THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 2, Issue of January 11, pp. 1477–1487, 2002

Printed in U.S.A.

Productive HIV-1 Infection of Primary CD4+ T Cells Induces Mitochondrial Membrane Permeabilization Leading to a Caspase-independent Cell Death*

Received for publication, March 26, 2001, and in revised form, August 9, 2001
Published, JBC Papers in Press, October 31, 2001, DOI 10.1074/jbc.M102671200

Frédéric Petit‡§, Damien Arnoult‡§, Jean-Daniel Lelièvre‡, Laure Moutouh-de Parseval¶, Allan J. Hance‖, Pascal Schneider, Jacques Corbeil¶, Jean Claude Ameisen‡ ‡‡, and Jérôme Estaquier‡ ‡‡§§**

*From ‡INSERM EMI-U 9922, CHU Bichat, Université Paris 7, 16 rue Henri Huchard, 75018 Paris, France, ‖INSERM U552, IMEA-INSERM, 46 rue Henri Huchard, 75018 Paris, France, ¶Departments of Pathology and Medicine, University of California San Diego, La Jolla, California 92161, and **Institute of Biochemistry, University of Lausanne, CH 1066 Epalinges, Switzerland*

We have explored *in vitro* the mechanism by which human immunodeficiency virus, type 1 (HIV-1) induces cell death of primary CD4+ T cells in conditions of productive infection. Although HIV-1 infection primed phytohemagglutinin-activated CD4+ T cells for death induced by anti-CD95 antibody, T cell death was not prevented by a CD95-Fc decoy receptor, nor by decoy receptors of other members of the TNFR family (TNFR1/R2, TRAILR1/R2/OPG, TRAMP) or by various blocking antibodies, suggesting that triggering of death receptors by their cognate ligands is not involved in HIV-induced CD4 T cell death. HIV-1 induced CD4 T cell shrinkage, cell surface exposure of phosphatidylserine, loss of mitochondrial membrane potential ($\Delta\psi m$), and mitochondrial release of cytochrome *c* and apoptosis-inducing factor. A typical apoptotic phenotype (nuclear chromatin condensation and fragmentation) only occurred in around half of the dying cells. Treatment with benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, a broad spectrum caspase inhibitor, prevented nuclear chromatin condensation and fragmentation in HIV-infected CD4+ T cells and in a cell-free system (in which nuclei were incubated with cytoplasmic extracts from the HIV-infected CD4+ T cells). Nevertheless, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone did not prevent mitochondrial membrane potential loss and cell death, suggesting that caspases are dispensable for HIV-mediated cell death. Our findings suggest a major role of the mitochondria in the process of CD4 T cell death induced by HIV, in which targeting of Bax to the mitochondria may be involved.

The depletion of CD4+ T cells is a major determinant of pathogenicity in human immunodeficiency virus type 1 (HIV-1)[1] infection. The finding that the level of the viral load established soon after infection correlates with the rate of CD4 T cell loss and the development of AIDS (1) supports the idea that active HIV-1 replication directly contributes to the depletion of CD4+ T cells. Accordingly, *in vitro* studies have shown that HIV-1 replication induces apoptosis in proliferating primary CD4+ T cells stimulated with PHA/IL-2 and in CD4+ T cell lines (2–6). Other findings, however, support the idea that death of uninfected T cells also contributes to AIDS pathogenicity. Both spontaneous and activation-induced apoptosis occur *in vitro* (7–10) and *in vivo* (11–13) in both infected and uninfected T cells from HIV-1-infected individuals.

Despite intensive investigations, several important questions remain about the mechanisms through which HIV infection induces CD4 T cell death. The first, as stated above, is whether HIV induces death in infected and/or uninfected CD4+ T cells. The second concerns the nature of the signal(s) that initiate cell death. T cells from HIV-1-infected individuals show enhanced cell surface expression of CD95 and exhibit increased susceptibility *in vitro* to CD95-mediated cell death, induced either by an agonistic anti-CD95 antibody, by soluble CD95 ligand (CD95L) (14–19), or by the engagement of other members of the tumor necrosis factor receptor (TNFR) family, including TRAILR and TNFR1 (20, 21). HIV-mediated death of productively infected CD4+ T cells *in vitro* has, however, been found to be independent of CD95/CD95L interactions (4–6), and the possible involvement of other members of the TNF receptor family has not been explored. Several findings suggest that viral proteins encoded by HIV-1 (gp120 envelope glycoprotein, Vpr) may induce death of either infected or uninfected CD4+ T cells in productively infected CD4 T cell cultures (22–28).

Finally, another area of uncertainty concerns the identification of the effector pathways that lead to cell death following HIV infection. It has been reported that treatment of HIV-

* This work was supported by INSERM, Université Paris 7, and grants from Agence Nationale de la Recherche sur le SIDA (ANRS), Sidaction, Université Paris 7, Fondation de la Recherche Médicale (to J. C. A.), a Human Science Frontier Program fellowship (to J. E.), a Sidaction fellowship (to F. P.), an ANRS fellowship (to J. D. L.), and a Délégation Générale de l'Armement fellowship (to D. A.). The part of the work performed at the University of California, San Diego (USCD), was supported by the UCSD Center for AIDS Research and Development Grant NIAID 5 P30 AI 36214; National Institutes of Health Grants AI 27670, AI 38858, AI 36214, and AI 29164; and the Research Center for AIDS and HIV Infection of the San Diego Department of Veterans Affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.
‡‡ These authors supervised this work.
§§ To whom correspondence should be addressed: INSERM EMI-U 9922, Faculté de Médecine Bichat, 16 rue Henri Huchard, 75018 Paris, France. Tel./Fax: 33 1 44 85 62 88; E-mail: estaquie@bichat.inserm.fr.

[1] The abbreviations used are: HIV, human immunodeficiency virus; TNF, tumor necrosis factor; TNFR, TNF receptor; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; IL, interleukin; MOI, multiplicity of infection; HM, heavy membrane; PS, phosphatidylserine; PI, propidium iodide; PHA, phytohemagglutinin; IAP, inhibitor of apoptosis protein; AIF, apoptosis-inducing factor; ROS, reactive oxygen species; $\Delta\psi m$, mitochondrial membrane potential; MIF, mean intensity of fluorescence; PARP, poly(ADP-ribose) polymerase; PBMC, peripheral blood mononuclear cell(s); zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; DDI, didanosine.

infected cells with caspase inhibitors prevents CD4 T cell death and results in increased viral production (4, 29), whereas other studies have found that caspase inhibitors did not prevent the death of infected CD4+ T cells (5). Two pathways are known to be important for transducing death signals to the apoptotic machinery. The "extrinsic" pathway involves activation of death receptors and recruit procaspase-8 through FADD (30, 31). Downstream of caspase-8, two pathways have been reported; caspase-8 may either directly activate caspase-3 or cleave Bid (a proapoptotic member of the Bcl-2 family), inducing the release of cytochrome *c* from mitochondria. Cytochrome *c*, together with Apaf-1, activates caspase-9, leading to the activation of the caspase-3 (32–34). The "intrinsic" pathway is death receptor-independent; stress signals activate proapoptotic members of the Bcl-2 family (Bax, Bak, etc.) and induce the permeabilization of the mitochondria and the release of apoptogenic factors (35). Although caspases are essential effectors of the nuclear apoptotic phenotype (chromatin condensation and fragmentation), evidence from experimental systems using broad spectrum caspase inhibitors support the notion that programmed cell death can proceed in a caspase-independent manner (36–39). Moreover, recent reports have suggested that caspase inhibitors, which inhibit apoptosis induced by diverse stimuli, lead to the appearance of dead cells expressing necrotic-like phenotype (40–42). Cellular mechanisms that account for caspase-independent programmed cell death are still elusive and may involve release by mitochondria of effectors such as apoptosis-inducing factor (AIF) (43).

To further characterize the mechanisms responsible for cell death induced by HIV-1, freshly isolated CD4+ T cells were infected with HIV-1 and stimulated with PHA/IL-2. We observed that only around half of the dying CD4+ T cells displayed a typical apoptotic phenotype (cell shrinkage, chromatin condensation, and fragmentation), the other half showing a nonapoptotic cell death phenotype (membrane permeabilization and intact nucleus) that only shared one feature with apoptosis (cell shrinkage). Treatment with zVAD-fmk, a broad caspase inhibitor, prevented the induction of an apoptotic phenotype (nuclear chromatin condensation and fragmentation) in the HIV-infected CD4+ T cells but did not prevent loss in mitochondrial membrane potential ($\Delta\Psi m$) and cell death. Our results support a scenario in which disruption of the mitochondria membrane permeability is a central event in cell death following HIV-1 infection.

MATERIALS AND METHODS

Reagents, Antibodies, and Cytokines—Murine monoclonal antibodies with the following specificities were used: CD14, CD19, CD56, and CD8 (Pharmingen, San Diego, CA); agonistic anti-CD95 mAb (CH11 and 7C11) (Coulter Corp., Miami, FL); antagonistic anti-CD95 mAb (ZB4) (Coulter Corp.); antagonistic anti-TNFR1 and -TNFR2 mAbs (R&D Systems); neutralizing anti-TNF antibody (R&D Systems). Soluble decoy proteins were human CD95-Fc immunoglobulin fusion protein (binds CD95L) purchased from Alexis Corp. (San Diego, CA), TRAILR1-Fc, TRAILR2-Fc (binds TRAIL), OPG-Fc (binds RANKL and TRAIL), TNFR1-Fc (binds TNF and lymphotoxin α), and TRAMP/DR3-Fc (orphan receptor) produced as described (44, 45). Labeled antibodies were as follows: PercP-labeled CD4 mAb (Leu 3a; Becton Dickinson, Mountain View, CA); PC5-labeled CD4 mAb (13B8.2; Coulter Corp.); phosphatidylethanolamine-labeled anti-p24 antigen (KC-57; Coulter Corp.); FITC-labeled anti-Bcl-2 (124; DAKO, Trappes, France). For Western blotting we used a rabbit polyclonal anti-caspase-3 (Pharmingen, San Diego, CA), a rabbit polyclonal anti-caspase-9 (Cayman Chemicals, Ann Arbor, MI), a mouse IgG2b anti-caspase-8 (5F7; Upstate Biotechnology, Inc., Lake Placid, NY), a mouse IgG1 anti-PARP (C2-10; Pharmingen), a mouse IgG1 anti-survivin (MAB747; R&D Systems), a mouse IgG1 anti-c-IAP1 (B75-1; Pharmingen), and a mouse anti-actin (AC40; Sigma). Cytochrome *c* was probed with a mouse IgG2b anti-cytochrome *c* (7H8.2C12; Pharmingen), and AIF was probed with a rabbit polyclonal anti-AIF obtained from rabbit immunized against a mixture of three different human AIF peptides (amino acids 106–120, 512–526, and 588–602). Bax and VDAC were probed with a mouse IgG1 anti-Bax (6A7; Pharmingen) and a mouse anti-VDAC (Calbiochem). Recombinant human IL-2 was kindly provided by Chiron Corp. (Emeryville, CA). Other reagents were annexin-V-FITC (Coulter Corp.), propidium iodide and DiOC6 (Molecular Probes, Inc., Eugene, OR), and Hoechst 33342 (Sigma). Caspase inhibitor zVAD-fmk was purchased from Calbiochem, while DDI, a reverse transcriptase inhibitor, was purchased from Sigma.

Cells and Culture Conditions—Heparinized venous peripheral blood was obtained from HIV-seronegative healthy donors. PBMC were isolated by Ficoll-Hypaque density gradient centrifugation and cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Summit Biotechnology, Greeley, CO), 2 mM L-glutamine, 1 mM sodium pyruvate (Invitrogen), and penicillin/streptomycin (Invitrogen). When indicated, purified CD4+ T cells were obtained by depleting PBMC of B cells, NK cells, and CD8+ T cells by negative selection, using CD19, CD56, and CD8 mAbs and magnetic beads coated with anti-mouse IgG (Dynal, Lake Success, NY). PBMC were incubated in the absence or presence of virus at the indicated multiplicity of infection (MOI) for 2 h at 37 °C. After two washes, the cells were resuspended in complete medium in the absence or presence of 1 μg/ml PHA-P (Sigma) and 100 IU/ml IL-2. When indicated, CD4+ T cells were incubated with either agonistic CD95 mAb or Fc decoy receptors. HIV p24 antigen was measured by an enzyme immunoassay as described by the manufacturer (Abbott). Intracellular p24 antigen was also assessed by flow cytometry after fixation and permeabilization of CD4+ T cells with Intraprep permeabilization reagent (Coulter Corp.).

Test of Decoy Receptors—Efficiency of decoy receptors was tested as described. Jurkat cells were incubated with FLAG-TRAIL (400 ng/ml) and cross-linked with anti-FLAG M2 (2 μg/ml) in the absence or presence of decoy receptors (TRAILR1-Fc, TRAILR2-Fc, OPG-Fc) (40 μg/ml) for 16 h, and apoptosis was monitored by flow cytometry using annexin-V-FITC. WEHI 164 cells were incubated with TNFα in the absence or presence of TNFR1-Fc (40 μg/ml) for 16 h. Living cells were stained with the PMS/MTS test (phenazine methosulfate/3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2*H*-tetrazolium, inner salt) as previously described (44).

Virus Preparation—High titer stocks of the laboratory strain HIV-1_{LAI} (10^6 $TCID_{50}$/ml) were prepared by inoculating CEM at an MOI of 0.001 and growing the cells for 10 days. 10 ml of this culture were added to 400 ml of uninfected CEM (5×10^5 cells/ml) and grown for 5–7 days until abundant syncytia were present. The cells were pelleted ($300 \times g$ for 10 min) and resuspended in one-one hundredth of the initial volume for 8 h. The supernatant was clarified by centrifugation ($800 \times g$ for 10 min).

Measurement of Cell Death—Nuclear condensation and fragmentation (typical morphological changes of apoptosis) was visualized by UV microscopy using Hoechst 33342 nuclear dye. Cells were also double-stained with propidium iodide and Hoechst 33342 to distinguish apoptotic cells from cells that lost membrane integrity. Intact blue nuclei, condensed fragmented blue-pink nuclei, and intact pink nuclei were considered viable, apoptotic (early and late), and nonapoptotic cells, respectively. Cells were also analyzed by light microscopy using trypan blue dye reagent (Sigma). Live cells showed normal refringent cytoplasm, apoptotic cells displayed typical chromatin condensation and fragmentation and excluded trypan blue, and nonapoptotic cells were trypan blue positive, as previously described (7, 15, 18, 46). By flow cytometry, we determined dying cells using FITC-conjugated annexin-V, and to evaluate $\Delta\psi m$, cells were stained with DiOC6. Nuclear condensation and fragmentation was also assessed by flow cytometry using propidium iodide.

Western Blotting—For total extracts, cells were incubated in SDS lysis buffer, boiled for 10 min, and centrifuged for 15 min at room temperature. Total protein was measured using the DC protein assay (Bio-Rad). Equal amounts of proteins were boiled for 5 min in 2× Laemmli sample buffer with β-mercaptoethanol and run on a 4/20% polyacrylamide gel (Bio-Rad). Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) and immunoblotted with specific antibodies. Western blots were then visualized using horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Inc.), followed by enhanced chemiluminescence (Amersham Biosciences).

For detection of cytochrome *c* and AIF, cells were incubated in cell extract buffer (50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM cytochalasin B, and 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4 °C, homogenized with a Dounce

homogenizer, and centrifuged at 14,000 × *g* for 30 min at 4 °C. The supernatant was removed and stored as cytosolic fraction.

The mitochondria-enriched heavy membrane (HM) fraction was prepared as follows. Cells were washed in isotonic buffer (10 mM HEPES, pH 7.5, 200 mM mannitol, 70 mM sucrose, 1 mM EGTA) supplemented with a mixture of protease inhibitors (Roche Molecular Biochemicals) and homogenized with a Dounce homogenizer. Nuclei and unbroken cells were separated at 120 × *g* for 5 min. The supernatant was centrifuged at 10,000 × *g* for 30 min to collect HM pellet.

Cell-free Extracts—Cytoplasmic extracts were derived from uninfected and HIV-infected primary CD4+ T cells stimulated with PHA/IL-2. Cytoplasmic extracts from Jurkat T cells incubated in the absence or presence of CD95 mAbs (7C11, Coulter Corp.) for 4 h were also used as a control. Cell-free extracts and nuclei from CEM cells were prepared as previously described (47). Briefly, cytoplasmic extracts were prepared as follows. Cells were washed twice in phosphate-buffered saline and incubated on ice for 20 min with cell extract buffer. Cells were lysed with a B-type pestle. Lysis was monitored by phase-contrast microscopy. The cell lysate was centrifuged at 4 °C for 15 min at 17,000 × *g*, and the clear cytosol was carefully removed. CEM nuclei were prepared as follows. CEM cells were washed twice in phosphate-buffered saline and once in nuclei isolation buffer (10 mM PIPES, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM cytochalasin B, and 1 mM phenylmethylsulfonyl fluoride), resuspended in nuclei isolation buffer, allowed to swell on ice for 20 min, and gently lysed with a Dounce homogenizer. Liberated nuclei were then layered over 30% sucrose in nuclei isolation buffer and centrifuged at 800 × *g* for 10 min, followed by washing in nuclei isolation buffer and resuspension in nuclei storage buffer (10 mM PIPES, pH 7.4, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM dithiothreitol, 0.5 mM spermidine, 0.2 mM spermine, 1 mM phenylmethylsulfonyl fluoride, and 50% glycerol) at 2 × 10^8 nuclei/ml. Nuclei were stored at −80 °C until use.

Cell-free reactions (25 μl) comprised 20 μl of cytoplasmic extract (2–10 mg/ml protein), 1 μl (2 × 10^5) of nuclei, and 4 μl of extract dilution buffer (10 mM HEPES, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 2 mM ATP, 10 mM phosphocreatine, and 50 μg/ml creatine kinase).

Cell Surface Staining—Two-color flow cytofluorometric analysis (FACScan; Becton Dickinson) was performed by co-staining cells with mAbs (including isotype controls) directly labeled with phosphatidylethanolamine or PercP. Lymphocytes were gated by forward and side scatter parameters.

Statistical Analysis—Statistical significance (*p*) was assessed using the paired Student's *t* test as indicated in the figure legends.

RESULTS

Productive HIV Infection of Primary CD4+ T Cells Induces both Typical Apoptosis and Nonapoptotic Cell Death—Resting PBMC from healthy donors were incubated for 2 h with the laboratory strain HIV-1_{LAI}. After removing unbound residual virus, T cells were stimulated with PHA and IL-2 for 4 days, and cell death and viral production were assessed. Both cell death and viral replication (assessed by p24 expression) increased with the inoculum (Fig. 1*A*). Absolute numbers of CD4+ T cells were decreased in the infected cultures, the extent of depletion being proportional to the viral input (Fig. 1*B*). Cytofluorimetric analysis of T cell populations indicated that after 4 days of PHA and IL-2 stimulation, ~10% of CD4+ T cells from six different donors inoculated with HIV-1_{LAI} remained in the culture, compared with 55% in the uninfected cultures (Fig. 1*C*). Cell death preceded any significant appearance of syncytia in the culture (data not shown).

In order to exclude the possibility that HIV-induced CD4 T cell death in the cultures resulted (at least in part) from CD8+ T cell-mediated killing, we purified CD4+ T cells by negative selection prior incubation with HIV-1_{LAI} and PHA/IL-2 stimulation (Fig. 1*D*). In these conditions (as in unfractionated PBMC), HIV-1_{LAI} induced CD4 T cell depletion. This strongly suggested that viral-induced CD4 T cell depletion occurred independently of participation of additional lymphoid cells, such as CD8+ T cells, NK cells, or B cells.

Approximately 50% of the HIV-infected cells displayed cell shrinkage (Fig. 2, *B* and *E*), phosphatidylserine (PS) exposure

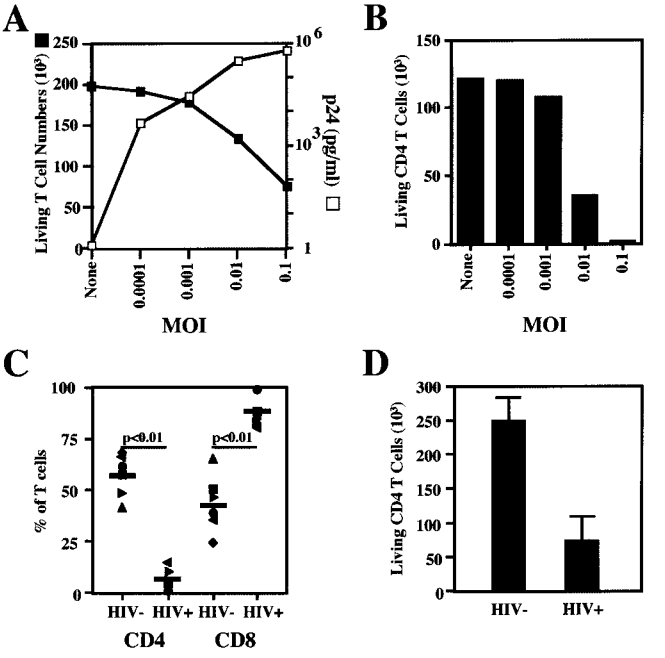

FIG. 1. **CD4 T cell depletion induced by productive infection of PBMC with HIV-1_{LAI} following PHA/IL-2 stimulation.** PBMC from normal donors were incubated for 2 h with either medium alone (*None*) or with HIV-1_{LAI} at various MOI (0.0001–0.1), washed, and cultured for 4 days in the presence of PHA and IL-2. After the 4-day culture, the following parameters were assessed. *A*, absolute numbers of surviving cells (■); viral replication (□) as assessed by p24 antigen in the cell supernatants. *B*, absolute numbers of surviving CD4+ T cells calculated by the counting of absolute numbers of surviving cells in a hemocytometer and analyzing the percentage of CD4+ T cells by flow cytometry. The experiment presented is representative of three experiments performed. *C*, percentages of CD4+ and CD8+ T cells, assessed using flow cytometry in PBMC from six different donors (each *symbol* represents results from one individual) cultured for 4 days in the presence of PHA/IL-2 after incubation with medium alone (*HIV*−) or with HIV-1_{LAI} (*HIV*+) at a MOI of 0.1. *Bars* represent mean values in each group. Statistical significance was assessed using the paired Student's *t* test. *D*, CD4+ T cells were purified from PBMC of normal donors using negative selection and then incubated for 2 h with either medium (*None*) or HIV-1_{LAI} (*HIV*+) at a MOI of 0.01, washed, and cultured for 6 days with PHA/IL-2. Viable cells were counted by microscopic analysis. Results are the mean of five independent experiments.

(Fig. 2, *C* and *E*) and loss in $\Delta\psi$m (Fig. 2, *D* and *E*). In contrast, nuclear chromatin condensation and fragmentation typical of apoptosis, as visualized by UV microscopy using Hoechst 33342 nuclear dye (Fig. 2*A*), was observed in only 20–30% of CD4+ T cells in the HIV-infected cultures (Fig. 2*E*). Thus, half of the dying CD4+ T cells displayed typical apoptosis, and the other half displayed a nonapoptotic cell death phenotype characterized by lack of nuclear chromatin condensation and fragmentation associated with a loss in membrane integrity (Fig. 2*E*). By double staining using annexin-V, which detects PS exposure, and propidium iodide (PI), which measures membrane integrity, HIV-infected cultures contained both annexin-V-positive/PI-negative CD4+ T cells (apoptotic) and annexin-V-positive/PI-positive CD4+ T cells (nonapoptotic) (20.5 ± 6 and 32.3 ± 8%, respectively; data not shown). Altogether, these data suggest that HIV induces both typical apoptosis and an additional phenotype of cell death. The latter was distinct from necrosis (membrane permeability and cell swelling), since CD4+ T cells displayed a membrane permeability associated with a cell shrinkage.

Antagonists of the TNFR Family Members Do Not Prevent HIV-1_{LAI}-mediated CD4 T Cell Death—As shown in Fig. 3, *A* and *B*, when CD4+ T cells were incubated with HIV-1_{LAI} and stimulated by PHA/IL-2, CD4+ T cells were primed for cell

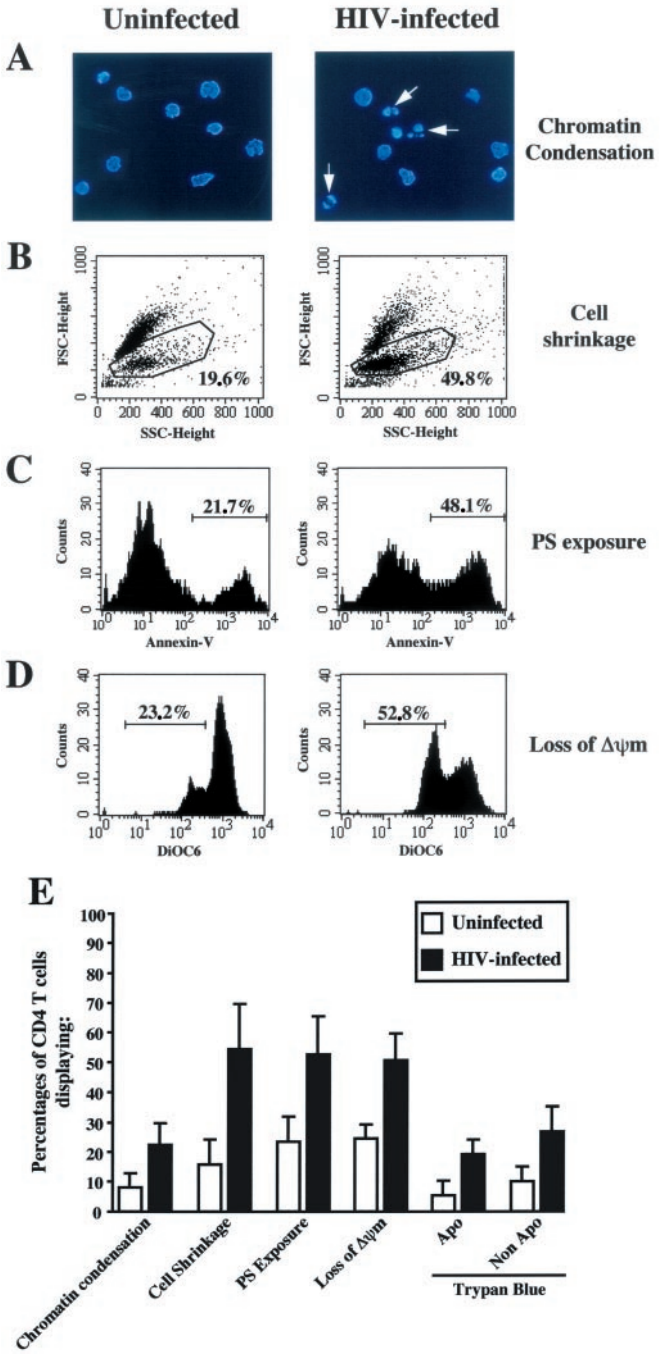

FIG. 2. **CD4 T cell depletion involved both apoptotic and non-apoptotic cell death.** CD4+ T cells were purified, incubated for 2 h with either medium (uninfected) or HIV-1_{LAI} (HIV-infected) at a MOI of 0.01, washed, and cultured with PHA/IL-2 for 6 days. *A*, chromatin condensation and fragmentation was visualized by UV fluorescence microscopy using Hoechst 33342. *B*, shrunken cells were visualized by flow cytometry with relatively high side scatter and low forward scatter properties. *C*, PS exposure was determined using annexin-V-FITC. *D*, $\Delta\psi$m was assessed using mitochondrial dye reagent DiOC6. *E*, mean of four independent experiments. Apoptotic and nonapoptotic cells were visualized by light microscopy using trypan blue dye reagent. Apoptotic cells (*Apo*) displayed translucent cytoplasm and condensed nuclei but excluded trypan blue dye reagent, nonapoptotic cells (*Non Apo*) were *blue* cells, and live cells displayed refringent cytoplasm. 300 cells were counted in duplicate.

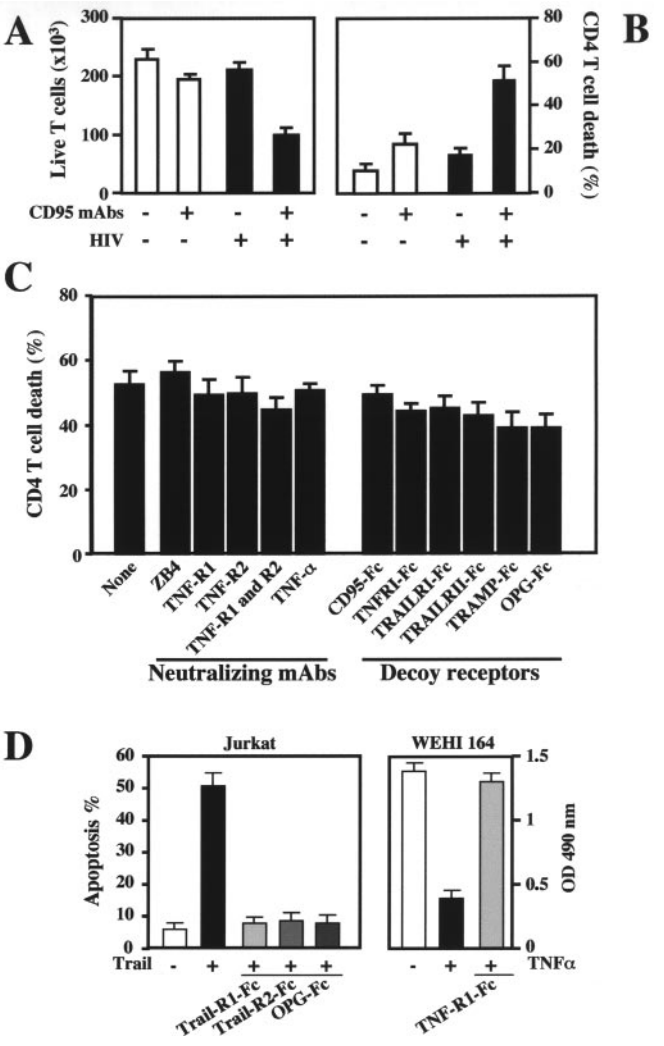

FIG. 3. **CD4 T cell depletion induced by HIV-1 infection following PHA/IL-2 stimulation does not involve members of the TNF receptor family.** To assess sensitivity of CD4+ T cells to CD95 ligation-mediated cell death, CD4+ T cells were purified, incubated for 2 h with either medium (□) or HIV-1_{LAI} (■) at a MOI of 0.005, washed, and cultured for 4 days with PHA/IL-2. Cells, isolated through Ficoll-Hypaque density gradient centrifugation, were then incubated for 6 h in the absence (−) or presence (+) of 1 μg/ml of an agonistic anti-CD95 antibody (*CD95 mAbs*). *A*, the absolute numbers of surviving CD4+ T cells remaining in the culture; *B*, the percentages of dying CD4+ T cells in the same cultures, as indicated by flow cytometric analysis using CD4 antibody and annexin-V double labeling. Results are the mean of three experiments performed. *C*, to assess the role of the death receptors, CD4+ T cells were purified, incubated for 2 h with HIV-1_{LAI} at a MOI of 0.01, washed, and cultured for 3 days with PHA/IL-2. Cells were then cultured for 72 h in the absence (*none*) or presence of neutralizing mAbs (20 μg/ml) or decoy receptors (40 μg/ml) that block receptor/ligand interactions. Shown are the percentages of dying CD4+ T cells as assessed by evaluation of annexin-V staining. Results are the mean of three independent experiments performed. *D*, Jurkat and WEHI 164 cells were used as control of efficiency for decoy receptors as described under "Materials and Methods."

death in response to antibody-mediated CD95 ligation at day 4. In contrast, uninfected activated CD4+ T cells became sensitive to CD95 ligation-mediated death at day 6, as previously described (48) (data not shown). Although HIV-infected CD4+ T cells became sensitive to CD95 ligation, this process appeared not to account for their subsequent death in the absence of any CD95 antibody treatment. Indeed, when cultures were treated 3 days after PHA/IL-2 stimulation with a neutralizing mAb (ZB4) or a CD95-Fc decoy receptor (which prevents interaction between CD95L and CD95 (49)), CD4 T cell death observed 3 days later was not prevented (Fig. 3*C*). Similarly, no preventive effect was observed when CD95-Fc was added at day 1 following PHA stimulation (data not shown). The cellular localization of CD95L was examined (50) by isolating cytosolic

and membrane fractions of CD4+ T cells after stimulation with PHA/IL-2 and evaluating the presence of CD95L by Western blotting. At both days 4 and 6, CD95L (35-kDa protein) was localized in the cytosolic fraction, not in the membrane fraction, of both uninfected and HIV-infected CD4+ T cells (data not shown).

CD95L/CD95 is a death ligand/receptor pair of the TNF/TNF receptor superfamily, which also includes other pairs, such as TRAIL/TRAILR1, TRAIL/TRAILR2, and the orphan death receptor TRAMP(DR3) (51). Using either neutralizing antibodies or decoy receptors that have been previously demonstrated to block the interaction between ligands and receptors (44, 45, 52) (Fig. 3*D*), we assessed whether HIV-mediated cell death of CD4+ T cells stimulated with PHA/IL-2 involved one or more of these death receptors. *In vitro* treatment with the decoy receptors or neutralizing antibodies 3 days after PHA/IL-2 stimulation did not prevent CD4 T cell death observed 3 days later (Fig. 3*C*). These data suggest that HIV-1 triggers a cell death pathway that is independent of the TNF receptor family (CD95, TNFR1/TNFR2, TRAILR1/R2/OPG, TRAMP).

HIV-1_{LAI}-mediated Cell Death Is Associated with Caspase Activation—Caspases, the main effectors of apoptosis, are synthesized as inactive procaspases that require processing to become active (53). The current model suggests that after apoptotic stimuli, activation of initiator caspases such as caspase-8 and caspase-9 leads to the proteolytic cleavage of effector caspases such as caspase-3. To assess the role of caspases in HIV-mediated cell death, caspases from CD4+ T cells were analyzed by Western blotting. We observed that in uninfected and HIV-infected PHA-stimulated CD4+ T cells, the amount of proenzymes detected was increased compared with that of unstimulated T cells at both 4 and 6 days. Caspase-3 (32-kDa proenzyme) was reproductively found to be processed into fragments of 20 kDa after PHA/IL-2 stimulation (Fig. 4). None of these fragments (17, 19, and 21 kDa) was observed in unstimulated CD4+ T cells. The observation of caspase cleavage in PHA-activated CD4+ T cells is in agreement with a recent report showing that CD4+ T cells, in contrast to transformed T cell lines, rapidly process caspases, including caspase-3, and caspase substrates following T cell activation (54). Although a similar pattern was observed comparing uninfected and HIV-infected CD4+ T cells, the amount of caspase-3 proenzyme in HIV-infected CD4+ T cells at days 4 and 6 after PHA/IL-2 stimulation was decreased compared with that in uninfected CD4+ T cells (Fig. 4), suggesting an increased processing of the proenzyme in the HIV-infected CD4+ T cells. Activation of the initiator caspases was also assessed using antibodies against caspase-8 and -9. Western blotting showed that after stimulation with PHA/IL-2 caspase-8 was processed into 43–45-kDa fragments, but the 18-kDa active subunit was detected at day 6 only in HIV-infected CD4+ T cells. In addition, the amount of proenzymes in HIV-infected CD4+ T cells drastically decreased at day 6 compared with that in uninfected CD4+ T cells (Fig. 4). In the same extracts, caspase-9 (46 kDa) was processed into a 37-kDa fragment following PHA/IL-2 stimulation in both uninfected and HIV-infected CD4+ T cultures. The amount of proenzyme decreased at days 4 and 6 in HIV-infected CD4+ T cells, compared with that of uninfected CD4+ T cells (Fig. 4). Altogether, these results suggest that HIV-1 infection induces procaspases processing. Since caspase-3 was activated after PHA/IL-2 stimulation, we determined whether caspase substrates were also processed in stimulated CD4+ T cells. PARP (115 kDa) is one of the caspase-3 substrates cleaved during apoptosis (55). Stimulation with PHA/IL-2 increased PARP protein levels, confirming previous reports showing an up-regulation of PARP and its cleavage in stimulated primary T cells (54). Concomitant with its induction, 80% of PARP was found processed into an 85-kDa fragment in PHA-stimulated CD4+ T cells, whether or not they were infected with HIV-1 (Fig. 4).

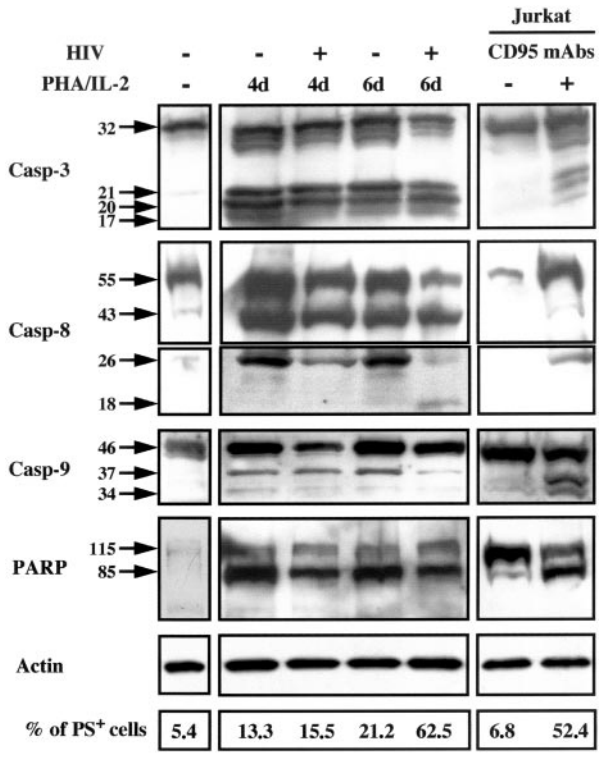

FIG. 4. **Procaspase processing in uninfected and HIV-infected CD4+ T cells following PHA/IL-2 stimulation.** CD4+ T cells were purified from PBMC of normal donors using negative selection and then incubated for 2 h with either medium (−) or HIV-1_{LAI} (+) at a MOI of 0.01, washed, and cultured for 4 (*4d*) or 6 days (*6d*) with PHA/IL-2 (+) or without stimulation (−). Extracts from uninfected and HIV-infected CD4+ T cells were analyzed for caspase-3, caspase-8, caspase-9, and PARP by Western blotting. Extracts from Jurkat cells cultured in the absence (−) or presence of CD95 mAbs (+) are shown as controls. For caspase-8, the Western blot was exposed for 3 min (proenzyme) and 25 min (cleaved products). As a control of loading, actin was used. Proenzymes and cleaved subunits are indicated on the *left* by *arrows*. The percentages of dying cells (*% of PS^+ cells*) determined by flow cytometry using annexin-V-FITC are indicated. The experiment is representative of three independent experiments performed.

Inhibitor of Apoptosis Proteins (IAPs) have been proposed to inhibit caspases in more distal portions of the cell death pathway, downstream of cytochrome *c* (56, 57). Western blotting of c-IAP1 and survivin (Fig. 5) revealed that the amount of c-IAP1 was much lower in the cytosolic fraction of HIV-infected CD4+ T cells compared with that in uninfected CD4+ T cells (c-IAP1 protein was decreased by 60% in two independent experiments). In contrast, no major difference in the expression of survivin protein was observed when comparing HIV-infected and uninfected CD4+ T cells (a difference of less than 10% was observed). These data indicate that cell death of productively HIV-infected CD4+ T cells is associated with a decrease in c-IAP1 expression.

We next determined the apoptogenic effect of cytoplasmic extracts on isolated CEM nuclei in a cell-free system. In these studies, cytosolic fractions of PHA-stimulated CD4+ T cells from uninfected and HIV-infected cultures were compared. The proportion of dying cells, as assessed by evaluation of PS exposure, was 20.2 and 66.5% in uninfected and HIV-infected cultures, respectively (data not shown). As a control, we used cytoplasmic extracts prepared from Jurkat T cells treated or not with CD95 mAbs. The cytosolic fraction of HIV-infected

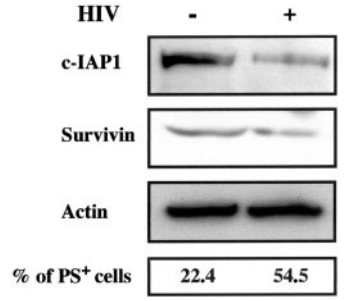

FIG. 5. **HIV-1-mediated cell death is associated with the down-regulation of c-IAP1 protein expression.** Cytosolic extracts from uninfected (−) and HIV-infected CD4+ T cells (+) at day 6 were analyzed by Western blotting for c-IAP1 and survivin expression. As a control of loading, actin was used. The percentages of dying cells (*% of PS^+ cells*), determined by flow cytometry using annexin-V-FITC, are indicated. The experiment is representative of two independent experiments performed.

CD4+ T cells mediated nuclear chromatin condensation and fragmentation as visualized by flow cytometric and microscopic analysis (Fig. 6), as was observed in nuclei incubated with the cytosolic fraction of CD95 mAb-treated Jurkat cells. In contrast, the activity of cytosol from uninfected CD4+ T cells was quite similar to the activity of cytosol from Jurkat T cells cultured in the absence of CD95 mAbs. Next, using zVAD-fmk, a broad caspase inhibitor, we explored whether the inhibition of caspases prevents nuclear chromatin fragmentation and condensation. When cytoplasmic extracts from Jurkat T cells treated with CD95 mAbs and HIV-infected CD4+ T cells were incubated with zVAD-fmk (50 μM), the degradation of CEM nuclei was inhibited (Fig. 6). Our data suggest that caspases are the main effectors involved in chromatin condensation and fragmentation during HIV-mediated cell death.

$HIV\text{-}1_{LAI}$ Mediated a Caspase-independent Cell Death—Since caspase inhibitor prevented chromatin condensation and fragmentation in a cell-free system, HIV-infected CD4+ T cells were cultured in the presence of zVAD-fmk to assess the role of caspases in HIV-mediated cell death. The apoptotic phenotype (chromatin condensation and fragmentation) was markedly decreased in cells maintained in the presence of zVAD-fmk (Fig. 7*A*). zVAD-fmk did not prevent $\Delta\psi$m loss (Fig. 7*B*), PS exposure (Fig. 7*C*), nor cell shrinkage (Fig. 7*D*). In fact, zVAD-fmk treatment turned the apoptotic cell death phenotype into a nonapoptotic cell death phenotype, as visualized by UV microscopic analysis using Hoechst 33342/PI double staining (Fig. 7*E*) and by light microscopic analysis using trypan blue dye reagent (Fig. 7*F*). Thus, our data suggest that HIV induces a caspase-independent cell death pathway that is associated with a disruption of mitochondrial membrane potential.

Involvement of Mitochondria during $HIV\text{-}1_{LAI}$-mediated Cell Death—A variety of key events during programmed cell death focus on mitochondria, including loss of $\Delta\psi$m and the release of apoptogenic factors into the cytosol (35). To evaluate the role of apoptogenic factors released by mitochondria in HIV-mediated cell death, cytosolic fractions prepared from uninfected and HIV-infected CD4+ T cells were analyzed using specific antibodies for the presence of cytochrome *c*, a 15-kDa protein that is involved in caspase activation, and AIF, a 57-kDa protein that is involved in caspase-independent cell death (43). Fig. 8 shows that the cytosolic fraction of HIV-infected CD4+ T cells at day 6 after PHA/IL-2 stimulation contains more cytochrome *c* and more AIF than uninfected CD4+ T cells, suggesting that mitochondria from HIV-infected CD4+ T cells release these two factors.

Members of the Bcl-2 family have been shown to be involved in the regulation of mitochondria permeability during apoptosis (35). Bcl-2 expression (an antiapoptotic protein) was assessed by flow cytometry after gating on live and dying cells. The mean intensity of fluorescence (MIF) of Bcl-2 expression in live cells (high forward scatter parameter) from uninfected (MIF = 46.3) and HIV-infected CD4+ T cells (MIF = 42.9) was similar (Fig. 9*A*) and was markedly decreased in dying cells (shrunken cells) of both uninfected and HIV-infected CD4+ T cells (MIF = 30.8 and 23.2, respectively), suggesting that loss of Bcl-2 is associated with cell death. By two-color flow cytometric analysis, we assessed Bcl-2 expression in live productively infected CD4+ T cells as determined by p24 antigen expression (Fig. 9*B*). Our data indicate that Bcl-2 expression was equivalent in CD4+ T cells in which HIV was or was not actively replicating, suggesting that HIV-mediated cell death is not associated with an early change in Bcl-2 expression. However, after cell fractionation, we observed that more Bax (a proapoptotic protein) was present in the mitochondria enriched HM fraction of infected CD4+ T cells (Bax protein was increased by 55% in two independent experiments) than that present in uninfected CD4+ T cells (Fig. 9*D*). *In vitro* treatment of infected CD4+ T cells with the reverse transcriptase inhibitor

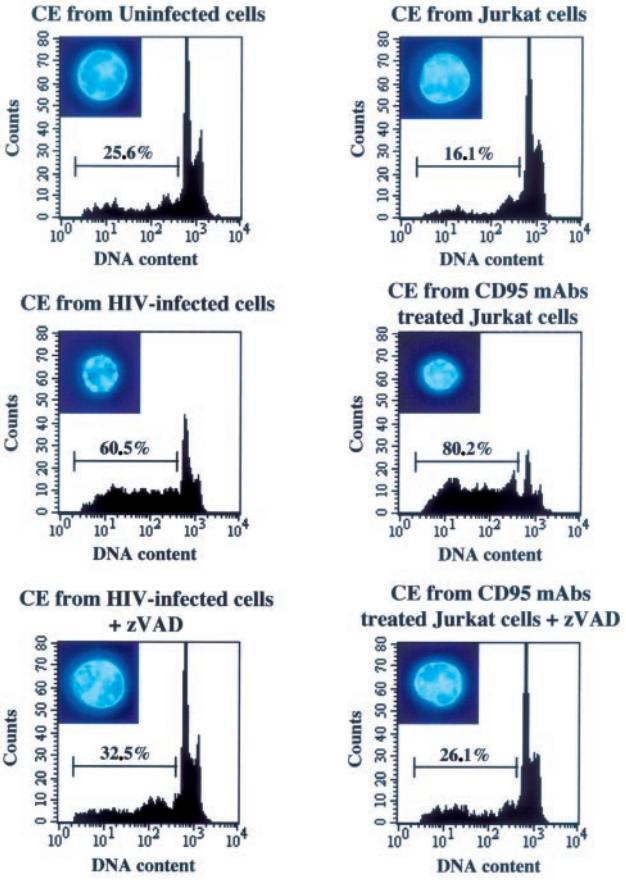

FIG. 6. **HIV-1 promotes apoptotic activity in cell-free extracts.** CEM nuclei were incubated for 2 h with cytolosic extracts (*CE*) from uninfected CD4+ T cells (*CE from Uninfected cells*) or from HIV-1-infected CD4+ T cells in absence (*CE from HIV-infected cells*) or presence of zVAD-fmk (50 μM; preincubated for 30 min) (*CE from HIV-infected cells + zVAD*). As a control, CEM nuclei were also incubated for 2 h with cytolosic extracts from Jurkat T cells (CE from Jurkat cells) or from Jurkat cells treated with CD95 mAbs (100 ng/ml for 4 h at 37 °C) in the absence (*CE from CD95 mAbs treated Jurkat cells*) or presence of zVAD-fmk (50 μM; preincubated 30 min) (*CE from CD95 mAbs treated Jurkat cells + zVAD*). The DNA content of the nuclei was then assessed by flow cytometry after propidium iodide staining. The *insets* show the most frequent morphology of the nuclei. Nuclear morphology was observed under UV fluorescence after Hoechst 33342 staining (magnification ×1000).

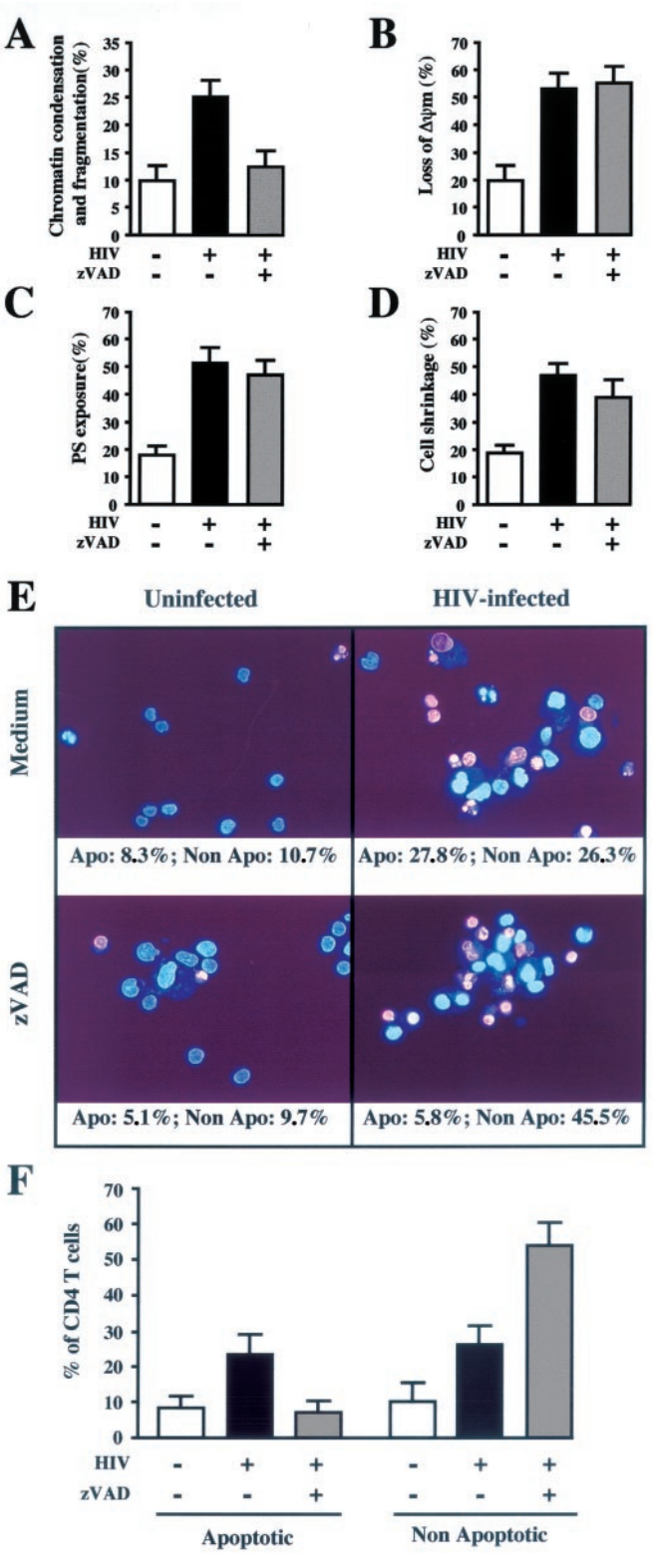

FIG. 7. **Caspase inhibitors prevent apoptosis but not HIV-induced cell death.** CD4+ T cells were purified, incubated for 2 h in the absence (*Uninfected*) or presence of HIV-1_{LAI} (*HIV-infected*) at a MOI of 0.01, washed, and cultured for 6 days with PHA/IL-2. At day 3, CD4+ T cells were treated in the absence (*Medium*) or presence (*zVAD*) of the broad caspase inhibitor zVAD-fmk (50 μM). *Histograms* represent the mean in chromatin condensation and fragmentation (*A*), loss in $\Delta\psi$m (*B*), PS exposure (*C*), or cell shrinkage (*D*) in three independent experiments performed. *E*, Hoechst 33342/PI double staining shows that blue intact nuclei were viable cells, whereas those with blue-pink condensed and fragmented nuclei were apoptotic cells (*Apo*). Cells with pink intact nuclei were considered as nonapoptotic cells (*Non Apo*). The percentages of apoptotic and nonapoptotic CD4+ T cells are indicated. The experiments is representative of two independent experiments performed. *F*, apoptotic and nonapoptotic cells were also determined by microscopic analysis using trypan blue dye exclusion as described in the legend to Fig. 2*E*. 200 cells were counted in duplicate. The data shown are the mean of three independent experiments performed.

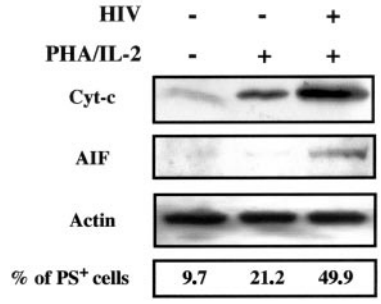

FIG. 8. **HIV-1-mediated cell death is associated with the release of cytochrome *c* and of AIF.** CD4+ T cells were purified from PBMC of normal donors using negative selection and incubated for 2 h with either medium (−) or HIV-1_{LAI} (+) at a MOI of 0.01, washed, and cultured for 6 days with PHA/IL-2 (+) or unstimulated (−). Cytosolic extracts of CD4+ T cells were analyzed for cytochrome *c* and AIF by Western blotting. As a control of loading, actin was used. The percentages of dying cells (*% of PS$^+$ cells*) determined by flow cytometry using annexin-V-FITC are indicated. The experiment is representative of three independent experiments.

DDI, which prevented cell death (PS exposure and loss in $\Delta\Psi$m) (Fig. 9*C*), also prevented targeting of Bax to the mitochondria (Fig. 9*D*). These data suggest that HIV-1 may induce CD4 T cell death via the targeting of Bax to the mitochondria.

DISCUSSION

Our results suggest that HIV-1 induces CD4 T cell death through a TNF receptor family-independent pathway. These findings confirm and extend previous findings indicating that productive infection of PHA/IL-2 stimulated primary CD4+ T cells, as well as productive infection of transformed CD4+ T cell lines, induces a process of CD4 T cell death that does not involve CD95/CD95L interactions (4–6). Other death pathways, such as TRAIL/DR4 or TNF/TNFR, have been suggested to play a role in apoptosis of T cells from HIV-infected persons (20, 21), but decoy receptors corresponding to these and other TNF receptor family members did not prevent HIV-mediated death of primary CD4+ T cells stimulated with PHA/IL-2, indicating that death occurred independently of these death receptors (CD95, TNFR1/R2/OPG, and TRAMP). In agreement with an absence of involvement of death receptors in HIV-mediated cell death, we did not observe an early full processing of caspase-8. Caspase-8 is generally considered to be an initiator caspase, which associates with the adaptator molecule FADD recruited to the death receptor. Full processing of caspase-8 in the 18-kDa active form was only observed at day 6, suggesting that caspase-8 activation may be a late event in the pathway leading to cell death in response to HIV infection (34).

Although HIV induced the cleavage of caspase-3, -8, and -9 proenzymes and markedly decreased the expression of c-IAP1, an inhibitor of caspase-3, our experiments indicate that caspases are dispensable for HIV-mediated programmed cell death. Nevertheless, these proteases do play a role in the nuclear apoptotic phenotype as evidenced by the effects of zVAD-fmk. Several recent reports have suggested that caspase inhibitors inhibit apoptosis induced by diverse stimuli but do not prevent further cell death (36–38, 40–42, 58, 59) and lead to the appearance of cells with a necrotic-like phenotype. Our data show that the nonapoptotic phenotype is characterized by membrane permeabilization and intact nuclei and shares with the apoptotic phenotype the presence of cell shrinkage. This

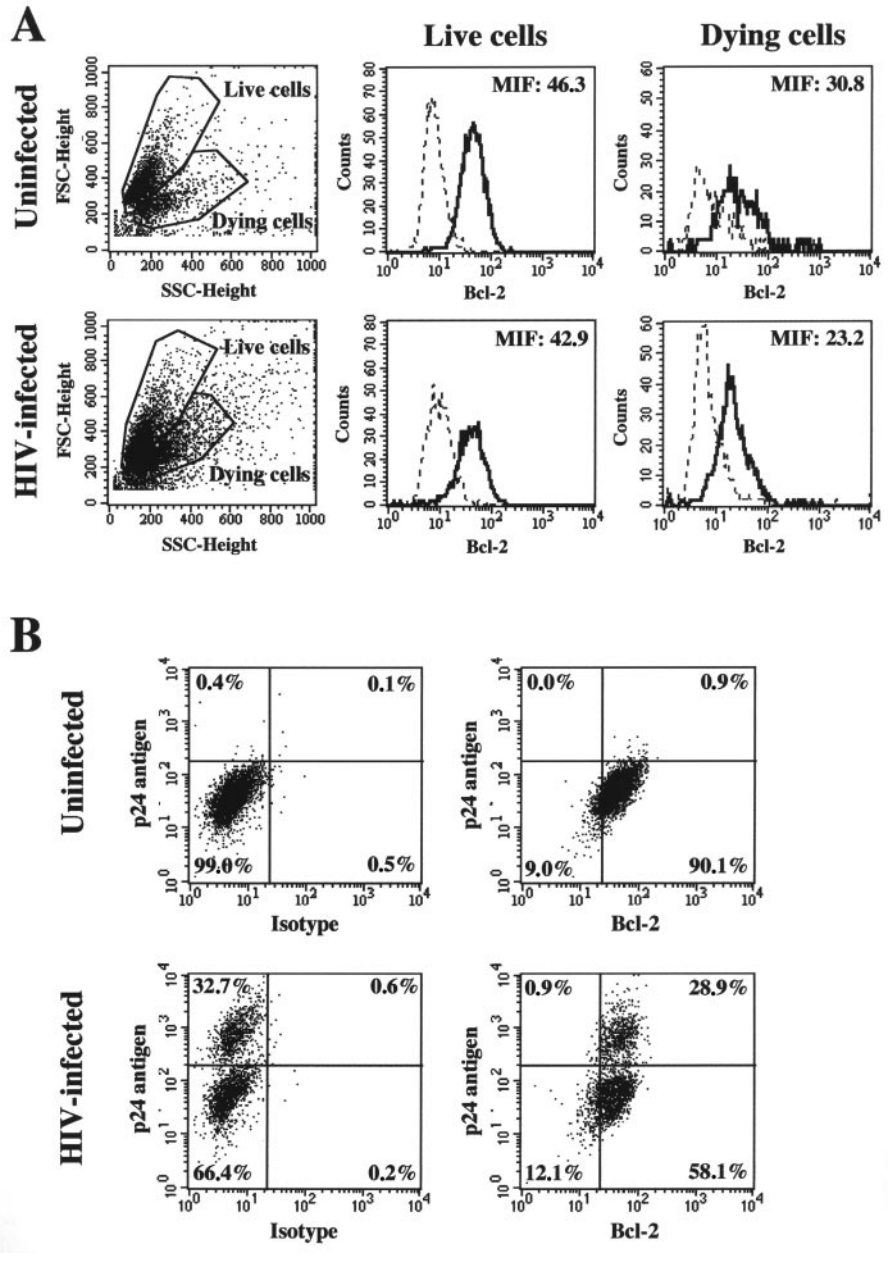

FIG. 9. **HIV-mediated cell death is not related to a down-regulation in Bcl-2 expression but to an increase of Bax to the mitochondrial-enriched HM fraction.** CD4+ T cells were purified, incubated for 2 h with either medium alone (*Uninfected*) or HIV-1_{LAI} (*HIV-infected*) at an MOI of 0.01, washed, and cultured with PHA and IL-2 for 6 days. *A*, Bcl-2 expression (*thick line*) was assessed by flow cytometry as compared with isotype mAb (*dashed line*), gated either on live (high forward scatter) or dying cells (low forward scatter). *B*, two-color flow cytometry was assessed to visualize Bcl-2 expression and p24 antigen in live (high forward scatter) CD4+ T cells. *C*, p24 expression (*thick line*) compared with isotype mAb (*dashed line*) and $\Delta\psi$m in CD4+ T cells either uninfected (*HIV*$^-$) or infected with HIV in the absence (*HIV*$^+$) or in the presence of DDI (5 μM) (*HIV*$^+$ + *DDI*). *D*, 15 μg of protein from mitochondria enriched HM fraction of uninfected (−), and HIV-infected CD4+ T cells (+) incubated in the absence (−) or presence (+) of DDI (5 μM) were analyzed for the presence of Bax by Western blotting. As control of loading, the presence of the voltage-dependent anion channel was determined. Bax was quantified by scan density using NIH Image 1.62. The percentages of dying cells (*% of PS*$^+$ *cells*), determined by flow cytometry using annexin-V-FITC, is indicated. The experiment is representative of two independent experiments.

suggests that the cell death program induced by HIV replication is distinct from necrosis (cell swelling) (60). Cell shrinkage, mediated by apoptotic inducers, is believed to be tightly linked to enhanced K^+ efflux (61–63). Thus, it would be interesting to determine whether cell volume is directly related to an efflux of K^+ during HIV-mediated cell death and if HIV deregulates the continuous activity of the Na^+/K^+ ATPase pump.

Cell death mediated by HIV is associated with a loss of $\Delta\psi$m, even when caspase activation is blocked, suggesting a crucial role for mitochondria in the regulation of this cell death. The loss of mitochondrial membrane potential is associated with the release of cytochrome *c* and AIF from the mitochondria. Among critical effectors, AIF, a flavoprotein, has been reported to directly cause chromatin condensation in isolated nuclei and participates in caspase-independent programmed cell death. Although, AIF protein was detected in the cytosolic fraction of HIV-infected CD4+ T cells along with cytochrome *c*, isolated nuclei treated with these extracts did not display the major features of AIF-induced nuclei morphology (64), even when the nuclei were incubated in the presence of zVAD-fmk. During preparation of this manuscript, Ferri *et al.* (28) reported that syncitia arising from the fusion of cells expressing gp120 with cells expressing the CD4 and CXCR4 molecules complex spontaneously undergo cell death, displaying evidence of caspase activation, loss of $\Delta\psi$m, and the release of cytochrome *c* and AIF from mitochondria. Although AIF has been assumed to be a potent activator of programmed cell death, recent work has suggested that AIF release from the mitochondria and translocation to the nucleus can occur in the absence of chromatin condensation and cell death (38, 65). Moreover, the study of AIF knockout mice suggests the existence of a third cell death pathway independent of caspases and AIF (66). Although dispensable for cell death, our data suggest that caspases are the main nuclear effectors involved in HIV-mediated DNA condensation and fragmentation, in which AIF is either not involved or not a major contributory factor. Whether AIF participates in other effector pathways of cell death remains to be investigated.

The mechanism responsible for mitochondrial membrane permeabilization has been reported to involve proapoptotic

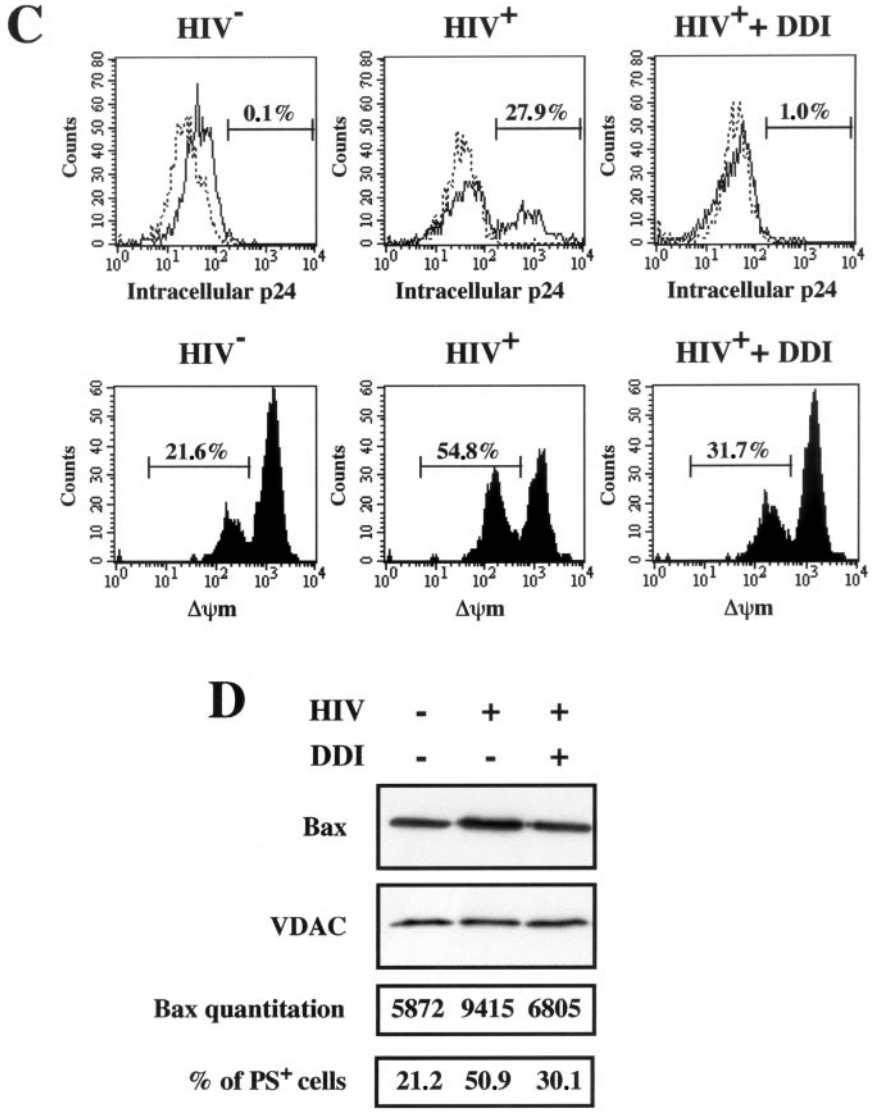

FIG. 9—*continued*

members of the Bcl-2 family, the permeability transition pore complex, the adenine nucleotide translocator, and/or the voltage-dependent anion channel (67). It has been proposed that the HIV-1 protease can cleave Bcl-2, thereby abolishing its mitochondrial membrane potential-inhibitory function (68). However, we observed that Bcl-2 expression was equivalent in CD4+ T cells, whether uninfected or HIV-infected, and Western blot analysis did not show the presence of fragmented Bcl-2 (data not shown), suggesting that the loss of Bcl-2 may be a consequence of cell death and not directly involved in the loss of $\Delta\psi$m. Upon death stimuli, Bax is rapidly translocated to the mitochondria and induces the loss of $\Delta\psi$m, cytochrome *c* release, and activation of caspases (69–72). Here, we showed that the amount of Bax was more important in the mitochondria-enriched heavy membrane fraction of HIV-infected CD4+ T cells than in that of uninfected and DDI-treated HIV-infected CD4+ T cells. It has been recently reported that HIV-1 induces an up-regulation of Bax via a p53 pathway (73). Thus, the result of Genini *et al.* (73) and our data suggest that HIV-1 may induce both Bax up-regulation and Bax targeting to the mitochondria. However, the nature of the signals that may lead to Bax translocation during HIV-1 infection remains an open question.

Studies by Xiang *et al.* (39) have shown that inducible Bax expression triggers rapid death even in the presence of caspase inhibitors, and nonapoptotic death proceeds through the generation of reactive oxygen species (ROS). In our model, HIV-infected CD4+ T cells die with a nonapoptotic phenotype when treated with the broad spectrum caspase inhibitor (zVAD-fmk). Preliminary data measuring intracellular oxidant levels using the oxidation-sensitive dye dihydroethidium indicate that the levels of ROS in HIV-infected CD4+ T cells were higher than in uninfected CD4+ T cells and that ROS levels decreased in the presence of DDI. Thus, independently of caspase activation, the ability of Bax to induce disruption of mitochondrial membrane permeability might be expected to result in the disruption of electron transport following cytochrome *c* release, loss of ATP and an increase in ROS may ultimately cause a nonapoptotic cell death (74).

At this stage, however, we cannot exclude other death pathways that result in increased mitochondrial membrane permeability. Indeed, it has been recently proposed that Vpr (a protein encoded by HIV) is directly targeted to the mitochondrial permeability transition pore complex and permeabilizes mitochondrial membranes in a cell-free system (27). The Vpr protein has been reported to kill lymphocytes, monocytes, and neurons (26, 75, 76) and to induce mitochondrial dysfunction in the yeast *Saccharomyces cerevisiae* (77). However, it may also act as a negative regulator of cell death in human cell lines and thymocytes (78, 79), and CEM T cells infected with a triple

mutant (*nef*, *vpr*, and *vpu* deleted) derived from HIV-1 also undergo cell death (80), suggesting that Vpr is dispensable for HIV-mediated CD4 T cell depletion. Thus, the role of HIV-1 proteins in inducing cell death through the loss of $\Delta\psi m$ remains to be elucidated, and other viral products may be involved in this process.

In conclusion, our data suggest that HIV-1 infection of cycling primary CD4+ T cells results in a cell death process associated with a mitochondrial deregulation (loss in $\Delta\psi m$). Caspases that mediate chromatin condensation and fragmentation are the main effectors of the apoptotic phenotype but are dispensable for cell death. Our data indicate a crucial role of the mitochondria in the regulation of cell death induced by HIV and suggest that the targeting of Bax to the mitochondria may be a major contributory factor.

Acknowledgments—We thank Sara Albanil for technical assistance and thank Judy Norberg and Michele Lutz for flow cytometric analysis. The help of Dr. David Looney (CFAR director molecular biology and the CFAR and VMRF Genomics core laboratory) is greatly acknowledged.

REFERENCES

1. Mellors, J. W., Rinaldo, C. R., Jr., Gupta, P., White, R. M., Todd, J. A., and Kingsley, L. A. (1996) *Science* **272,** 1167–1170
2. Laurent-Crawford, A. G., Krust, B., Muller, S., Riviere, Y., Rey-Cuille, M. A., Bechet, J. M., Montagnier, L., and Hovanessian, A. G. (1991) *Virology* **185,** 829–839
3. Terai, C., Kornbluth, R. S., Pauza, C. D., Richman, D. D., and Carson, D. A. (1991) *J. Clin. Invest.* **87,** 1710–1715
4. Glynn, J. M., McElligott, D. L., and Mosier, D. E. (1996) *J. Immunol.* **157,** 2754–2758
5. Gandhi, R. T., Chen, B. K., Straus, S. E., Dale, J. K., Lenardo, M. J., and Baltimore, D. (1998) *J. Exp. Med.* **187,** 1113–1122
6. Moutouh, L., Estaquier, J., Richman, D. D., and Corbeil, J. (1998) *J. Virol.* **72,** 8061–8072
7. Estaquier, J., Idziorek, T., de Bels, F., Barre-Sinoussi, F., Hurtrel, B., Aubertin, A. M., Venet, A., Mehtali, M., Muchmore, E., Michel, P., Mouton, Y., Girard, M., and Ameisen, J. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 9431–9435
8. Gougeon, M. L., Garcia, S., Heeney, J., Tschopp, R., Lecoeur, H., Guetard, D., Rame, V., Dauguet, C., and Montagnier, L. (1993) *AIDS Res. Hum. Retroviruses* **9,** 553–563
9. Oyaizu, N., McCloskey, T. W., Coronesi, M., Chirmule, N., Kalyanaraman, V. S., and Pahwa, S. (1993) *Blood* **82,** 3392–3400
10. Lewis, D. E., Tang, D. S., Adu-Oppong, A., Schober, W., and Rodgers, J. R. (1994) *J. Immunol.* **153,** 412–420
11. Finkel, T. H., Tudor-Williams, G., Banda, N. K., Cotton, M. F., Curiel, T., Monks, C., Baba, T. W., Ruprecht, R. M., and Kupfer, A. (1995) *Nat. Med.* **1,** 129–134
12. Muro-Cacho, C. A., Pantaleo, G., and Fauci, A. S. (1995) *J. Immunol.* **154,** 5555–5566
13. Su, L., Kaneshima, H., Bonyhadi, M., Salimi, S., Kraft, D., Rabin, L., and McCune, J. M. (1995) *Immunity* **2,** 25–36
14. Gougeon, M. L., Lecoeur, H., Boudet, F., Ledru, E., Marzabal, S., Boullier, S., Roue, R., Nagata, S., and Heeney, J. (1997) *J. Immunol.* **158,** 2964–2976
15. Estaquier, J., Idziorek, T., Zou, W., Emilie, D., Farber, C. M., Bourez, J. M., and Ameisen, J. C. (1995) *J. Exp. Med.* **182,** 1759–1767
16. Katsikis, P. D., Wunderlich, E. S., Smith, C. A., and Herzenberg, L. A. (1995) *J. Exp. Med.* **181,** 2029–2036
17. Baumler, C. B., Bohler, T., Herr, I., Benner, A., Krammer, P. H., and Debatin, K. M. (1996) *Blood* **88,** 1741–1746
18. Estaquier, J., Tanaka, M., Suda, T., Nagata, S., Golstein, P., and Ameisen, J. C. (1996) *Blood* **87,** 4959–4966
19. Sloand, E. M., Young, N. S., Kumar, P., Weichold, F. F., Sato, T., and Maciejewski, J. P. (1997) *Blood* **89,** 1357–1363
20. Katsikis, P. D., Garcia-Ojeda, M. E., Torres-Roca, J. F., Tijoe, I. M., Smith, C. A., and Herzenberg, L. A. (1997) *J. Exp. Med.* **186,** 1365–1372
21. Jeremias, I., Herr, I., Boehler, T., and Debatin, K. M. (1998) *Eur. J. Immunol.* **28,** 143–152
22. Laurent-Crawford, A. G., Krust, B., Riviere, Y., Desgranges, C., Muller, S., Kieny, M. P., Dauguet, C., and Hovanessian, A. G. (1993) *AIDS Res. Hum. Retroviruses* **9,** 761–773
23. Laurent-Crawford, A. G., Coccia, E., Krust, B., and Hovanessian, A. G. (1995) *Res. Virol.* **146,** 5–17
24. Corbeil, J., Tremblay, M., and Richman, D. D. (1996) *J. Exp. Med.* **183,** 39–48
25. Corbeil, J., and Richman, D. D. (1995) *J. Gen. Virol.* **76,** 681–690
26. Stewart, S. A., Poon, B., Jowett, J. B., and Chen, I. S. (1997) *J. Virol.* **71,** 5579–5592
27. Jacotot, E., Ravagnan, L., Loeffler, M., Ferri, K. F., Vieira, H. L., Zamzami, N., Costantini, P., Druillennec, S., Hoebeke, J., Briand, J. P., Irinopoulou, T., Daugas, E., Susin, S. A., Cointe, D., Xie, Z. H., Reed, J. C., Roques, B. P., and Kroemer, G. (2000) *J. Exp. Med.* **191,** 33–46
28. Ferri, K. F., Jacotot, E., Blanco, J., Este, J. A., Zamzami, N., Susin, S. A., Xie, Z., Brothers, G., Reed, J. C., Penninger, J. M., and Kroemer, G. (2000) *J. Exp. Med.* **192,** 1081–1092
29. Chinnaiyan, A. M., Woffendin, C., Dixit, V. M., and Nabel, G. J. (1997) *Nat. Med.* **3,** 333–337
30. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) *Cell* **85,** 817–827
31. Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996) *Cell* **85,** 803–815
32. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) *Cell* **94,** 481–490
33. Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998) *Cell* **94,** 491–501
34. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998) *EMBO J.* **17,** 1675–1687
35. Green, D. R., and Reed, J. C. (1998) *Science* **281,** 1309–1312
36. McCarthy, N. J., Whyte, M. K., Gilbert, C. S., and Evan, G. I. (1997) *J. Cell Biol.* **136,** 215–227
37. Quignon, F., De Bels, F., Koken, M., Feunteun, J., Ameisen, J. C., and de The, H. (1998) *Nat. Genet.* **20,** 259–265
38. Dumont, C., Durrbach, A., Bidere, N., Rouleau, M., Kroemer, G., Bernard, G., Hirsch, F., Charpentier, B., Susin, S. A., and Senik, A. (2000) *Blood* **96,** 1030–1038
39. Xiang, J., Chao, D. T., and Korsmeyer, S. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14559–14563
40. Vercammen, D., Brouckaert, G., Denecker, G., Van de Craen, M., Declercq, W., Fiers, W., and Vandenabeele, P. (1998) *J. Exp. Med.* **188,** 919–930
41. Matsumura, H., Shimizu, Y., Ohsawa, Y., Kawahara, A., Uchiyama, Y., and Nagata, S. (2000) *J. Cell Biol.* **151,** 1247–1256
42. Holler, N., Zaru, R., Micheau, O., Thome, M., Attinger, A., Valitutti, S., Bodmer, J.-L., Schneider, P., Seed, B., and Tschopp, J. (2000) *Nat. Immunol.* **1,** 489–495
43. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999) *Nature* **397,** 441–446
44. Holler, N., Kataoka, T., Bodmer, J. L., Romero, P., Romero, J., Deperthes, D., Engel, J., Tschopp, J., and Schneider, P. (2000) *J. Immunol. Methods* **237,** 159–173
45. Schneider, P. (2000) *Methods Enzymol.* **322,** 325–345
46. Idziorek, T., Estaquier, J., De Bels, F., and Ameisen, J. C. (1995) *J. Immunol. Methods* **185,** 249–258
47. Martin, S. J., Newmeyer, D. D., Mathias, S., Farschon, D. M., Wang, H. G., Reed, J. C., Kolesnick, R. N., and Green, D. R. (1995) *EMBO J.* **14,** 5191–5200
48. Peter, M. E., Kischkel, F. C., Scheuerpflug, C. G., Medema, J. P., Debatin, K. M., and Krammer, P. H. (1997) *Eur. J. Immunol.* **27,** 1207–1212
49. Buzyn, A., Petit, F., Ostankovitch, M., Figueiredo, S., Varet, B., Guillet, J. G., Ameisen, J. C., and Estaquier, J. (1999) *Blood* **94,** 3135–3140
50. Bossi, G., and Griffiths, G. M. (1999) *Nat. Med.* **5,** 90–96
51. Ashkenazi, A., and Dixit, V. M. (1999) *Curr. Opin. Cell Biol.* **11,** 255–260
52. Rennert, P., Schneider, P., Cachero, T. G., Thompson, J., Trabach, L., Hertig, S., Holler, N., Qian, F., Mullen, C., Strauch, K., Browning, J. L., Ambrose, C., and Tschopp, J. (2000) *J. Exp. Med.* **192,** 1677–1684
53. Thornberry, N. A., and Lazebnik, Y. (1998) *Science* **281,** 1312–1316
54. Alam, A., Cohen, L. Y., Aouad, S., and Sekaly, R. P. (1999) *J. Exp. Med.* **190,** 1879–1890
55. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) *Nature* **371,** 346–347
56. Deveraux, Q. L., and Reed, J. C. (1999) *Genes Dev.* **13,** 239–252
57. Ambrosini, G., Adida, C., and Altieri, D. C. (1997) *Nat. Med.* **3,** 917–921
58. Leist, M., Single, B., Castoldi, A. F., Kuhnle, S., and Nicotera, P. (1997) *J. Exp. Med.* **185,** 1481–1486
59. Haraguchi, M., Torii, S., Matsuzawa, S., Xie, Z., Kitada, S., Krajewski, S., Yoshida, H., Mak, T. W., and Reed, J. C. (2000) *J. Exp. Med.* **191,** 1709–1720
60. Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980) *Int. Rev. Cytol.* **68,** 251–306
61. Yu, S. P., Yeh, C. H., Sensi, S. L., Gwag, B. J., Canzoniero, L. M., Farhangrazi, Z. S., Ying, H. S., Tian, M., Dugan, L. L., and Choi, D. W. (1997) *Science* **278,** 114–117
62. Beauvais, F., Michel, L., and Dubertret, L. (1995) *J. Leukocyte Biol.* **57,** 851–855
63. Bortner, C. D., Hughes, F. M., and Cidlowski, J. A. (1997) *J. Biol. Chem.* **272,** 32436–32442
64. Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K. F., Irinopoulou, T., Prevost, M. C., Brothers, G., Mak, T. W., Penninger, J., Earnshaw, W. C., and Kroemer, G. (2000) *J. Exp. Med.* **192,** 571–580
65. Zhou, G., and Roizman, B. (2000) *J. Virol.* **74,** 9048–9053
66. Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L., Ferri, K. F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y. Y., Mak, T. W., Zuniga-Pflucker, J. C., Kroemer, G., and Penninger, J. M. (2001) *Nature* **410,** 549–554
67. Kroemer, G., and Reed, J. C. (2000) *Nat. Med.* **6,** 513–519
68. Strack, P. R., Frey, M. W., Rizzo, C. J., Cordova, B., George, H. J., Meade, R., Ho, S. P., Corman, J., Tritch, R., and Korant, B. D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 9571–9576
69. Finucane, D. M., Bossy-Wetzel, E., Waterhouse, N. J., Cotter, T. G., and Green, D. R. (1999) *J. Biol. Chem.* **274,** 2225–2233
70. Wolter, K. G., Hsu, Y. T., Smith, C. L., Nechushtan, A., Xi, X. G., and Youle, R. J. (1997) *J. Cell Biol.* **139,** 1281–1292
71. Goping, I. S., Gross, A., Lavoie, J. N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S. J., and Shore, G. C. (1998) *J. Cell Biol.* **143,** 207–215
72. Gross, A., Jockel, J., Wei, M. C., and Korsmeyer, S. J. (1998) *EMBO J.* **17,** 3878–3885

73. Genini, D., Sheeter, D., Rought, S., Zaunders, J. J., Susin, S. A., Kroemer, G., Richman, D. D., Carson, D. A., Corbeil, J., and Leoni, L. M. (2001) *FASEB J.* **15,** 5–6
74. Reed, J. C. (1997) *Cell* **91,** 559–562
75. Yao, X. J., Mouland, A. J., Subbramanian, R. A., Forget, J., Rougeau, N., Bergeron, D., and Cohen, E. A. (1998) *J. Virol.* **72,** 4686–4693
76. Piller, S. C., Jans, P., Gage, P. W., and Jans, D. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4595–4600
77. Macreadie, I. G., Thorburn, D. R., Kirby, D. M., Castelli, L. A., de Rozario, N. L., and Azad, A. A. (1997) *FEBS Lett.* **410,** 145–149
78. Ayyavoo, V., Mahboubi, A., Mahalingam, S., Ramalingam, R., Kudchodkar, S., Williams, W. V., Green, D. R., and Weiner, D. B. (1997) *Nat. Med.* **3,** 1117–1123
79. Conti, L., Rainaldi, G., Matarrese, P., Varano, B., Rivabene, R., Columba, S., Sato, A., Belardelli, F., Malorni, W., and Gessani, S. (1998) *J. Exp. Med.* **187,** 403–413
80. Rapaport, E., Casella, C. R., Ikl, Mustafa, F., Isaak, D., and Finkel, T. H. (1998) *Virology* **252,** 407–417