Engagement of CD4 Before TCR Triggering Regulates Both Bax- and Fas (CD95)-Mediated Apoptosis

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Engagement of CD4 Before TCR Triggering Regulates Both Bax- and Fas (CD95)-Mediated Apoptosis

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In the present study, we have aimed at clarifying the CD4-dependent molecular mechanisms that regulate human memory T cell susceptibility to both Fas (CD95)-dependent and Bcl-2-dependent apoptotic pathways following antigenic challenge. To address this issue, we used an experimental system of viral and alloantigen-specific T cell lines and clones and two ligands of CD4 molecules, Leu-3a mAb and HIV gp120. We demonstrate that CD4 engagement before TCR triggering suppresses the TCR-mediated neosisynthesis of the Flice-like inhibitory protein and transforms memory T cells from a CD95-resistant to a CD95-susceptible phenotype. Moreover, evidence that the apoptotic programs were executed while Fas ligand mRNA expression was inhibited led us to analyze Bcl-2-dependent pathways. The data show that the engagement of CD4 separately from TCR influences the expression of the proapoptotic protein Bax independently of the anti-apoptotic protein Bcl-2, whereas Ag activation coordinately modulates both Bax and Bcl-2. The increased expression of Bax and the consequent dissipation of the mitochondrial transmembrane potential (ΔΨm) suggest a novel immunoregulatory function of CD4 and demonstrate that both passive cell death and activation-induced cell death are operative in CD4+ memory T cells. Furthermore, analysis of the mechanisms by which IL-2 and IL-4 cytokines exert their protective function on CD4+ T cells in the presence of soluble CD4 ligands shows that they were able to revert susceptibility to Bax-mediated but not to CD95-dependent apoptotic pathways. The Journal of Immunology, 2000, 164: 5078 –5087.

Abbreviations used in this paper: AICD, activation-induced cell death; PCD, passive cell death; Fasl, Fas ligand; FLIP, Flice-like inhibitor protein; ICE, IL-1-converting enzyme; HA, hemagglutinin; ZVAD-fmk, Z-Val-Ala-Asp-fluoromethilketone; I, 1-100 mycin; pAPC, peptide APC; PI, propidium iodide.
At present, a lot of data suggest the ability of anti-CD4 Abs to activate apoptotic pathways in Ag-activated human CD4+ T cells (20–23). Since anti-CD4 therapy has also been considered a valid approach to control autoimmune diseases (24–26) and organ graft rejection (27, 28), the use of anti-CD4 Abs can be considered a sound approach to revert resistance to apoptosis in Ag-activated T cells. A good model for the activation of apoptosis by CD4 engagement in vivo derives from HIV infection, and although this sound approach to revert resistance to apoptosis in Ag-activated T cells for rejection (27, 28), the use of anti-CD4 Abs can be considered a sound approach to control autoimmune diseases (24–26) and organ graft rejection (20–23). Since anti-CD4 therapy has also been considered a valid approach to control autoimmune diseases or in transplants or to fight apoptosis in AIDS, we need a detailed understanding of the mechanisms underlying CD4 function of CD4.

Mitochondrial damage, suggesting a novel immunoregulatory pathway, are activated by the increase of Bax expression and mitochondrial pathways are activated by the increase of Bax expression and mitochondrial damage, suggesting a novel immunoregulatory function of CD4.

Materials and Methods

Abs and reagents

 Synthetic peptides corresponding to residues 100–115 and 307–319 of influenza hemagglutinin (HA) were used in this study (Neosystem, Paris, France). The anti-human CD95 (CH11) and anti-human ZAP-70 mAbs were purchased from Upstate Biotechnology (Lake Placid, NY); the anti-CD4 was obtained from Kamiya Biomedical (Seattle, WA) and incubated at 37°C for 15 min. Both cell cycle distribution and apoptosis were measured by a Becton Dickinson FACStar flow cytometer as previously described (38), and 10,000 events were recorded for each sample. The amplification scale was linear for all parameters.

Cytotoxicity assay

The G12 effector T cell was activated with a mixture of 0.05 μM PMA and 0.5 μM ionomycin (PMA+I) for 4 h. Target cells, previously labeled with 51Cr for 1 h, were incubated with 20 μg/ml of gp120 and cultured after 2 h with APC in a 96-well round-bottom plate with varying numbers of effector T cells with M3 or not. After 4 h, the 51Cr release in the supernatants was determined on a ME Plus gamma scintillation counter (Micromedic Systems, Huntsville, TN). The percentage of specific apoptosis was calculated as follows: % specific apoptosis = 100 × (% PI−untreated cells − % spontaneous PI−untreated cells)/100 − % spontaneous PI−untreated cells.

PCR amplification

Gene expression was determined by RT-PCR (35). The PCR mixture, containing 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.2 μM 5’ and 3’ oligonucleotide primers, and 2.5 U Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT), was amplified in 0.5 ml GeneAmp tubes to a final volume of 50 μl. PCR reactions were amplified by 35 cycles at 94°C for 1 min, 60°C for 30 s, and 72°C for 30 s. PCR was performed in the automated DNA thermal cycler GeneAmp PCR System 2400 (Perkin-Elmer/Cetus). Primer sequences for β-actin, CD95, Bcl-2, Bcl-xL, FasL, and FLIP have been described previously (18). The primer sequence of Bax was as follows: Bax, 5’-AGCTCTGAGCAGATCATGAAG and Bax, 3’-CTCCCCGGAGGAAGCCCTAATG. Primer sequences for IL-2, IL-4, IL-10, FAS, and IFN-γ were obtained from the study by Butch et al. (39). PCR products were size fractionated by agarose electrophoresis and normalized according to the amount of β-actin detected in the same mRNA sample.

Transfection of COS cells and preparation of soluble human Fasl

Monkey COS cells (2 × 106 cells) were transiently transfected in 100-mm petri plates with 5 μg of the mammalian expression plasmid pEX-hFL1 carrying the full-length human FasL cDNA (kindly provided by Dr. S. Nagata, Department of Genetics, Osaka University Medical School, Osaka, Japan) using the DEAE-dextran method as reported previously (40). After 72 h, the soluble FasL was concentrated from the supernatant with Centricon filters (Amicon, Beverly, MA) and was used in apoptosis induction assay.

Immunoblotting

T cells were lysed at 106 cell/ml for 30 min on ice in 1% Nonidet P-40 lysis buffer containing 10% glycerol, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and CD45 RO+. Moreover, CD4 phenotypes of HC3 and HC6 and G12 were Th0 and Th1, respectively. DR1-expressing L cells (5–3.1), used as APC, were generated and cultured as described elsewhere (37).
and 1 mM EGTA in the presence of inhibitors of proteases and phosphatases: 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Pefabloc-SP (Pentapharm, Basel, Switzerland), 50 mM NaF, 10 mM Na$_4$P$_2$O$_7$, and 1 mM NaVO$_4$. Postnuclear lysates, obtained after centrifugation at 14,000 rpm for 10 min at 4°C, were boiled in SDS sample buffer before gel electrophoresis. After equilibration in transfer buffer (25 mM Tris base, 192 mM glycine, and 20% ethanol), gels were transferred to nitrocellulose membranes. Blots were blocked in PBST (10 mM phosphate buffer (pH 7.4), 2.7 mM KCl, 137 mM NaCl, and 0.1% Tween 20) in the presence of 10% nonfat milk for at least 1 h and incubated overnight at 4°C with primary Abs. At the end of the incubation, blots were extensively washed, incubated with 1/2000 dilution of peroxidase-conjugated goat anti-mouse or anti-rabbit (Amersham, Amersham, Buckshire, U.K.) and developed with the enhanced chemiluminescence detection system (Amersham). The densitometric analysis was performed by using a molecular imager (ImageQuant; Molecular Dynamics, Sunnyvale, CA).

Flow cytometric analysis of Bax and Bcl-2

Cells were fixed with PBS containing 2% paraformaldehyde and subsequently permeabilized in PBS containing 0.5% BSA, 0.02% sodium azide, and 0.5% saponin. Then cells were incubated for 15 min at room temperature with mouse anti-human Bax (Immunotech, Luminy, France) or FITC-conjugated hamster anti-human Bcl-2 (PharMingen, San Diego, CA) mAbs. Species- and isotype-matched irrelevant Abs used as background staining controls were: mouse IgG2b (Coulter Clone, Miami, FL) and FITC-conjugated hamster Ig mAbs (PharMingen). FITC-labeled goat F(ab’)$_2$ anti-mouse IgG and IgM (BioSource International, Camarillo, CA). Flow cytometric analysis was performed on a Becton Dickinson FACStar flow cytometer. Data are presented as mean fluorescence intensities over irrelevant control staining.

Analysis of variation in the mitochondrial transmembrane electrical potential ($\Delta \psi_{m}$)

Variations in $\Delta \psi$ at the single mitochondrial level was detected by using the lipophilic cation 5,5'6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1). Briefly, cells were incubated at 10^6 cells/ml in RPMI 1640 with 10% FCS for 15 min at 37°C with 10 μg/ml JC-1 (Molecular Probes, Eugene, OR). At the end of the incubation, cells were washed twice with PBS, resuspended in PBS, and $\Delta \psi_{m}$ was analyzed by a Becton Dickinson FACStar flow cytometer as described previously (41). Depolarization of mitochondrial membrane is accompanied by a change of JC-1 color from greenish orange (analyzed in FL-2) to green (analyzed in FL-1).

Results

CD4-mediated suicide of CD4$^{+}$ T cell clones is a CD95-independent phenomenon

To further characterize the effect of CD4 cross-linking on CD4$^{+}$ T cells activated by the specific Ag, we analyzed both apoptosis induction and the expression of proapoptotic and antiapoptotic genes, as well as type 1 and 2 cytokine genes, in CD4$^{+}$ T cell clones treated with Leu-3a mAb (Fig. 1). We found that cross-linking of CD4 2 h before TCR stimulation by Ag-pulsed APC

![Figure 1](http://www.jimmunol.org/): CD4-mediated suicide of CD4$^{+}$ T cell clones is a Fas-independent phenomenon. A, HC6 clone was incubated for 2 h with or without Leu-3a at 37°C. At the end of the incubation, it was cultured with prepulsed APC (CTR) in the presence (Leu3a + M3) or absence (Leu3a) of M3 mAb (10 μg/ml). After 72 h of incubation, apoptosis was evaluated by FACS analysis of PI-stained T cells. The graphs report DNA content measured by fluorescence-2 vs cell granularity measured by side scatter. The percentages of apoptotic cells are indicated in the squares. B, Total cellular RNA extracted from unstimulated T cells (T cells), activated by DR1$^{+}$ murine fibroblasts, Ag-pulsed (T + pAPC) or not (T + APC), and treated with Leu3a (T + pAPC + Leu3a) was reverse transcribed and amplified for 30 cycles with CD95, Fasl, Bcl-2, Bcl-xL, IL-2, IL-4, IL-10, and IFN-γ primers; β-actin primers were added in the same tubes. The reported results are representative of similar results obtained with the alloreactive T cell clone G12.
induced, after 3 days of culture, a massive apoptosis of T cells whereas Ag-stimulated T cells in the absence of CD4-cross-linking did not show any detectable effect (Fig. 1A). RT-PCR analysis of CD95, FasL, Bcl-2, and Bcl-xL (Fig. 1B), performed after 4 h of culture, revealed that only FasL, which was expressed in T cells correctly activated by Ag-pulsed APC, disappeared following the dissociation of TCR from CD4-mediated activation signals. Bcl-2 expression, undetectable in resting T cells and detectable only after Ag recognition, was not modified by CD4 cross-linking. CD95 and Bcl-xL did not show any modifications in all of the experimental conditions, suggesting that TCR triggering, either in association or in dissociation with CD4 triggering, does not modify their expression. The evidence that T cells induced by anti-CD4 to apoptosis were CD95<sup>+</sup> and FasL<sup>-</sup> suggested that the CD95-mediated apoptotic pathways were not directly activated and that T cells died due to CD95-independent phenomena. To verify this assumption, we added a soluble anti-CD95 antagonistic mAb (M3), shown in our previous experiments to inhibit CD95/FasL-mediated apoptosis, to the cultures of these cells (Fig. 1A and Ref. 42). The apoptotic features of T cells treated with anti-CD4 were not obstructed by M3 masking of FasL binding sites, confirming our hypothesis that a different apoptotic pathway from CD95 must be activated by CD4 cross-linking. RT-PCR of IL-2, IL-4, IL-10, and IFN-γ gene expression clearly show that the treatment with Leu-3a of Ag-activated T cells totally suppressed their expression (Fig. 1B).

**CD4-mediated suicide of CD4<sup>+</sup> T cell clones is a Bax-dependent phenomenon**

It has become more and more evident that a central checkpoint of apoptotic pathways is represented by mitochondria to such a point that many authors divide apoptosis into mitochondrion dependent and independent (43). Moreover, both CD95-independent and CD95-dependent apoptosis use mitochondria to mediate or to amplify, respectively, the protease cascade involved in the apoptotic machinery. Thus, to determine which of these two pathways was...
directly involved in apoptosis mediated by CD4 cross-linking, we studied the effect of the tripeptide pan-ICE inhibitor, zVAD-fmk, described as inhibiting only CD95-mediated apoptosis, (44) on our system of apoptosis. Thus, zVAD-fmk was added to the cultures of T cells programmed to apoptosis by Leu-3a or by anti-CD95 mAb CH11 at different times. The results reported in Fig. 2 show that apoptosis still occurred in zVAD-fmk-treated cultures when CD4 engagement by Leu-3a was used as an apoptotic stimulus. However, apoptotic features were significantly inhibited in the same cells when CD95 programs were activated by CH11. Since it has been demonstrated that hyperexpression of Bax causes mitochondrial depolarization and cytolysis, even in the presence of zVAD-fmk (44), we proceeded to analyze the levels of both Bax and Bcl-2 proteins by immunoblotting. The data reported in Fig. 2 clearly demonstrate that CD4 cross-linking before activation via TCR results in up-regulation of the Bax level present in Ag-activated CD4 T cell clones without affecting either Bcl-2 (middle panel) or ZAP-70 (lower panel) expressions. It is interesting to note that although Ag activation coordinately modulates Bax and Bcl-2, the engagement of CD4 by Leu-3a influences the expression of the proapoptotic protein Bax independently of the anti-apoptotic protein Bcl-2. The increased expression of Bax in T cells programmed to apoptosis suggested measuring the mitochondrial transmembrane potential (ΔΨm) by the lipophilic cationic probe JC-1 (41). In Ag-activated T cell clones, the ΔΨm presents a very low alteration, correlating with the scanty presence of apoptotic cells observed in the control of Fig. 1A. When the cells were treated with Leu-3a, a consistent percentage of cells showed significant ΔΨm alterations. These results are the first to provide evidence that the dissociation of the signals mediated by CD4 and TCR engagement results in up-regulation of Bax and consequent ΔΨm dissipation.

**FIGURE 3.** CD4 cross-linking favors CD95-dependent killing of CD4+ T cell clones by suppressing the expression of the Flice inhibitor FLIP. PALP T cell lines, treated or untreated with Leu-3a, were activated (D) or not (A–C) by the specific APC for 24 h. Different concentrations of CH11 (A) and APO1 (B) mAbs and soluble rFasL (C) were added to unstimulated cultures while 50 ng/ml of CH11, 10 ng/ml of APO1, and 1:5 soluble rFasL were added to Ag-stimulated cultures. Apoptosis was evaluated by FACS analysis and the values are expressed as a percentage of specific apoptosis. The data are representative of four independent experiments. Mean comparison was conducted using the unpaired Student’s t test. Differences between the data of pAPC + T and pAPC + T + Leu3a cultures untreated or treated with CH11, APO-1, and rFasL are significant (p < 0.0001). E, Total cellular RNA, extracted from unstimulated HC3 T cells (T cells), HC3 T cells activated by peptide-pulsed (T + pAPC), or unpulsed (T + APC) APC, and HC3 T cells activated by peptide-pulsed APC and treated with Leu-3a (T + pAPC + Leu3a), was reverse transcribed and amplified for 35 cycles; β-actin primers were added in the same tubes. Similar results were obtained for PALP T cell lines.

**FIGURE 4.** Effect of exogenous IL-2 and IL-4 on the expression of proapoptotic and antiapoptotic genes in T cells treated with Leu-3a. Leu-3a-treated HC6 clone was cultured with unpulsed (T + APC) or pulsed (T + pAPC) APC in the presence or absence of IL-2 and IL-4. After 4 h, total cellular RNA was reverse transcribed and amplified for 35 cycles. β-actin primers were added in the same tubes.

CD4 cross-linking favors CD95-dependent killing of CD4+ T cell clones

The evidence that T cells from HIV-infected subjects resemble a preactivated T cell phenotype (30) extremely susceptible to apoptosis upon CD95 ligation (45, 46), and recent data demonstrating that CD4 regulates the susceptibility to FasL-mediated apoptosis in primary T lymphocytes (23), suggested verifying the effect of CD4
engagement on the susceptibility of memory T cells, activated or not by an Ag, to CD95-dependent apoptosis. To this aim, T cells, treated or untreated with Leu-3a, were cultured in the absence (Fig. 3, A and B) or presence (Fig. 3D) of Ag-pulsed APC and one of the two agonistic anti-CD95 mAbs, CH11 or APO-1. Recombinant FasL was also used (Fig. 3C). The data in A–C of Fig. 3 clearly demonstrate that in the absence of Ag, treatment with Leu-3a only did not modify the susceptibility of CD4+ T cell clones to CD95-mediated apoptotic programs. In accordance with our recent observations (18), TCR activation with Ag-pulsed APC significantly rescues T cells from CD95-mediated apoptosis. On the contrary, CD4 engagement dissociated from TCR activation abolishes the rescue phenomenon (Fig. 3D). We have recently demonstrated that the ability of human memory T cells to regulate susceptibility to CD95-mediated apoptosis is dependent on the expression of FLIP. Since FLIP expression characterizes the differential responsiveness of resting or activated T cells to CD95 pathways (47), we compared the expression of FLIP in Leu-3a-treated or -untreated Ag-activated T cells. As shown in Fig. 3E, Ag-pulsed APC induce a strong induction of FLIP expression that completely disappears when the same cells are treated with Leu-3a. In conclusion, these results evidence the ability of CD4 to transform memory T cells from CD95-resistant to CD95-susceptible phenotypes.

Exogenous IL-2 and IL-4 protect T cells treated with Leu-3a from Bax-dependent but not from CD95-dependent apoptosis

Although we and others have demonstrated that apoptotic features mediated by the engagement of CD4 can be reverted by exogenous cytokines such as IL-2 and IL-4 (37, 48, 49), all of these data do not clarify the mechanisms by which these cytokines exert their protective function. Our data on the CD4-mediated regulation of Bax- and CD95-dependent apoptosis have made it possible to define which of these two apoptotic pathways was influenced by IL-2 and IL-4. To this aim, we analyzed first the effect of rIL-2 and rIL-4 on the expression of proapoptotic and antiapoptotic genes in apoptosis-induced cultures (Fig. 4). Analysis by RT-PCR revealed that both cytokines did not modify the expression of the proapoptotic genes, CD95 and FasL, in CD4-treated and Ag-activated cultures when compared with cultures activated in the absence of IL-2 and IL-4 (see Fig. 1A). Moreover, IL-2 and IL-4 were unable to restore the expression of the antiapoptotic gene, FLIP, suggesting that these cytokines were unable to revert the susceptibility to the CD95-mediated apoptotic pathway (Fig. 4). It is interesting to note that both IL-2 and IL-4 up-regulate the expression of Bcl-2 in T cells cultured in the absence of Ag stimulation, confirming the ability of these cytokines to regulate Bcl-2 expression (50, 51). No modification of Bcl-xL level in all culture conditions was observed (Fig. 4). Although the above results suggest that IL-2 and IL-4 rescue CD4-treated T cells from CD95-independent but not -dependent apoptotic pathways, we have confirmed these data in functional experiments where these two mechanisms could be dissected. First, we focused on determining whether IL-2 modifies the susceptibility of Ag-activated T cells treated with Leu-3a to anti-CD95 Ab. As shown in Fig. 5A, IL-2 completely rescues T cells from Leu-3a-induced apoptosis without modifying the susceptibility to anti-CD95, thus confirming the need of FLIP to antagonize CD95 pathways.

The next step was to investigate whether IL-2, able to up-regulate Bcl-2, could modify Bax expression and inhibit the mitochondrial changes associated with apoptosis. As shown in Fig. 5B, the presence of IL-2 in Ag-activated T cells treated with Leu-3a results in a significant decrease of Bax levels and a slight increase

**FIGURE 5.** Exogenous IL-2 protects Leu-3a-treated T cells from Bax-dependent but not from Fas-dependent apoptosis. A, PALP T cell lines preincubated with Leu-3a and activated by APC in the presence (+) or absence (−) of IL-2 (20 U/ml) were cultured in the absence of CH11 for 72 h or in the presence of CH11 (50 ng/ml) for 18 h, and apoptosis was evaluated by FACS analysis. The reported results represent the means ± SD of the percentage of specific apoptosis from four independent experiments. Differences between IL-2-untreated and -treated cultures in the absence of CH11 are significant (p < 0.0001). B, PALP cells were activated as in B, and Bax and Bcl-2 expressions were evaluated by FACS analysis. The reported values, expressed as mean fluorescence intensity, are the means ± SD of three independent experiments. C, PALP cells were activated for 72 h with APC after preincubation for 2 h with medium alone (CTR) or Leu-3a in the presence (Leu3a + IL-2) or absence (Leu3a) of IL-2 (20 U/ml). At the end of the incubation, mitochondrial ΔΨm, was analyzed by flow cytometry after staining with JC-1.
FIGURE 6. HIV gp-20 kills CD4+ T cells directly by a Fas-independent mechanism and indirectly by the interaction with FasL+ bystander T cells. A. G12 T cell clone was incubated for 2 h with or without gp120 at 37°C. At the end of the incubation, it was cultured with APC (CTR) in the presence (gp120 + M3) or absence (gp120) of M3 mAb (10 μg/ml). After 72 h of incubation, apoptosis was evaluated by FACS analysis as reported in Fig. 1A. B, G12, treated with gp120 was activated with APC in the absence (lanes 1–5) or presence of IL-2 (lanes 6–10). After 4 h total cellular RNA was reverse transcribed and amplified for 35 cycles with CD95 (lanes 1 and 6), FasL (lanes 2 and 7), FLIP (lanes 3 and 8), Bcl-2 (lanes 4 and 9), and Bcl-xL (lanes 5 and 10); β-actin primers were added in the same tubes. C, Lysis of 51Cr-labeled G12 target cells Ag-activated (T + pAPC) and treated with gp120 (T + pAPC + gp120) or with gp120 + M3 (T + pAPC + gp120 + M3) by PMA + I-activated G12 effector clone. The lysis of the T + pAPC + M3 target clone G12 was negative and is not shown. The results are expressed as a percentage of 51Cr release. Similar results were obtained when the HA-specific HC6 T cell clone, expressing the Vβ11 chain, was used as a target and the HC3 T cell clone, expressing the Vβ8 chain, was used as an effector.

of Bcl-2 levels observed in the same cultures in the absence of the cytokine. No significant differences in both Bax and Bcl-2 were observed between Ag-activated T cells and in the presence of Leu-3a and IL-2. The analysis of mitochondrial transmembrane potential by the lipophilic cationic probe JC-1 clearly shows that the presence of IL-2 results in a consistent reduction of ΔΨm alterations, confirming that hyperexpression of Bax causes mitochondrial depolarization (Fig. 5C). We believe that taken together these data offer for the first time an explanation of the mechanism by which IL-2 exerts its protective function on CD4-mediated apoptosis and can be of support to a rational use of IL-2 in therapy.

HIV gp120 kills CD4+ memory T cells directly by a CD95-independent mechanism and indirectly by interaction with FasL+ bystander T cells

We have previously demonstrated that the engagement of CD4 by gp120 without cross-linking with specific Abs and before TCR ligation programmed CD4+ memory T cells for apoptosis (35). The apoptotic programs were executed in the absence of accessory signals such as those mediated by the costimulatory molecules CD2 and CD28 and the cytokines IL-2 and IL-4 (37). These results prompted us to verify whether the same apoptotic mechanisms used by Leu-3a could also be used by gp120 to generate an in vitro system that could mimic the apoptotic phenomena induced in vivo by gp120. To this aim, we tested both the expression of proapoptotic and antiapoptotic genes and the susceptibility to CD95-mediated apoptosis, in the presence and absence of IL-2 and IL-4, by adopting the experimental conditions described in Figs. 1 and 3–5. The only difference was that T cells were incubated with gp120 (10 μg/ml) rather than Leu-3a. We found that gp120 treatment induced a relevant apoptosis of T cells that was not modified by the addition of anti-CD95 antagonistic mAbs (M3), confirming that, as observed with Leu-3a, gp120-mediated apoptosis is a CD95-independent phenomenon (Fig. 6A). Moreover, the interaction of gp120 with CD4 before TCR ligation inhibited FasL and FLIP expression that were expressed in T cells correctly activated by APC (data not shown) without modifying the levels of CD95, Bcl-2, and Bcl-xL mRNA, and this effect was not modified by the presence of rIL-2 (Fig. 6B). The addition of rIL-2 to the cultures was also unable to modify the increase of the susceptibility of Ag-activated T cells treated with gp120 to both agonistic mAbs (CH11 and APO-1) and rFasL (data not shown). The evidence that both CD95-dependent and CD95-independent programs are operative in CD4+ T cell clones treated with gp120 prompted us to mimic in vitro the scenario that could happen in vivo. We hypothesized that Ag-activated T cells, programmed to apoptosis by gp120, may easily be killed by Ag-activated FasL+ bystander T cells before executing their suicide. To confirm this hypothesis, we set up cocultures of T cell clones specific for alloantigen (G12) or for viral Ags (HC3 and HC6). Both G12 and HC6 T cell clones were treated with gp120 and activated for 24 h with the specific APC. At the end of the incubation, both G12 and HC6 T cells were cocultured either with the same clonotype or with a different clonotype and activated with PMA + I for 4 h as described previously.
(42). In particular, gp120-treated G12 cells and gp120-treated HC6 cells were cocultured with PMA + I-activated G12 and PMA + I-activated HC3, respectively. The possibility that activated bystander T cells were able to mediate apoptotic events in gp120-treated and Ag-activated T cells derived from the evidence that although both target and effectors were CD95 only the effector cells were FasL+ (Fig. 6B). Lysis of gp120-treated target cells (G12 and HC6) was evaluated using 51Cr release assay. Fig. 6B shows the results obtained with the alloreactive G12 T cell clone. It is clear that the cognate interaction of CD95+ and Fas+ T cells both expressing a resistant phenotype after TCR triggering (18) does not result in any lysis. However, the interaction of gp120 with CD4 molecules expressed on Ag-activated T cells that transforms these cells from AICD-resistant phenotypes to AICD-susceptible phenotypes favors their lysis. The lack of cytotoxicity in the presence of anti-CD95 antagonistic Mab (M3) confirms that the observed phenomenon is CD95/FasL dependent. Similar results were obtained in cocultures of HC3 and HC6 T cell clones (data not shown).

**Discussion**

The goal of this study was to characterize in a human experimental system the effect of CD4 engagement in regulating both PCD and AICD. The need for this information derives from the observation that peripheral T cells often acquire death-resistant phenotypes contrasting with the necessity to reduce the number of activated T cells after they have accomplished their task. Despite the large amount of evidence that CD4 regulates apoptosis both in vivo and in vitro, many questions still remain unanswered. We demonstrate that two ligands of CD4 molecules, Leu-3a mAb and gp120, regulate CD95 and Bcl-2 pathways in memory T cells. In fact, our data show for the first time that CD4 engagement before TCR triggering influences either the Bcl-2:Bax ratio or the transcription of FLIP. As a consequence, anti-CD4-treated T cells acquire both a PCD- and an AICD-susceptible phenotype. However, treatment with IL-2 or IL-4 rescues T cells from PCD, but fails to restore FLIP expression. Consequently, susceptibility to CD95-mediated apoptosis is maintained and cells become an easy prey of FasL+ bystander T cells.

To accomplish the analysis of the effect of CD4 engagement on apoptosis of human memory T cells, we have considered many parameters such as the quality of antigenic signaling and the cell cycle phase. Thus, we chose viral peptides or alloantigens because they are physiological stimuli and respond to the essential requirements for mediating full activation (52). Since a differential expression has been described for proapoptotic and antiapoptotic genes during the cell cycle (47), cell cycle progression is also a very critical parameter. Before setting up each experiment, we consequently waited for cell growth arrest and checked that almost all cells were accumulated in G0/G1. In this phase, T cell clones are negative for the antiapoptotic genes Bcl-2 and FLIP and for the cytokines IL-2, IL-4, IL-10, and IFN-γ and positive for CD95 and Bcl-xL (see Figs. 1 and 3).

The critical activator of AICD in mature T cells is FasL and therefore it has been proposed as the prime mediator of the peripheral T cell deletion and maintenance of peripheral self-tolerance (53). Recently, it has also been shown that FasL signals can partially overcome activation signals, thus preventing CD4+ T cell clonal expansion (54). Moreover, Oberg et al. (22) showed that inhibition of FasL expression in anti-CD3- or superantigen-stimulated T cells, by ligation of CD4 with anti-CD4 mAbs or HIV gp120, prevents AICD. The observed inhibition of AICD allowed the authors to establish that AICD can be inhibited in activated T cells. Although our data on the suppression of FasL by either Leu-3a or monomeric gp120 confirm these results, we cannot assert that these cells can be protected from undergoing AICD. In fact, CD4 engagement can protect T cells from CD95/FasL-mediated autolysis but not from bystander phenomena.

Recent data suggest that T cells acquire an AICD resistant phenotype only when correctly activated by an Ag. This kind of resistance can be attributed to high levels of intracellular FLIP which competitively inhibit the binding of caspase-8 to the CD95 receptor complex, thus shutting off the downstream CD95-signaling pathway (15, 18). Although the mechanisms controlling FLIP levels have not been elucidated, substantial data suggest that either TCR and costimulatory molecules (18) or cell cycle progression (47) or IL-2 (55) regulate the transcription of FLIP. In the present study, the evidence that also CD4 regulates FLIP expression suggests another mechanism to control susceptibility to apoptosis, both in vitro and in vivo (29, 56, 57). Moreover, the evidence that this condition of susceptibility is not reverted by IL-2 confirms the role of IL-2 in suppressing FLIP and in potentiating CD95-mediated apoptosis (58).

Several studies indicate the requirement of p56lck (58, 59) and Ras and calcineurin activation (60) for an optimal expression of FasL. The contemporary inhibition of FasL and FLIP expression mediated by the engagement of CD4 before TCR triggering suggests that these molecules may use common regulatory pathways. These results, along with our previous evidence that protein tyrosine kinase and calcineurin inhibitors inhibit both FasL and FLIP expression (18), reinforce this hypothesis.

The balance of interactions between pro- and antiapoptotic members of the Bcl-2 family is described to regulate Bcl-2-dependent apoptosis (5) and the ratio of Bcl-2:Bax determines whether a cell will respond to apoptotic stimuli. In particular, although changes in the levels of the Bcl-2 proteins probably do not contribute to the induction of apoptosis after IL-2 withdrawal (61), a predominance of Bax has been found to accelerate apoptosis in response to cytokine removal (5). However, it has also been shown that Bax proteins change modestly during T cell differentiation and activation as compared with Bcl-2 and Bcl-xL (61). Thus, Bax appears to be more sensitive to apoptotic stimuli than Bcl-2 and can be considered the executioner of PCD programs. The ability of Bax dimers to directly kill cells is consistent with genetic evidence that Bax can function independently of Bcl-2 (62). More recently, it has been demonstrated that a physiological death stimulus, the withdrawal of IL-3, resulted in the activation of Bax (63). The authors suggest a model where the translocation of monomeric Bax from the cytosol to the mitochondria, where it could be cross-linked as a Bax homodimer, induces mitochondrial dysfunction, resulting in cell death. Our data suggest that growth factors and Ag activation represent survival signals that, modulating Bax and Bcl-2 coordinately, sustain a PCD-resistant phenotype in both resting and activated T cells. Inhibition of the protective cytokines IL-2 and IL-4 following separated CD4 and TCR triggering represents a proapoptotic stimulus that induces the up-regulation of Bax independently of Bcl-2 and mitochondrial dysfunction. Evidence that exogenous IL-2 was able to down-regulate Bax expression and to reconstitute the mitochondrial function indicates that cytokine-mediated signals are necessary to regulate Bax expression (64).
alterations (65), an increased expression of CD95 on their membranes, and are more susceptible to CD95-mediated killing (21, 32, 45, 46, 66–68), although an increase of FasL is not clear (22). Our data on the mechanism by which gp120 regulates the susceptibility to FasL+ bystander cells reconcile the discrepancies on the role of CD95 in HIV infection. Moreover, our evidence that HIV gp120 is also able to induce CD95-independent apoptotic pathways is consistent with the observation that HIV kills T cells from patients with a genetically defective CD95 pathway or T cells in which the downstream effectors of the CD95 apoptotic pathway are blocked (57, 69) and the expression of the survival genes Bcl-2 and Bcl-xL are decreased (48, 70). Finally, our observations that T cells treated with gp120 acquire susceptibility to Fas-mediated apoptotic pathways in just a few hours, whereas it takes more than 24 h for the Fas-independent pathway to be operative, strongly suggests that the two distinct pathways of apoptosis, PCD and AICD, can be executed in HIV patients.

In conclusion, our studies have identified the molecular mechanisms that allow CD4 to control the balance between survival and apoptotic signals favoring both clonal amplification and tolerance in mature T cells. The detailed analysis of the effect of CD4 engagement by anti-CD4 mAb or gp120 on the susceptibility of T cells to PCD and AICD has clear implications for their use in mediating the loss of CD4+ T cells improperly activated by auto- and alloantigens.

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