[23], rescues the CD4XL-induced CD4$^+$ T lymphocyte apoptosis by preventing Bcl-2 downmodulation.

MATERIALS AND METHODS

Cells and culture conditions. PBMC from healthy individuals were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Accu-Prep, Accurate Chemical, Westbury, NY) as recently described.[3] RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), 2 mmol/L L-glutamine (BIOWHITTAKER, Inc, Walkersville, MD), 100 U/mL penicillin G, and 100 μg/mL streptomycin was used for all cultures.

Antibodies and reagents. Reagents and sources were as follows: monoclonal antibody (MoAb) to CD4 (Leu3a; IgG1; Becton Dickinson, Mountain View, CA); goat antimouse Ig (GAM; Tago, Inc, Burlingame CA); mouse Igs from nonimmunized animals (nonimmune IgGs; ChromPure IgG; Jackson ImmunoResearch Lab, West Grove, PA); phytohemagglutinin (PHA, 10.5 μg/ml, Difco, Detroit, MI); recombinant human IL-2 (Boehringer Mannheim, Indianapolis, IN). The native HIV-1 envelope protein gp120 was purified from supernatants of human T-cell leukemia virus type III451-infected 6D5 cells, as described previously.[24] Rabbit anti-gp120 (HIV-1IIIB) polyclonal antibodies were obtained from American Bio Technologies (Cambridge, MA).

Induction of CD4 cross-linking. Cells were treated as described[3] with Leu3a or control antibody at concentrations of 3 μg/2 $\times$ 10^6 cells for 40 minutes at 4°C and cultured in round-bottom 12 $\times$ 75 mm polystyrene tubes (Falcon 2054, Becton Dickinson Lab Ware, Bedford, MA) coated with GAM. For coating of GAM to the tubes, 100 μL of GAM in coating buffer (100 μg/mL in 30 mmol/L $NaHCO_3$, 15 mmol/L Na_2CO_3, pH 9.6) was added to each tube, incubated 1 hour at 37°C, and washed five times with Hanks' Balanced Salt Solution (HBSS). Treatment of cells with gp120 (5 μg/mL) followed by culture with immobilized anti-gp120 antibodies (10 μg/mL) was performed in a procedure identical to that described for Leu3a/GAM.

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Lymphocyte surface phenotype and cytoplasmic staining. Lymphocyte surface markers were identified by triple-color immunofluorescence using various combinations of MoAbs. The MoAbs were conjugated with phycoerythrin (PE) or peridinin chorophyII A (PerCP) as indicated: PerCP-CD8 (IgG2a), PE-CD14 (IgG2b), PerCP-CD45 (IgG1), and PE- or PerCP-CD3 (IgG1); all MoAbs and relevant isotype-matched control MoAbs were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). PE-Fas (clone UB2, IgG1) was obtained from Immunotec Inc (Westbrook, ME). Surface staining was performed at room temperature for 10 minutes in cells suspended in staining buffer (HBSS containing 1% FCS and 0.1% sodium azaide). For intracytoplasmic staining, cells were fixed following surface staining with 1% paraformaldehyde in HBSS, washed, and permeabilized for 10 minutes with saponin (Sigma Chemicals, St Louis, MO) at a concentration of 0.1% (wt/vol) in permeabilization buffer (PB; 10 mmol/L HEPES in HBSS). fluorescein isothiocyanate (FITC)-labeled antihuman Bcl-2 (IgG1, DAKO, Carpinteria, CA) was used for cytoplasmic direct staining. Bax (Bax N-20; Santa Cruz Biotechnology, Santa Cruz, CA) was used for indirect staining with PE-labeled goat antirabbit IgG (Caltag Laboratories, Burlingame, CA). Normal mouse and rabbit IgG were used as negative controls for staining. Cells were washed with PB, resuspended in 1% paraformaldehyde HBSS, and analyzed by flow cytometry using the Epics Elite instrument (Coulter Corp, Hialeah, FL).

Analysis of lymphocyte apoptosis. Two methods were used to identify apoptotic cells at 72 hours after culture: (1) light scatter of cells gated for CD3 or CD45 and subpopulation-specific marker, which allowed the analysis of surface phenotype of cells undergoing apoptosis,[3,25,26] and (2) propidium iodide (PI) staining, which was performed as described.[3,13] In brief, cells were fixed in 75% ethanol for 1 hour at 4°C, washed and resuspended in HBSS containing PI (50 μg/mL, Molecular Probes, Eugene, OR) and ribonuclease A (type I-A, Sigma, 250 μg/mL). The PI fluorescence of cells in the lymphocyte-gate was measured at 48 to 72 hours poststaining by flow cytometry.

RESULTS

CD4 cross-linking downmodulates Bcl-2 expression. To determine the effect of CD4XL on Bcl-2 expression, sequential analysis of Bcl-2 expression was performed after CD4XL in PBMC from HIV-seronegative healthy volunteers. As shown in Fig 1, CD4XL clearly reduced Bcl-2 expression in $CD3^+$T cells on days 2, 3, and 5 (Bcl-2^{dim} cells 20.7%, 23.3%, and 24.6%, respectively). In contrast, cells in medium or those treated with mouse Ig/GAM showed minimal downmodulation of Bcl-2 on day 3 (2.3% and 5.6%, respectively). The net percentages of Bcl-2^{dim} population in the CD4XL samples after subtracting out mIg controls were 13.8%, 17.7%, and 10.7% on days 2, 3, and 5, respectively. Moreover, the Bcl-2 downmodulation was not observed with Leu3a treatment or GAM treatment alone on day 3 (4.1% and 8.4%, respectively). In subsequent experiments, we evaluated CD4XL-PBMC on day 3 of culture.

In our previous study, we observed that a loss of cellular volume (forward scatter: [FSC]) and an increase in cellular density (side scatter: [SSC]) is a characteristic feature of cells undergoing apoptosis.[6,25] Thus, we examined the Bcl-2 expression in low FSC/high SSC fraction (Apoptotic lymphocyte fraction: [Apo Fr.]) and in the high FSC/low SSC (Intact lymphocyte fraction: [Int Fr.]). As shown in Fig 2A, cells in the Apo Fr. were found to be composed principally of Bcl-2^{dim} cells, while the Int Fr. clearly expressed a single high Bcl-2 peak. Mean fluorescence intensity (MFI) in Apo Fr. was significantly lower as compared to MFI in the Int Fr. (Fig 2B). These results suggest that downmodulation of Bcl-2, which is known to act as an apoptosis-repressor, might play a critical role in CD4XL-induced apoptosis.

CD4 cross-linking does not change the Bax expression even in the Bcl-2^{dim} population. Bax is known to antagonize the survival promoting effects of Bcl-2[27] and forms a heterodimer with Bcl-2. Thus, we sought to determine Bax expression in CD4XL-PBMC. To detect both Bcl-2 and Bax proteins at the single cell level, we employed simultaneous cytoplasmic staining of both Bcl-2 and Bax proteins, as well as CD3 surface staining. As shown in Fig 3, 95% of $CD3^+$ cells cultured in medium alone coexpressed both Bcl-2 and Bax proteins. Upon CD4XL, Bcl-2 expression was downmodulated in approximately 20% of $CD3^+$ cells. In contrast, Bax protein was expressed in high levels even in the Bcl-2 downmodulated population. However, we failed to detect high Bcl-2 expression in Bax negative cells.

Bcl-2 expression is reduced in $CD4^+$ T cells but not in $CD8^+$ T cells. We next examined the effect of CD4XL on Bcl-2 expression in different T-cell subsets. As previously reported, $CD4^+$ cells are undetectable by staining with anti-CD4 MoAb in the CD4XL samples.[3] Thus, we examined intracytoplasmic Bcl-2 with FITC-Bcl−2 in conjunction with PE-CD3 and Per-CP-CD8 for evaluating cell surface antigens. We observed Bcl-2 downmodulation only in the $CD8^-$ $CD3^+$ lymphocyte subset, whereas Bcl-2 was expressed as a single high peak in the $CD3^+CD8^+$ lymphocyte subset (Fig 4). Moreover, we confirmed that Bcl-2 downmodulation occurs predominantly in $CD4^+$ T cells following CD4XL using HIV-gp120, as well. Bcl-2 downmodulation was noted to occur in approximately 40% to 50% of the $CD4^+$ T cells, which is in agreement with the percentage of $CD4^+$ T cells undergoing CD4XL-induced apoptosis.[7]

Bcl-2 downmodulation was observed in Fas positive population. We previously reported that Fas antigen expression is upregulated in lymphocytes by CD4XL.[6] To investigate the distribution of Fas in relation to Bcl-2 expression, we examined surface Fas antigen expression in Bcl-2^{dim} and Bcl-2^{high} populations in the CD4XL-PBMC. As shown in Fig 5, we confirmed the upregulation of Fas antigen expression even in the Bcl-2^{high} population in CD4XL-PBMC as compared with medium-treated cells. Moreover, the single peak indicating high expression of Fas antigen was clearly demonstrated in the Bcl-2^{dim} population. Cells stimulated by PHA expressed high Fas antigen, but with minimal downmodulation of Bcl-2 expression.

Exogenous IL-2 blocks CD4 cross-linking–induced apoptosis. It has been previously reported that IL-2 receptor-dependent signaling pathway induces Bcl-2.[28] Moreover, exogenous IL-2 induces Bcl-2 and rescues cells from apoptosis.[29-32] We previously reported that CD4XL induces IFN-γ and TNF-α, but not IL-2 secretion. Thus, we sought to determine the effects of IL-2 on CD4XL-induced

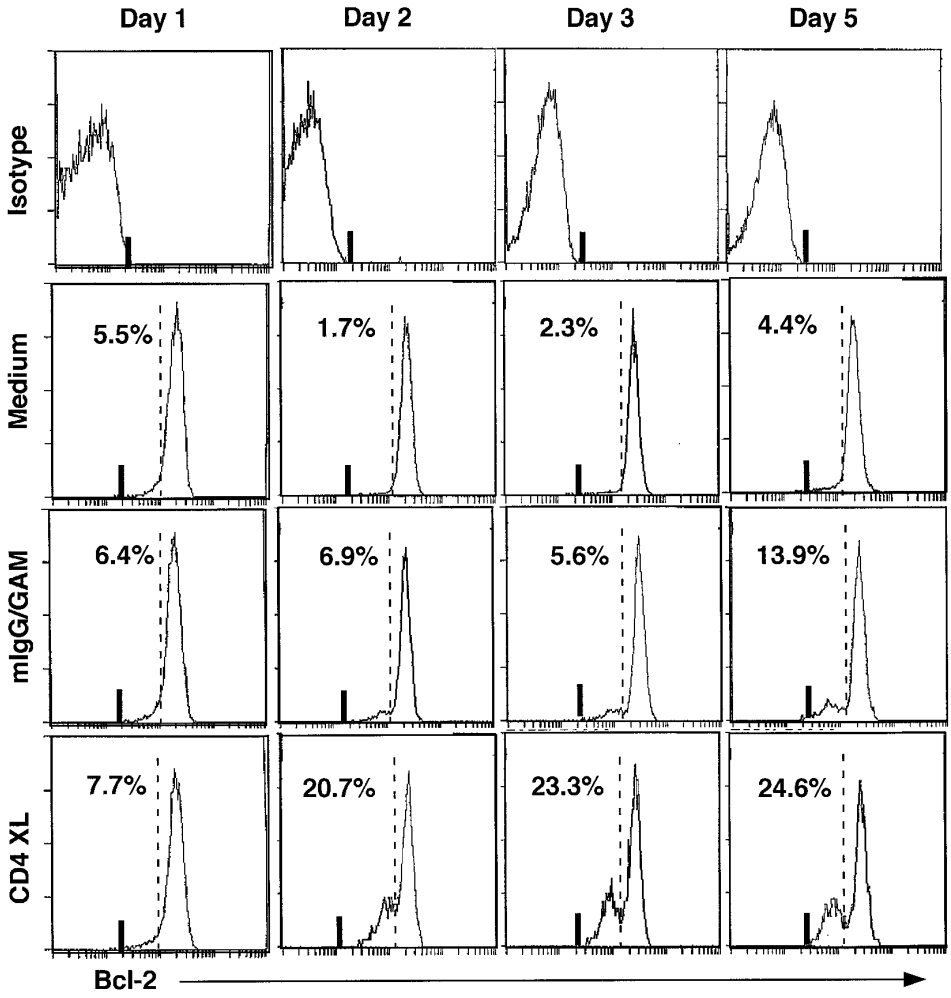


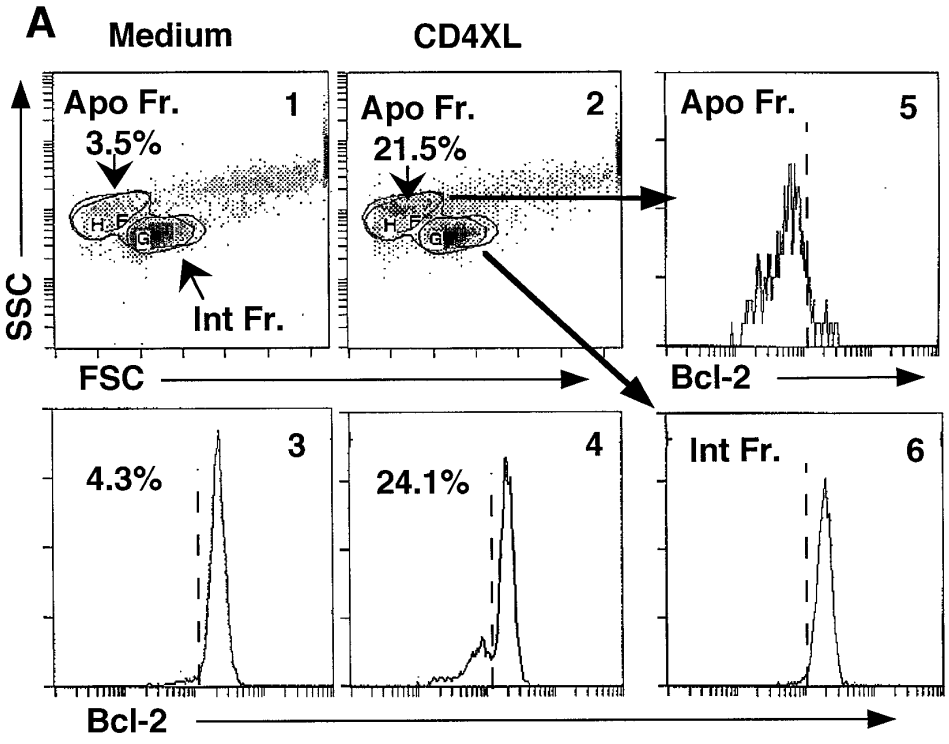
Fig 1. Time course of Bcl-2 downmodulation after CD4 cross-linking. PBMC, treated as indicated, were cultured for 1, 2, 3, and 5 days. Cells were analyzed by flow cytometry for intracytoplasmic Bcl-2 expression in conjunction with surface staining with PE-labeled CD3 MoAb and per-CP–labeled CD45 MoAb. Broken and solid lines indicate the notch of the Bcl-2 downmodulation and the uppermost boundary of the isotype control, respectively. Percentages indicated in the figure are percentages of Bcl-2^{dim} plus Bcl-2 negative cells (almost all were Bcl-2^{dim}).

apoptosis. IL-2 was added to PBMC at initiation of CD4XL and the emergence of subdiploid nuclei (% A0 cells) was evaluated in the PI-stained sample. As shown in Fig 6A, exogenous IL-2 significantly decreased the % A0 cells in CD4XL-PBMC cultures on day 2 and day 3, but no increase was observed in the S phase in PI staining at this time. In addition, exogenous IL-2 did not increase net cell counts during the 3-day culture period of CD4XL (data not shown). Thus, the significant decrease in the % A0 cells (Fig 6B), is attributable to the apoptosis blocking effect of IL-2 and does not occur as a result of cell division. As shown in Fig 7, IL-2 also prevented the CD4XL-related Bcl-2 downmodulation in the peripheral T cells. Moreover, the prevention of the Bcl-2 downmodulation was clearly associated with the decrease in the % A0 cells. Thus, the rescue of cells from apoptosis by IL-2 occurs in association with a reduction in the Bcl-2^{dim} lymphocyte population.

DISCUSSION

We previously demonstrated that CD4XL in PBMC results in induction of CD4$^+$ T-cell apoptosis in conjunction with generalized Fas antigen upregulation in lymphocytes[6] and have recently shown that CD4XL leads to enhanced FasL expression in monocytes.[7] Although Fas and FasL expression reached high levels as early as day 1 after CD4XL, numbers of lymphocytes undergoing apoptosis were still low on day 1 and increased to high levels by day 3 (Fig 6A). This discrepancy between the time of Fas upregulation and induction of apoptosis led us to examine the expression of Bcl-2 protein, which is known to be an inhibitor of apoptosis. In this report, we show that the Bcl-2 expression is reduced on day 3 after CD4XL, but not on day 1 (Fig 1), and that the cells undergoing apoptosis are composed primarily of the Bcl-2^{dim} population (Fig 2). Furthermore, despite the augmentation of Fas expression, the number of apoptotic cells resulting from CD4XL were significantly decreased in the presence of exogenous IL-2 (Fig 6), and the reduction in apoptosis correlated with a reversal of Bcl-2 downmodulation. These results suggest that although the upregulation of Fas and FasL expression facilitates CD4XL-induced apoptosis, the downmodulation of Bcl-2 is essential for the conduct of apoptosis.

Recently, Desbarats et al[33] reexamined CD4XL-induced apoptosis in murine CD4$^+$ T cells. They noted that although



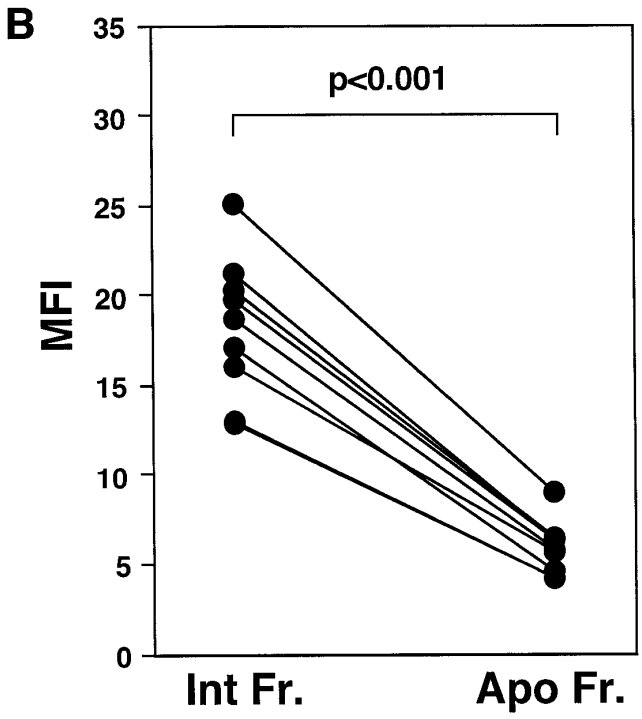


Fig 2. Bcl-2 downmodulated lymphocyte population is undergoing apoptosis. The Bcl-2 expression of cells in CD4XL-PBMC was analyzed on day 3. (A) Based on the light scatter profiles, the low FSC/high SSC Apo Fr. was compared with high FSC/low SSC Int Fr. Histograms of Bcl-2 in the total lymphocyte fraction [box 3 (medium), box 4 (CD4XL)], and after CD4XL in Apo Fr. (box 5) and Int Fr. (box 6) are indicated. (B) Comparison of the MFI level of Bcl-2 between the apoptotic and intact lymphocyte fractions (n = 9). Values of statistical significance determined by paired *t* test are indicated.

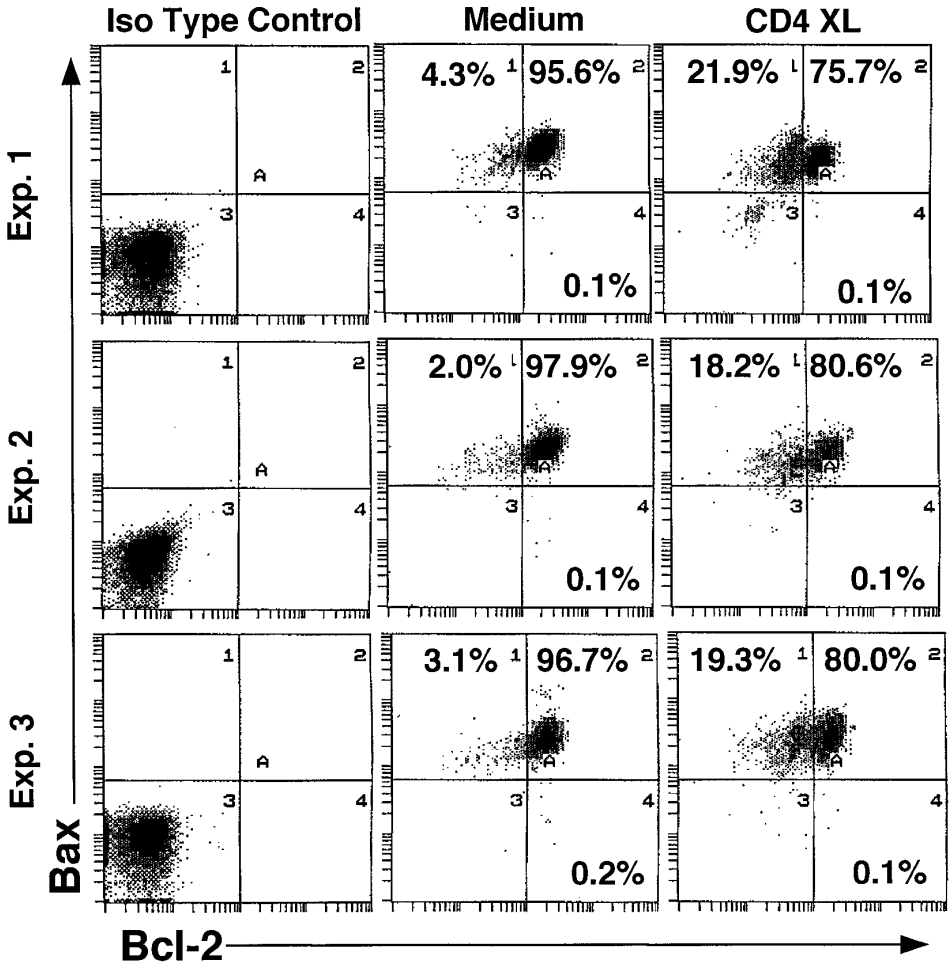


Fig 3. Bax expression is unchanged in CD4 cross-linked PBMC. Intracytoplasmic staining of Bcl-2 and Bax was performed with surface CD3 staining on day 3. Three representative results of Bcl-2 and Bax expression in $CD3^+$ T cells are demonstrated.

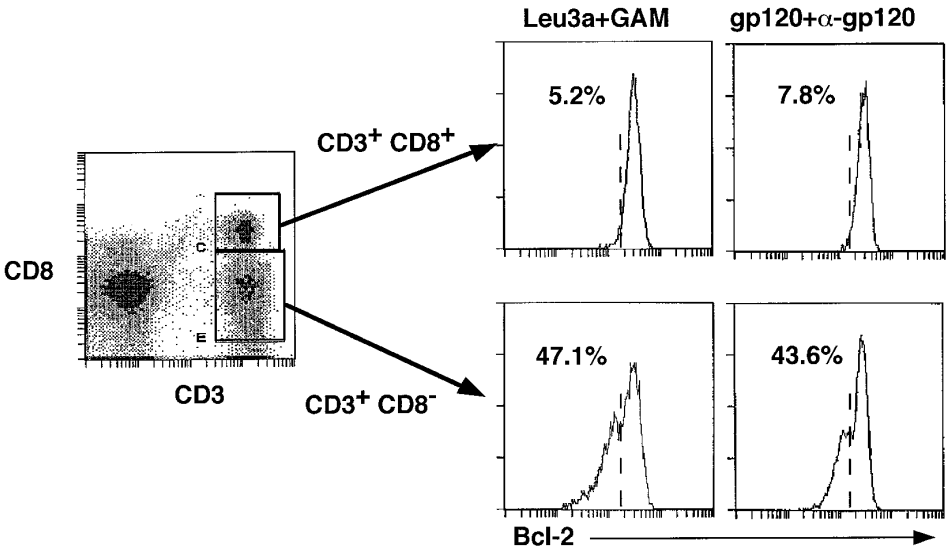


Fig 4. Bcl-2 downmodulation by CD4XL in PBMC is restricted to $CD4^+$ T cells. Intracytoplasmic Bcl-2 staining was performed on day 3 in combination with surface CD8 and CD3 staining in the CD4XL-PBMC. Histograms representing the Bcl-2 expression in ($CD3^+CD8^+$, and $CD3^+CD8^-$) gated populations are shown. The vertical line indicates the notch of the Bcl-2 downmodulation in $CD3^+CD8^-$ cells.

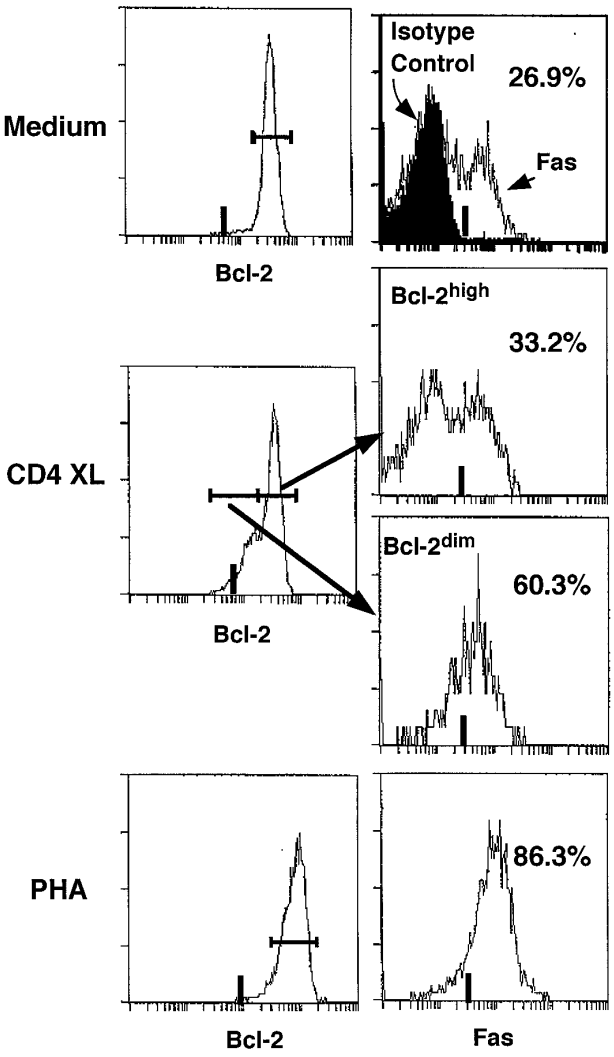


Fig 5. Fas expression is increased in CD4XL-PBMC in the Bcl-2^{dim} population. PBMC were treated with medium, Leu3a/GAM, or PHA for 3 days and intracytoplasmic Bcl-2 staining was performed in combination with surface Fas antigen and CD3 staining. Fas expression was evaluated separately in Bcl-2^{dim} and Bcl-2^{high} cells gated on the $CD3^+$ cell population.

anti-CD4 MoAb-treated and untreated cells expressed equivalent amounts of surface Fas antigen, only the CD4 MoAb-treated cells were susceptible to anti-Fas–mediated increase in cell death. The investigators concluded that CD4XL alters the sensitivity of $CD4^+$ cells to Fas-induced death. Our data suggests that Bcl-2 downmodulation in CD4XL-cells renders them sensitive for Fas-induced apoptosis.

The capacity of Bax and Bcl-2 to compete for one another suggests a reciprocal relationship in which Bcl-2 monomers or homodimers favor survival and Bax homodimers favor death.[27] Our results show that most of the normal peripheral T cells were Bcl-2^+ Bax^+ in day 3-medium–treated cultures (Fig 3) and in freshly isolated PBMC (data not shown). Also, we found that Bax expression was still high even in the Bcl-2^{dim} population. Thus, in normal PBMC, almost all peripheral T cells appear to be in a stable state of Bcl-2/Bax heterodimers, whereas the Bcl-2^{dim} population may be indicative of formation of Bax/Bax homodimers in cells undergoing apoptosis. However, we failed to observe an increase in Bcl-2^+/Bax^- cell population even with the addition of IL-2 (data not shown). Although it remains controversial whether Fas-induced apoptosis can be rescued by overexpression of Bcl-2,[34,35] we did not observe a cell population in our system that expressed higher Bcl-2 than that in resting cells.

The biologic relevance of our studies is particularly applicable to HIV disease. Uninfected and latently infected $CD4^+$ cells in HIV-infected subjects have the potential to undergo CD4 cross-linking in vivo by HIV envelope protein, either as expressed on infected cells or by free gp120 or via virions trapped within the processes of dendritic cells in lymph nodes. In this study, we show that the Bcl-2^{dim} lymphocyte cells, which undergo apoptosis on in vitro CD4XL, are contained in the $CD4^+$ T-cell subset, but not in the $CD8^+$ T-cell population. Furthermore, we confirmed this restriction of Bcl-2 distribution in cells subjected to CD4XL with HIV gp120. This finding should be considered in the context of observations made in infected individuals. Patients with HIV infection manifest augmented lymphocyte apoptosis of $CD4^+$ T, $CD8^+$ T, and B lymphocytes. Moreover, we have recently observed that the Bcl-2 downmodulation of cultured PBMC from HIV^+ patients was not restricted to $CD4^+$ T cells, but was observed in $CD8^+$ T cells and also in B cells.[25] These apparent disparities between results in patients' peripheral blood and the effect of in vitro CD4XL of normal $CD4^+$ T cells can be explained by the possibility that in HIV infection, at least two mechanisms are involved in induction of generalized lymphocyte apoptosis. Superimposed on the mechanism of apoptotic $CD4^+$ T-cell death induced by CD4XL, is another mechanism for apoptosis attributable to Fas/FasL upregulation and Bcl-2 downmodulation in terminally differentiated- and senescent lymphocytes of HIV^+ patients as a result of chronic persistent infection. The importance of the CD4XL mechanism as a primary driving force for lymphocyte apoptosis is nevertheless underscored by our previous demonstration that CD4XL resulted in augmented Fas antigen expression, not only in $CD4^+$ T cells, but also in $CD8^+$ T cells and in B cells as well.[6]

The downmodulation of Bcl-2 expression, which was restricted to $CD4^+$ T cells in CD4XL-PBMC could not, of itself, be attributed to induced cytokines TNF-α and IFN-γ, which were shown to play a central role in the generalized upregulation of Fas antigen on various lymphocytes subsets.[6] One explanation for the observed downmodulation of Bcl-2 is that attributable directly to CD4 signaling effects; another possibility is that CD4 ligation results in a decrease in IL-2 production in $CD4^+$ T cells but not in $CD8^+$ T cells, thereby leading to Bcl-2 downmodulation in an autocrine manner. Although this issue is under investigation, the absence of IL-2 induction by CD4XL[3] and the inability of gp120-treated $CD4^+$ T cells to secrete IL-2 in response to antigenic stimulation,[36] implicate an important role for IL-2 deficiency in the induction of lymphocyte apoptosis. Finally, the observed

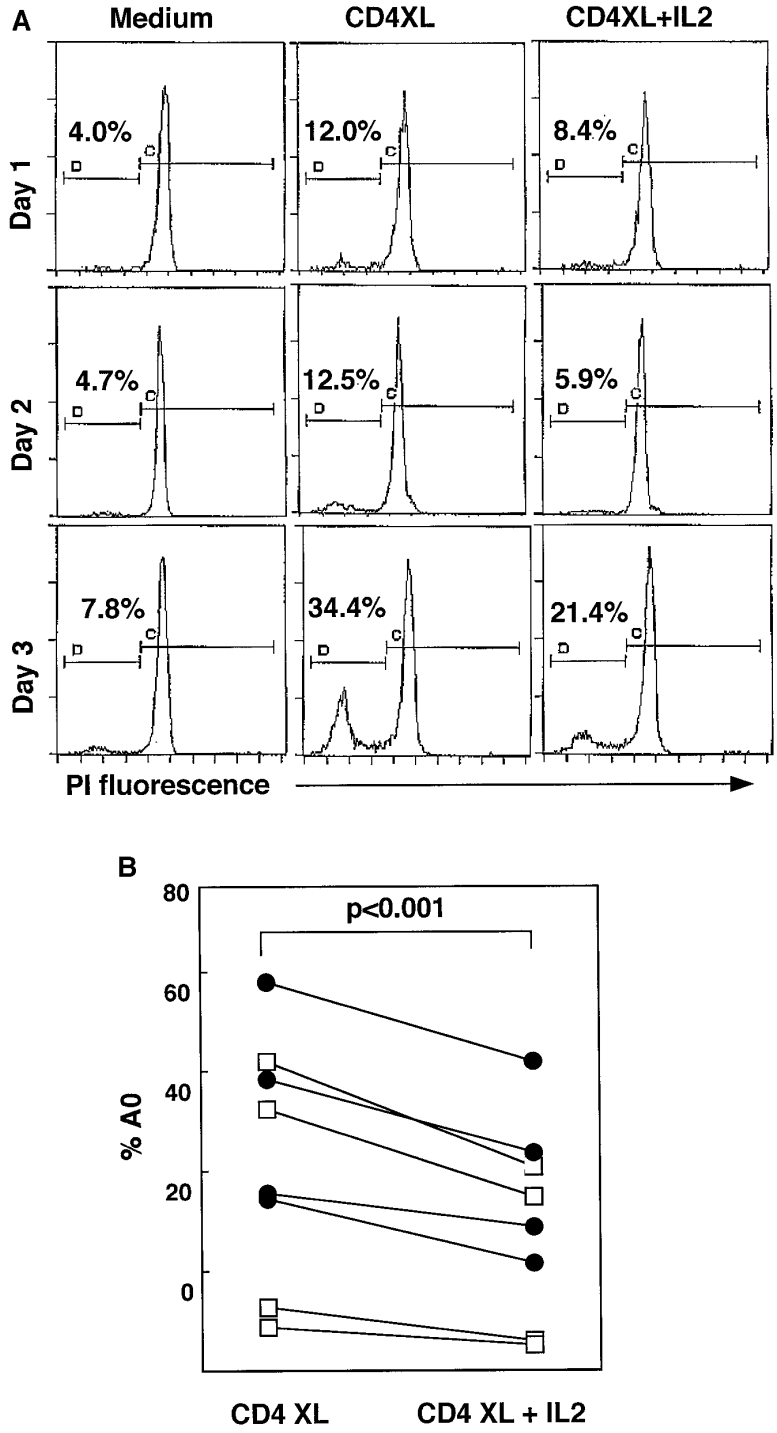


Fig 6. IL-2 rescues CD4XL cells from apoptosis. (A) Time course study of the effect of IL-2 on CD4 cross-linking induced-apoptosis. PBMC treated with or without Leu3a/GAM were cultured in the absence or presence of IL-2 (30 U/mL). Cells were harvested on days 1, 2, and 3 after treatment and analyzed for the percent of A0 cells by flow cytometry. The percentages of A0 cells (% A0) are indicated in the histograms. (B) Comparison of the % A0 cells in CD4XL samples with and without addition of IL-2. The (□) indicate results on day 2 (n = 4) and the (●) indicate results on day 3 (n = 4) after CD4XL. Values of statistical significance determined by paired *t* test are indicated.

effects of IL-2 in blocking CD4XL-induced apoptosis were closely associated with blocking of the CD4XL-induced downmodulation of Bcl-2 expression (Fig 7). As recent reports show,[29,31] this finding strongly suggests that IL-2 mediates its apoptosis-blocking effects via prevention of the downmodulation of Bcl-2 protein. Recent clinical trials of IL-2 in HIV-positive individuals[37,38] have showed that although no significant change occurred in plasma HIV concentrations with IL-2 therapy, $CD4^+$ T cells increased in a dose-dependent manner. The data reported herein support the findings of the clinical trial and implicate that rescue from apoptosis may be a mechanism for the observed increase in $CD4^+$ T cells. However, the addition of exogenous IL-2 in our experimental system only partially blocked the apoptosis

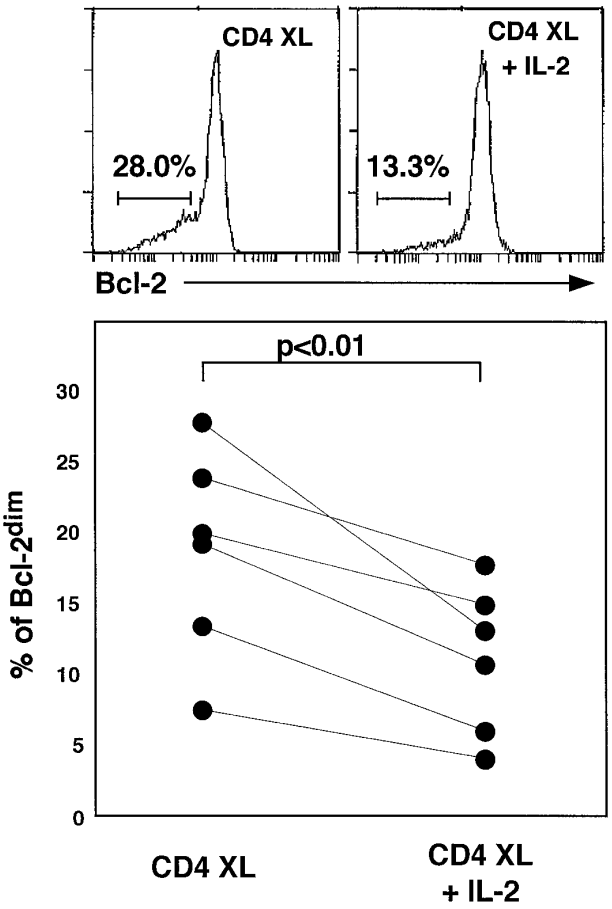


Fig 7. Downmodulation of Bcl-2 expression is prevented by IL-2. PBMC treated with Leu3a/GAM were cultured in the absence or presence of IL-2 (30 U/mL) for 3 days. The percentages indicated in the figure are percentages of the Bcl-2 dim plus Bcl-2 negative cells. Values of statistical significance determined by paired *t* test are indicated (n = 6).

resulting from CD4XL. Additional mechanisms underlying the CD4XL-induced apoptosis, such as failure of Bcl-x_L induction and measures to counteract these effects need further investigation.

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