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# Caspase-3-Like Activity Is Necessary for IL-2 Release in Activated Jurkat T-cells

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**The caspase family of proteases has previously been implicated in the biochemical cascade leading to apoptotic cell death. Recently caspase-3 was reported to be cleaved into its catalytically active subunits (17 and 13 kDa) following phytohemagglutinin (PHA) activation of peripheral blood mononuclear cells (C. Miossec *et al., J. Biol. Chem.* 272, 13459–13462). More recently, J. M. Zapata and colleagues (*J. Biol. Chem.* 273, 6916–6920, 1998), however, proposed that caspase-3 activity detected during T-cell activation was due to a methodological artifact related to the composition of the cell lysis buffer. Here we show that in PHA-activated Jurkat T-cells using the recommended lysis buffer detailed by Zapata *et al.,* a caspase-3-like protease is activated and is accompanied by cleavage of PARP and α-spectrin into cleavage products suggestive of caspase-3 proteolytic activation. LDH release did not increase following PHA stimulation in this paradigm. Two caspase inhibitors, carbobenzoxy-Asp-$CH_2OC(O)$-2,6-dichlorobenzene (Z-D-DCB) and acetyl-Asp-Glu-Val-Asp-CHO, blocked IL-2 release in a dose-dependent manner. Caspase-3-like protease-generated PARP and α-spectrin breakdown product formation was also reduced by Z-D-DCB. In addition, Jurkat T-cells costimulated with anti-CD3 plus anti-CD28 produced significant levels of IL-2 that were also blocked by these caspase inhibitors. Importantly, IL-2 was determined in cell culture supernatants, thus avoiding a cell lysis step that might have enabled activation of caspase-3 by granzyme B. Collectively, these data support the role of caspase-3-like protease activity in Jurkat T-cell activation and demonstrate that caspase-3 like activity is necessary for IL-2 release in PHA-activated and anti-CD3/anti-CD28 costimulated Jurkat T-cells.** © 1998 Academic Press

***Key Words:*** **apoptosis; caspase-3; Jurkat; Interleukin-2; activation.**

## INTRODUCTION

The participation of the caspase family (previously called the interleukin-1β converting enzyme family) of cysteine proteases in programmed cell death (apoptosis) is well established [3–6]. Apoptosis is required for the development and maintenance of cells in many organ systems, including neuronal cells [7–9], cardiac cells [10], and immune cells [11–15]. In addition apoptosis has been implicated in many disease processes, such as Alzheimer's disease [16], Huntingtons disease [17], and amyotrophic lateral sclerosis [18]. However, two recent studies have shown that high levels of caspase-3-like activity can occur in the absence of apoptotic cell death [1, 19]. In the first study, Miossec and colleagues [1] showed that caspase-3-like activity occurred in PHA activated T-cells. In the second report [19] it was demonstrated that when epithelial cells terminally differentiate into enucleated lens fibers, caspase-3-like activity was markedly enhanced. These observations suggested that caspase-3-like proteases may not be exclusively death effector proteases but may indeed be involved in other cellular processes not accompanied by cell death. However, a recent report from Zapata and colleagues [2] claims that the caspase-3 activity previously described by Miossec *et al.* [1] was a methodological artifact related to the cell lysis buffer. Currently, the involvement of caspase-3-like protease activation in T-cell activation and other nonapoptotic events has yet to be established.

Several apoptotic challenges have been shown to activate caspase-3 from a 32-kDa pro-enzyme into 20- [20, 21], 17-, and 13-kDa fragments [15, 22, 23]. The latter two form the active enzyme complex. Caspase-3 has a conserved pentapeptide active site QACXG (where X is R, Q, or G) common to the caspase family and a preference for proteins that contain a DXXD motif at $P_4$–$P_1$ [23–25]. Activated caspase-3 can proteolytically cleave a number of intracellular, membrane, and nuclear proteins that are associated with apoptotic cell death [22, 26, 27] including poly(ADP-ribose) polymerase (PARP) [23, 28], α-spectrin [29, 30], sterol regulatory element binding proteins [31], the 70-kDa protein component of the U1-ribonucleopro-

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tein [32], and the catalytic subunit of DNA-dependent protein kinase [32].

T-cell activation is initiated by the binding of foreign antigens to the T-cell receptor complex (TCR) [33]. Subsequent signal transduction events include the activation of protein tyrosine kinases (i.e., Fyn, Lck, and ZAP-70) [33], PKC [34, 35], and Ras-related G proteins [36], which contribute to the expression of several cytokines including interleukin-2 (IL-2) [37, 38]. IL-2 is required for growth and differentiation of B-cells [39] and of T-cells [40]. In the present study we explored the physiological significance of caspase-3-like proteases in normal T-cell activation. First, we confirmed that caspase-3 processing occurs in activated T-cells using the preferred sample processing methodology of Zapata and colleagues [2]. Second, we demonstrated that caspase-3-like protease activation accompanies PHA stimulated and anti-CD3/anti-CD28 costimulated IL-2 release in the absence of cell death. Third, and importantly, we also show that caspase inhibitors (carbobenzoxy-Asp-$CH_2OC(O)$-2,6-dichlorobenzene (Z-D-DCB) [41] and acetyl-aspartate-glutamate-valine-aspartate-CHO (Ac-DEVD-CHO)) can markedly attenuate IL-2 release in PHA-activated and anti-CD3/anti-CD28 costimulated Jurkat T-cells. We conclude that caspase-3-like activity is necessary for IL-2 release in activated Jurkat T-cells.

## METHODS

*PHA challenge and anti-CD3/anti-CD28 costimulation of human Jurkat T-cells.* Jurkat T-cells (Clone E6-1, ATCC TIB 152, American Type Culture Collection, [Rockville, MD]) were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum, 100 i.u./ml penicillin, and 100 $\mu$g/ml streptomycin (all reagents used in cell maintenance were obtained from GibcoBRL [Grand Island, NY]). Cells were washed three times with RPMI prior to PHA (5 $\mu$g/ml; DIFCO laboratories [Detroit, MI]) stimulation, anti-fas (Upstate Biotechnologies [Lake Placid, NY]) challenge (0.5 $\mu$M), or anti-CD3 /anti-CD28 (0.5 $\mu$g/ml; 1.0 $\mu$l/ml, respectively [Biosource, Camarillo, CA]) costimulation for 6, 16, or 24 h. The ability of the pan caspase inhibitor Z-D-DCB (Z-Asp-$CH_2OC(O)$-2,6-dichlorobenzene) synthesized at Warner–Lambert Co. (5, 15, 50, or 100 $\mu$M), Ac-DEVD-CHO (100 $\mu$M, Peptide International [Louisville, KY]), or rolipram (30 $\mu$M) to attenuate IL-2 release from Jurkat T-cells was determined by adding these reagents to cultures 1 h prior to PHA activation or anti-CD3/anti-CD28 costimulation. Z-D-DCB has an $IC_{50}$ of 0.08, 0.2, and 0.1 $\mu$M for recombinant human caspase-1, caspase-3, and caspase-4, respectively (K. Brady, BASF, unpublished data). The concentration of 50 $\mu$M Z-D-DCB was selected based on past studies showing effective inhibition of caspase-3 following various apoptotic challenges at this concentration [42, 43]. All assays were performed in Falcon 12 well plates (Becton–Dickinson [Lincoln Park, NJ]) at $2 \times 10^6$ cells per 2 mL per well in serum-free medium.

*IL-2 immunoassay.* Human IL-2 was detected using R&D Systems Quantikine Human IL-2 immunoassay detection kits (Minneapolis, MN). Supernatants were collected from Jurkat T-cell cultures at 6, 16, and 24 h post PHA administration.

*LDH measurement.* Cell death was assessed in Jurkat T-cells by measuring the release of the cytosolic enzyme, lactate dehydrogenase (LDH) using a Cytotox kit 96 (Promega [Madison, WI]).

*Protein extraction, SDS–PAGE, and Western blot methodology.* On completion of the experimental protocols, Jurkat T-cells were washed twice with TBS–EDTA (20 mM Tris–HCl, pH 7.4, 155 mM NaCl, and 1 mM EDTA). SDS–protein extractions of cell lysates were performed in the presence of protease inhibitors containing 2% SDS as previously described [44]. The extraction procedure was completed by precipitating the proteins with trichloroacetic acid and resolubilization of the pellet was achieved with 3 M Tris base. Protein determination of samples was performed using a modified Lowry assay and bovine serum albumin standards. Protein samples containing 5–15 $\mu$g of protein were run on 4–20% acrylamide gradient gels (Novex [San Diego, CA]) employing a Tris glycine running buffer. Following separation of proteins by SDS–PAGE, proteins were transferred onto a PVDF membrane using a Tris glycine buffer (48 mM Tris base, 39 mM glycine, and 10% MeOH, pH 8.3) and semi-dry electrotransferring unit (Bio-Rad) at 20 mV for 2 h. All lanes contained identical amounts of protein. To insure consistency of loading, gels were routinely stained with Coomassie blue dye. Following transfer of the proteins to PVDF membrane, nonspecific sites were blocked by soaking in 5% nonfat dry milk in 20 mM Tris–HCl (pH 7.4), 0.05% Tween 20, and 0.02% $NaN_3$ at room temperature. Incubation of the primary antibody was performed with either anti-PARP (Santa Cruz Biotechnologies [Santa Cruz, CA]), anti-$\alpha$-spectrin (Clone AA6; Affinity Research Products [Nottingham, UK]), or anti-caspase-3 (polyclonal; Pharmingen [San Diego, CA]). Visualization of proteins was performed with biotinylated secondary antibodies and streptavidin–alkaline phosphatase (Amersham [Heightsville, IL]) using 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (BCIP/NBT [Sigma, St. Louis, MO]) as the active chromagen.

*Triton X-100 protein extraction for assaying caspase-3-like activity.* On completion of the experimental protocols, Jurkat T-cells were collected from 3 wells of a 12-well plate, placed in 15-ml centrifuge tubes, and washed twice with 5 ml of TBS–EDTA buffer by centrifugation for 5 min at 4°C. Cell pellets were resuspended and lysed in a buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM dithiothreitol (DTT), 5 mM EDTA, 5 mM EGTA, and 1% Triton X-100 for 60 min at 4°C. Cell lysates were then recovered after centrifugation and stored in 50% glycerol at −70°C. All cell lysates were assayed for protein concentration with a modified Lowry assay. Caspase-3-like and caspase-1-like proteolytic activities were assayed using the fluorometric peptide substrates Ac-DEVD-MCA or Ac-YVAD-MCA, respectively (where MCA represents 7-amido-4-methylcoumarin) (Bachem Bioscience, Inc. [King of Prussia, PA]). Cell lysates were added to a buffer (200 $\mu$l) containing 100 $\mu$M peptide substrate, 100 mM Hepes, 10% glycerol, 1 mM EDTA, 10 mM DTT, and 10 $\mu$M of the general caspase inhibitor Z-D-DCB. Fluorescence (excitation 380 ± 15 nm and emission 460 ± 15 nm) was measured every 15 min up to 60 min with a Millipore Cytoflor 2300 fluorescence plate reader.

## RESULTS

*PHA stimulation of Jurkat T-cells results in the production of proteolytic activity suggestive of caspase-3-like proteolysis.* Protein extracts from PHA-stimulated Jurkat T-cells were collected at 0, 6, and 16 h and analyzed using SDS–PAGE and Western blotting methodology to establish the temporal profile for caspase-3-like protease activation, as well as the hydrolysis of preferred intracellular substrates of caspase-3. The Western blots were probed with antibodies against caspase-3, the nuclear enzyme PARP, and $\alpha$-spectrin to detect the presence of lower molecu-

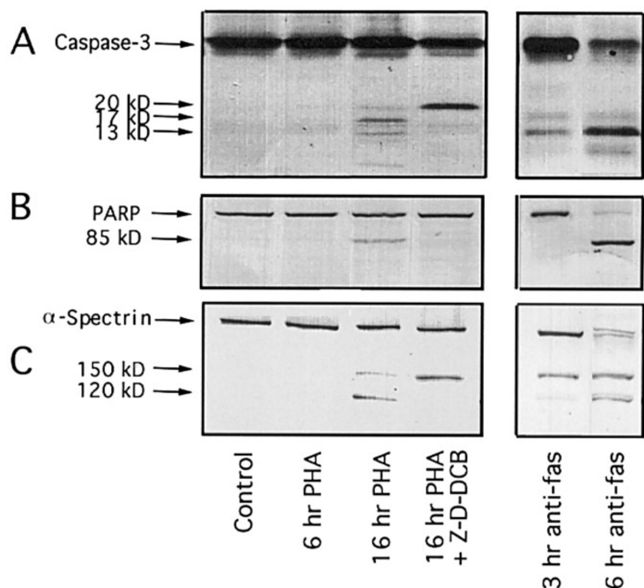


**FIG. 1.** PHA-activated T-cells exhibit caspase-3-mediated cleavage product formation. SDS–PAGE and Western blot analyses were performed on cell lysates using anti-caspase-3 (A), anti-PARP (B), and anti-α-spectrin (C). Immunoblots shown are from untreated Jurkat T-cell lysates or Jurkat cultures activated by PHA alone for 6 or 16 h, with PHA, but pretreated with 50 μM Z-D-DCB, or with anti-fas at 3 and 6 h posttreatment.

lar weight break down products (BDPs) suggestive of caspase-3-like proteolysis. The production of the 17 and 13-kDa caspase-3 fragments was maximal at 16 h following PHA stimulation (Fig. 1A). Anti-fas, an established inducer of apoptosis and caspase activation in T-cells [45], also produced the active lower molecular weight caspase-3 fragments. The caspase-3 cleavage products detected following anti-fas treatment had molecular weights approximating those seen in PHA-treated Jurkat cultures (Fig. 1A).

Cleavage of the parent PARP from a 110-kDa protein to an 85-kDa BDP characteristic of caspase-3-like protease cleavage was also detected in cell lysates at 16 h following PHA activation (Fig. 1B). Anti-fas-treated Jurkat T-cells (at 6 h) expectedly produced the 85-kDa PARP BDP detected in PHA-treated cultures. Western blots on PHA-activated Jurkat T-cell protein extracts were also probed for the appearance of the caspase-3-generated 120-kDa α-spectrin BDP (Fig. 1C). PHA-activated T-cells contained a 120-kDa fragment at 16 h post activation. Anti-fas apoptotic controls at 6 h post challenge contained the identical 120-kDa α-spectrin BDP detected in PHA-treated Jurkat T-cells. Immunoblots also revealed that the caspase inhibitor Z-D-DCB (50 μM) significantly blocked caspase-3 processing (Fig. 1A), PARP cleavage (Fig. 1B), and the accumulation of the 120-kDa spectrin BDP (Fig. 1C) when Z-D-DCB was used as a Jurkat pretreatment.

To examine further the type of protease activity present in PHA-activated Jurkat T-cells, cell lysates were extracted for enzymatic assay. Two fluorogenic peptide substrates, Ac-DEVD-MCA and Ac-YVAD-MCA, were used to examine the activity of caspase-1-like and caspase-3-like proteases, respectively, in cell lysates (Fig. 2). The activity units expressed in this assay are the average relative fluorescent units detected at 460 ± 15 nm per μg of protein for the given experimental conditions ($n$ = 3). Cleavage of Ac-DEVD-MCA was used to infer caspase-3-like activity because it is preferred over Ac-YVAD-MCA [25]: caspase-1 cleaves both substrates at a similar rate [23]. Ac-DEVD-MCA hydrolysis from cell lysates obtained from PHA-stimulated Jurkat T-cells demonstrated a slight but statistically significant increase in hydrolytic activity at 16 h (0.322 fluorescent units) and at 24 h (0.522 fluorescent units) compared with control (0.160 fluorescent units) (2.02- and 3.27-fold control, respectively) (Fig. 2). Ac-DEVD-MCA hydrolysis at 6 h post PHA challenge in Jurkat T cells was comparable to that at 16 h but was not significant in comparison to untreated controls. PHA-stimulated Jurkat T-cells demonstrated nonsignificant ($P > 0.05$) levels of Ac-YVAD-MCA hydrolytic activity at all time points ex-

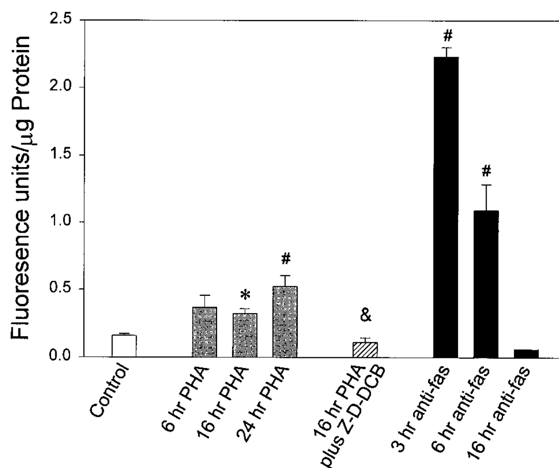


**FIG. 2.** Effects of PHA administration on caspase activity in Jurkat T-cells. Hydrolysis of the caspase-3-preferred substrate Ac-DEVD-MCA in control, PHA-treated, and anti-fas-treated Jurkat T-cells is expressed as relative fluorescent units per microgram of protein. Cell lysates were collected from Jurkat T-cells at 6, 16, and 24 h post PHA challenge, or at 3, 6, and 16 h post anti-fas challenge, in the absence or presence of 50 μM Z-D-DCB as indicated. Hydrolysis of the caspase-1-preferred substrate Ac-YVAD-MCA in PHA-treated cultures was not significant at any time point examined in this study ($P > 0.05$; data not shown). The data shown are the averages of three experiments (mean ± SEM). "#" indicates significance at $P < 0.05$ in comparison to untreated controls using Tukey-HSD ANOVA; "*" indicates significance ($P < 0.05$) in comparison to untreated controls using Student's $t$ test; "&" denotes statistical difference between the 16-h PHA value and the 16-h PHA plus Z-D-DCB value using Student's $t$ test ($P < 0.05$).

amined in these studies (data not shown). The pan caspase inhibitor, Z-D-DCB (50 $\mu$M), completely blocked Ac-DEVD-MCA hydrolysis at 16 h post PHA stimulation (0.70-fold increase compared with control). As expected, anti-fas-treated cell cultures demonstrated marked increases in Ac-DEVD-MCA hydrolysis at 3 h (2.231 fluorescent units; 13.97-fold control) and at 6 h (1.091 fluorescent units; 6.83-fold control) post PHA stimulation in comparison to untreated controls (Fig. 2). Ac-YVAD-MCA hydrolysis was not significantly increased 6 and 16 h after anti-fas treatment ($P > 0.05$, data not shown).

*Jurkat T-cell activation results in IL-2 release that is inhibited by the caspase inhibitors Z-D-DCB and Ac-DEVD-CHO.* Jurkat T-cells were pretreated with the pan caspase inhibitor Z-D-DCB and challenged with PHA to determine if caspase activation is necessary for IL-2 release following PHA-induced T-cell activation. Jurkat T-cells produced a marked increase in IL-2 release over untreated controls as early as 16 h (174.6 ± 12.1 pg/ml; $P < 0.001$) and sustained increases in IL-2 release for at least 24 h (75.5 ± 8.2 pg/ml; $P < 0.05$) following PHA administration (Fig. 3A). Rolipram (Rol, 30 $\mu$M), a selective PDE-4 inhibitor previously reported to inhibit IL-2 release [46], significantly reduced IL-2 levels by 66% at 16 h (58.6 ± 13.8 pg/ml vs 174.6 ± 12.1 pg/ml; $P < 0.001$) and 51% at 24 h (37.0 ± 3.0 pg/ml vs 75.5 ± 8.2; $P < 0.001$) post PHA stimulation (Fig. 3A). The pan caspase inhibitor, Z-D-DCB (Z-D, 50 $\mu$M), also markedly attenuated IL-2 release at 16 h (93% reduction; 11.5 ± 3.3 pg/ml vs 174.6 ± 12.1 pg/ml; $P < 0.001$) and 24 h (57% reduction; 32.7 pg/ml ± 3.3 vs 75.5 ± 8.2 pg/ml; $P < 0.001$) following stimulation (Fig. 3A). Anti-fas-treated Jurkat T-cells failed to induce a significant increase in IL-2 release at any time point examined following PHA challenge ($P > 0.05$) (Fig. 3A).

LDH release was not enhanced in PHA-stimulated Jurkats at any time point evaluated ($P > 0.05$) (Fig. 3B). As a control, anti-fas treatment of Jurkat T-cells did result in increased LDH release at 16 h (0.876 ± 0.04 LDH units) and 24 h (1.344 ± 0.06 LDH units) compared to untreated controls (0.204 ± 0.01 and 0.227 ± 0.00 LDH units, respectively). Cultures pretreated with Z-D-DCB plus PHA also did not exhibit an increase in LDH release ($P > 0.05$). Morphologically, PHA-treated cells were devoid of all apoptotic phenotypes as described previously [30, 43] (data not shown).

We further examined the Z-D-DCB concentration-dependent reduction of IL-2 release in PHA-activated Jurkat T-cells (Fig. 4A). The dose–response effects of Z-D-DCB were determined at 16 h post PHA challenge because IL-2 release was greatest at this time point (Fig. 3A). Jurkat T-cells cotreated with as little as 5 $\mu$M Z-D-DCB had significantly less PHA-induced IL-2 released than cultures treated with PHA only (216.1 ± 5.7 vs 296 ± 6.8 pg/ml). Increasing concentrations of Z-D-DCB (15, 50, and 100 $\mu$M) further decreased IL-2 release (158.0 ± 25.1, 112.9 ± 19.4, and 12.9 ± 1.6 pg/ml, respectively; Fig. 4A). Again, there were no significant increases in LDH release at any dose of Z-D-DCB used following PHA-induced T-cell activation ($P > 0.05$, data not shown). We also examined another

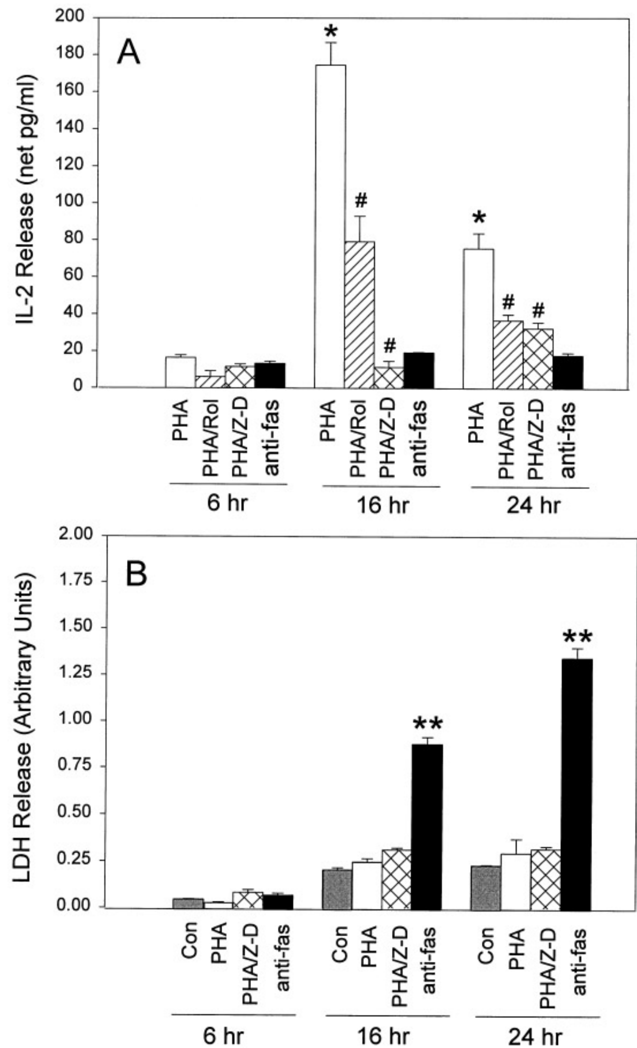


**FIG. 3.** IL-2 release and LDH determination in PHA-treated Jurkat T-cells. (A) Supernatants were assayed for IL-2 by ELISA in Jurkat T-cells treated with PHA (open bars) or with PHA in the presence of either rolipram (Rol) or Z-D-DCB (Z-D) at 6, 16, and 24 h post activation. Effects of anti-fas alone on IL-2 release in Jurkat T-cells were also evaluated at 6, 16, and 24 h post challenge (solid bars). "#" indicates significance in comparison to corresponding PHA-treated cultures. (B) Supernatants were assayed for LDH release from untreated Jurkat T-cells (Con, gray-shaded bars), PHA treated, PHA treated in the presence of 50 $\mu$M Z-D-DCB (PHA/Z-D), or anti-fas-treated Jurkat T-cells at 6-, 16-, and 24-h time points. The data shown are the averages of three experiments (mean ± SEM). Tukey–HSD ANOVA was performed to determine group differences ($P < 0.05$). "**" indicates significance in comparison to the untreated control.

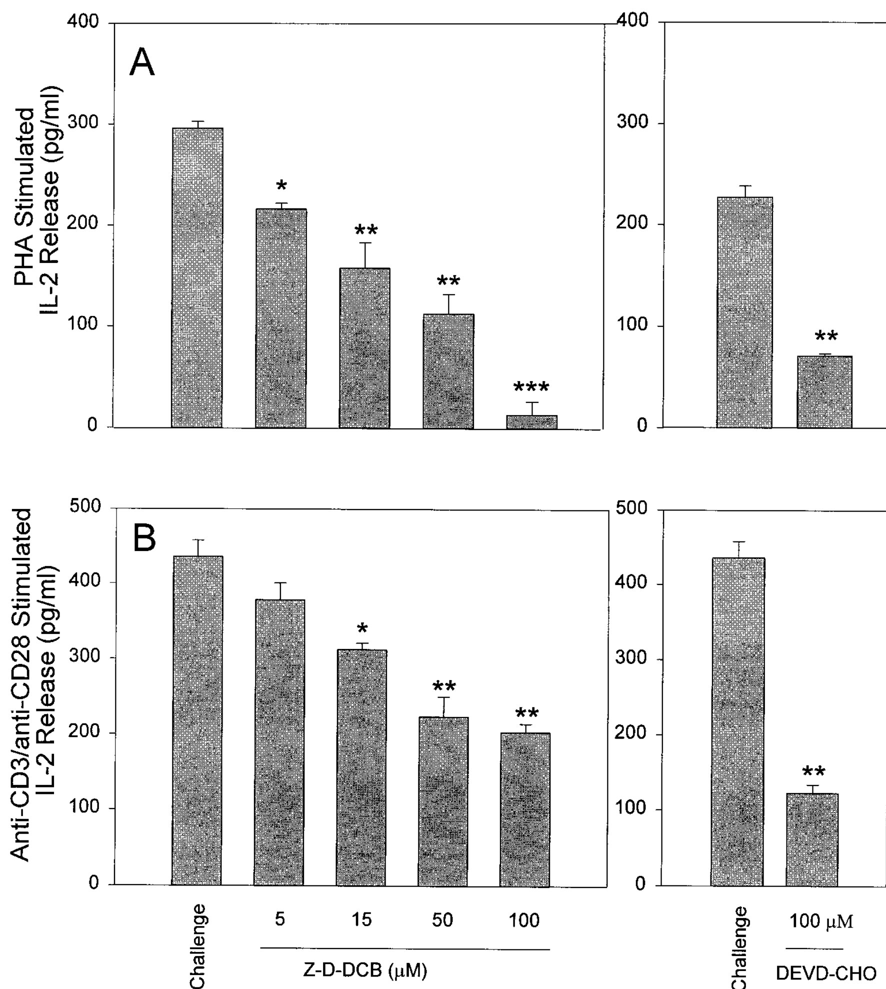


**FIG. 4.** Z-D-DCB dose-related inhibition of PHA and anti-CD3/anti-CD28 induced IL-2 release in Jurkat T-cells. Supernatants were assayed for IL-2 by ELISA and were obtained from Jurkat T-cells cultured in the absence or presence of increasing concentrations of Z-D-DCB (5, 15, 50, and 100 $\mu$M) or Ac-DEVD-CHO (100 $\mu$M) for 16 h after challenge with PHA (A) or anti-CD3/anti-CD28 costimulation (B). The data shown are the averages of three experiments (mean $\pm$ SEM). Tukey–HSD ANOVA was performed to determine group differences ($P < 0.05$). "*" indicates significance at $P < 0.05$, "**" indicates significance at $P < 0.01$, and "***" indicates significance at $P < 0.001$ in comparison to the IL-2 released from corresponding PHA- (A) or anti-CD3/anti-CD28 (B) treated cultures.

caspase inhibitor (Ac-DEVD-CHO, 100 $\mu$M) which also markedly reduced IL-2 release at 16 h following PHA stimulation (71.1 $\pm$ 2.2 vs 227.1 $\pm$ 11.1 pg/ml, $P < 0.01$) (Fig. 4A).

Next we extended our investigation utilizing another pathway of T-cell activation, namely, anti-CD3 plus anti-CD28 costimulation. IL-2 release was detected at 16 h following the costimulation of Jurkat T-cells with anti-CD3/anti-CD28 (Fig. 4B; 435.9 $\pm$ 22.2 pg/ml). Z-D-DCB produced a concentration-dependent reduction of IL-2 release following anti-CD3/anti-CD28 costimulation (15 $\mu$M [312.4 $\pm$ 8.5], 50 $\mu$M [223.8 $\pm$ 26.6], and 100 $\mu$M [203.1 $\pm$ 12.0.] vs 435.9 $\pm$ 22.2 pg/ml; $P <$ 0.05). Similarly, Ac-DEVD-CHO (100 $\mu$M) significantly reduced IL-2 release at 16 h following anti-CD3/anti-CD28 costimulation (122.2 $\pm$ 10.9 vs 435.9 $\pm$ 22.2 pg/ml) ($P < 0.01$, Fig. 4B).

## DISCUSSION

In this study we examined a possible role for caspase-3-like proteases in PHA or anti-CD28 plus anti-CD3 activated Jurkat T-cells. The data in this study confirm that caspase-3-like protease activity is present in activated T-cells as first described by Miossec and colleagues in peripheral blood mononuclear cells (PBMC) [1]. Our paper also represents the first evidence that caspase-3-like protease activation is necessary for IL-2 production in PHA-activated T-cells. Moreover, we observed caspase-3 processing to the active 17- and 13-kDa caspase-3 subunits (Fig. 1A) in

activated Jurkats, in contrast to the finding of Miossec and colleagues [1], who did not detect caspase-3 processing in Jurkat T cells at 24 h following PHA stimulation. We also showed that the caspase-3 substrates PARP and $\alpha$-spectrin are indeed proteolyzed in PHA-activated T-cells (Figs. 1A and 1B). Importantly, the appearance of the active 17-kDa caspase-3 cleavage product (Fig. 1A), the 85-kDa PARP cleavage product [23, 28] (Fig. 1B), and the exclusive caspase-3-generated 120-kDa $\alpha$-spectrin BDP [29, 30] (Fig. 1C), in the absence of apoptotic cell death, suggests that caspase-3-like activation is involved in intracellular signaling following T-cell activation. In addition, cleavage of the fluorometric caspase-3-like substrate Ac-DEVD-MCA [23] was observed without Ac-YVAD-MCA hydrolysis (preferred caspase-1 substrate; Fig. 2) in lysates from PHA-activated Jurkat T-cells. Collectively, these findings support a role for caspase-3-like proteases in Jurkat T-cell activation.

Recently, questions have been raised as to the validity of caspase-3 processing and its upregulated activity in activated T-cells. A recent paper from Zapata *et al.* [2] suggests that previous evidence of caspase-3 processing and activation [1] was due to a sample-processing artifact. Specifically, Zapata *et al.* claim that when activated T-cells are lysed in the absence of SDS, resident granzyme B is released into the lysate and subsequently cleaves and activates caspase-3. These events were demonstrated even if samples were kept at 0–4°C. The authors [2] then reported that when the lysis buffer contained ≥2% SDS, or when an alternate lysis buffer such as a RIPA buffer containing anti-granzyme B was used, caspase-3 processing and subsequent activity were not detected. However, the lines of evidence supporting caspase-3 processing in Jurkat T-cell activation in our current study were obtained using a lysis buffer that contained 2% SDS. In addition, our protocol used a trichloroacetic acid protein extraction step that would further limit enzymatic activity during sample processing.

Significant enhancement of IL-2 release was detected following PHA activation in Jurkat T-cells, peaking at 16 h (Fig. 3A). Caspase-3-like activity and IL-2 release were each significantly elevated at 16 and 24 h after PHA treatment (Fig. 2 vs Fig. 3A). We demonstrated almost a complete blockade of IL-2 release 16 h following pretreatment with 50 $\mu$M Z-D-DCB (Fig. 3A), suggesting that caspase-3-like activity might be required for the expression of IL-2 protein and/or its subsequent release, since IL-2 is not processed before secretion [47]. The inhibition of IL-2 release by Z-D-DCB (Fig. 4) was dose dependent and occurred in the absence of any increase of LDH. The ability of Z-D-DCB to attenuate IL-2 release following T-cell activation was similar to that of rolipram, a selective PDE4 inhibitor previously reported to inhibit IL-2 release [46]. In addition, the blockade of IL-2 release by Z-D-DCB in PHA-activated Jurkat T-cells paralleled the retention of a less active caspase-3 form (20 kDa) and the blockade of the 85-kDa PARP [23, 28] and 120-kDa $\alpha$-spectrin [29, 30] BDP formation generated by caspase-3-like proteases (Fig. 1). This reduction of IL-2 is caspase-inhibition specific as another caspase-3 inhibitor (Ac-DEVD-CHO) [48] also markedly reduced IL-2 release following PHA stimulation (Fig. 4A). Moreover, caspase-3-like protease dependent IL-2 release was not restricted to PHA-activated Jurkat T-cells because Jurkat T-cells costimulated by anti-CD3 and anti-CD28 yielded IL-2 release that was also attenuated by Z-D-DCB in a dose-dependent manner and Ac-DEVD-CHO (Fig. 4B).

The finding that caspase-3 inhibitors can prevent IL-2 release in activated Jurkat T-cells is significant since sample preparation for measurement of IL-2 by ELISA does not involve cell lysis. That is, exposure to and possible activation of caspase-3 by granzyme B should not occur under these conditions. These data then independently support the role of a caspase-3-like protease during T-cell activation. However, since IL-2 is not directly activated by proteolytic cleavage [47], the role of caspase-3 in the signal transduction pathway that leads to IL-2 release represents an enigma. Nevertheless, new findings in the literature support the participation of caspase-3-like proteases in T-cell activation. A recent paper has provided confirmatory data that caspase-3-like proteases participate in T-cell proliferation upon activation by mitogens and IL-2 [49]. Another paper has shown that mice deficient in FADD, an adaptor protein that was previously shown to be involved in caspase-mediated apoptotic cell death, have an unexpected impairment in T-cell activation [50]. Moreover, other recent studies have shown that several substrates of caspase-3, when cleaved, can increase their overall activity [51]. These substrates include members of the MAP kinase family [51, 52] and Ras-related G-proteins [50, 53], both of which contain proteins that are necessary but not sufficient for IL-2 release following T-cell activation. Future studies are clearly needed to establish a role for caspase-3 in T-cell activation.

The finding of significant levels of caspase-3-like activity (Figs. 1 and 2) associated with neither elevated LDH levels (Fig. 3B) nor morphological correlates of apoptosis (data not shown) is in stark contrast to caspase-3-mediated apoptotic cell death observed in anti-fas-treated T-cells [45] (Fig. 3B). The findings reported here suggest that Jurkat T-cells have alternative pathways for activating caspase-3 physiologically which do not result in apoptotic cell death. Levels of caspase-3-like protease-mediated cleavage products and the hydrolysis of Ac-DEVD-MCA following PHA-stimulated Jurkat T-cells were much lower than that

achieved with anti-fas. The low levels of caspase-3-like activation possibly contribute to the lack of LDH release in PHA-activated Jurkat T-cells. The reason that Miossec and colleagues [1] did not detect caspase-3-like activity in Jurkat T-cells treated with PHA, and we were consistently able to do so, is not precisely clear. However, one possibility is that Miossec *et al.,* [1] did not detect caspase-3-like activity due to the different lengths of time required for caspase-3 activation in Jurkat T-cells and PBMC cells treated with PHA. That is, in our studies the time course of caspase-3 activation in Jurkat T-cells following PHA treatment suggests that the highest amounts of caspase-3 processing occurred at around 16 h post PHA challenge, which is before the earliest time point examined in the Miossec *et al.* study [1]. Thus, a T-cell line can potentially be used to examine the role of caspase-3-like proteases in T-cell activation.

In closing, we demonstrated with several lines of evidence that caspase-3-like protease activation does occur in activated Jurkat T-cells and that this activation parallels IL-2 release. Moreover, caspase inhibition significantly reduced IL-2 release in both PHA-activated and anti-CD3/anti-CD28 costimulated Jurkat T-cells, suggesting that IL-2 release is dependent on caspase-3-like activity. Importantly, the evidence for caspase-3-like protease activation in Jurkat T-cell activation in this study was obtained using the preferred methodologies of Zapata *et al.* [2]. The evidence that the increase of caspase-3-like activity can occur in activated T-cells in the absence of overt cell death necessitates a reclassification of caspase-3-like proteases from being exclusively death proteases to enzymes that may have several functions in different cellular processes. Most recently, a report by Zhang *et al.* [54] has shown that caspase-3 can process IL-16, a proinflammatory cytokine, into a more active form. Since a large number of proteins can potentially be cleaved by caspase-3-like proteases, future studies are needed to clarify the target substrates that contribute to IL-2 release following T-cell activation.

## REFERENCES


1. Miossec, C., Dutilleul, V., Fassay, F., and Diu-Hercend, A. (1997). Evidence for CPP32 activation in the absence of apoptosis during T lymphocyte stimulation. *J. Biol Chem.* **272,** 13459–13462.
2. Zapata, J. M., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1998). Granzyme release and caspase activation in activated human T-Lymphocytes. *J. Biol Chem.* **273,** 6916–6920.
3. Fraser, A., and Evan, G. (1996). A license to kill. *Cell* **85,** 781–784.
4. Vaux, D. L., and Strasser, A. (1996). The molecular biology of apoptosis. *Proc. Natl. Acad. Sci. USA* **93,** 2239–2244.
5. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996). Human ICE/CED-3 protease nomenclature. *Cell* **87,** 171.
6. Muzio, M., Salvesen, G. S., and Dixit, V. M. (1997). FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. *J. Biol. Chem.* **272,** 2952–2956.
7. Homma, S., Yaginuma, H., and Oppenheim, R. W. (1994). Programmed cell death during the earliest stages of spinal cord development in the chick embryo: A possible means of early phenotypic selection. *J. Comp. Neurol.* **345,** 377–395.
8. Zamenhof, S., and Guthrie, D. (1995). Programmed cell death enhances uniformity in rat cerebral hemispheres. *Dev. Neurosci.* **17,** 264–266.
9. Yaginuma, H., Tomita, M., Takashita, N., McKay, S. E., Cardwell, C., Yin, Q. W., and Oppenheim, R. W. (1996). A novel type of programmed neuronal death in the cervical spinal cord of the chick embryo. *J. Neurosci.* **16,** 3685–3703.
10. Kirshenbaum, L. A., and de Moissac, D. (1997). The bcl-2 gene product prevents programmed cell death of ventricular myocytes. *Circulation* **96,** 1580–1585.
11. Kappler, J. W., Roehm, N., and Marrack, P. (1987). T-cell tolerance by clonal elimination in the thymus. *Cell* **49,** 273–280.
12. von Boehmer, H. (1994). Positive selection of lymphocytes. *Cell* **76,** 219–228.
13. Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* **267,** 1456–1462.
14. Jenne, D. E., and Tschopp, J. (1989). Granzymes: A family of serine proteases in granules of cytolytic T Lymphocytes. *Curr. Top. Microbiol. Immunol.* **140,** 33–34.
15. Darmon, A. J., Ehrman, N., Caputo, A., Fujinaga, J., and Bleackley, R. C. (1994). The cytotoxic T cell proteinase granzyme B does not activate interleukin-1-beta-converting enzyme. *J Biol. Chem.* **269,** 32043–32046.
16. Yamatsuji, T., Matsui, T., Okamoto, T., Komatsuzaki, K., *et al.* (1996). G-protein-mediated neuronal DNA fragmentation induced by familial Alzheimers disease-associated mutants of APP. *Science* **272,** 1349–1352.
17. Goldberg, Y. P., Nicholson, D. W., Rasper, D. M., Kalchman, M. A., Koide, H. B., Graham, R. K., Bromm, M., Kazemi-Esfarjani, P., Thornberry, N. A., Vaillancourt, J. P., and Hayden, M. R. (1996). Cleavage of Huntington by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. *Nat. Gen.* **13,** 442–449.
18. Marx, J. (1996). Mutant enzyme provides new insights into the cause of ALS. *Science* **271,** 446–447.
19. Ishizaki, Y., Jacobson, M. D., and Raff, M. C. (1998). A role for caspases in lens fiber differentiation. *J. Cell Biol.* **140,** 153–158.
20. Fernandes-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L., Tomaselli, K. J., Wang, L., Yu, Z., Croce, C. M., Salveson, G. *et al.* (1995). Mch3, a novel human apoptotic cysteine protease highly related to CPP32. *Cancer Res.* **55,** 6045–6052.
21. Schlegel, J., Peters, I., Orrenius, S., Miller, D. K., Thornberry, N. A., Yamin, T. T., and Nicholson, D. W. (1996). CPP32/Apopain is a key interleukin $1\beta$ converting enzyme-like protease involved in Fas-mediated apoptosis. *J. Biol. Chem.* **271,** 1841–1844.
22. Cerretti, D. P., Kozlosky, C. J., Mosley, B., Nelson, N., Van Ness, K., Greenstreet, T. A., March, C. J., Kronheim, S. R., Druck, T., Cannizzaro, L. A., Huebner, K., and Black, R. A. (1992). Molecular cloning of the interleukin-$1\beta$ converting enzyme. *Science* **256,** 97–100.
23. Nicholson, D. W., Ambereen, A. N., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T. T., Yu, V. L., and Miller, D. K. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376,** 37–43.

24. Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., Aunins, J., Elliston, K. O., Ayala, J. M., Casano, F. J., Chin, J., Ding, G. F., Egger, L. A., Gaffney, E. P., Limjuco, G., Palyha, O. C., Raju, S. M., Rolando, A. M., Salley, J. P., Yamin, T. T., Lee, T. D., Shively, J. E., MacCross, M., Mumford, R. A., Schmidt, J. A., and Tocci, M. J. (1992). A novel heterodimeric cysteine protease is required for interleukin-1$\beta$ processing in monocytes. *Nature* **356,** 768–774.

25. Talanian, R. V., Quinlan, C., Trautz, S., Hackett, M. C., Mankovich, J. A., Banach, D., Ghayur, T., Brady, K. D., and Wong, W. W. (1997). Substrate specificities of caspase family proteases. *J. Biol. Chem.* **272,** 9677–9682.

26. Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995). Yama/CPP32$\beta$, a mammalian homolog of CED-3, is a crmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81,** 801–809.

27. Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1994). CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian 1-$\beta$ converting enzyme. *J. Biol. Chem.* **269,** 30761–30764.

28. Lazebnik, Y. A., Kaufman, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994). Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371,** 346–347.

29. Martin, S. J., O'Brien, G. A., Nishioka, W. K., McGahon, A. J., Mahboubi, A., Saido, T. C., and Green, D. R. (1995). Proteolysis of fodrin during apoptosis. *J. Biol. Chem.* **270,** 6425–6428.

30. Nath, R., Raser, K. J., Stafford, D., Hajimohammadreza, I., Posner, A., Allen, H., Talanian, R. V., Yuen, P. Y., Gilbertsen, R. B., and Wang, K. K. W. (1996). Non-erythoid $\alpha$-spectrin breakdown by calpain and interleukin 1$\beta$-converting-enzyme-like-protease(s) in apoptotic cells: Contributory roles of both protease families in neuronal apoptosis. *Biochem. J.* **319,** 683–690.

31. Wang, X., Zelenski, N. G., Yang, J., Sakai, J., Brown, M. S., and Goldstein, J. L. (1996). Cleavage of sterol regulatory element binding proteins (SREBPs) by CPP32 during apoptosis. *EMBO J.* **15,** 1012–1020.

32. Casciola-Rosen, L., Nicholson, D. W., Chong, T., Rowan, K. R., Thornberry, N. A., Miller, D. K., and Rosen, A. (1996). Apopain/CPP32 cleaves proteins that are essential for cellular repair: A fundamental principle of apoptotic death. *J. Exp. Med.* **183,** 1957–1964.

33. Cantrell, D. (1996). T cell antigen receptor signal transduction pathways. *Annu. Rev. Immunol.* **14,** 259–274.

34. Tsutsumi, A., Kubo, M., Fujii, H., Freire-Moar, J., Turck, C. W., and Ransom, J. T. (1993). Regulation of protein kinase C isoform proteins in phorbol ester-stimulated Jurkat T lymphoma cells. *J. Immunol.* **50,** 1746–1754.

35. Barja, P., Alavi-Nassab, A., Turck, C. W., and Freire-Moar, J. (1994). Inhibition of T cell activation by protein kinase C pseudosubstrates. *Cell Immunol.* **153,** 28–38.

36. Pastor, M. I., Woodrow, M., and Cantrell, D. (1995). Regulation and function of P21 Ras in T-lymphocytes. *Cancer Surv.* **22,** 75–83.

37. Smith, K. A. A. (1984). Interleukin 2. *Annu. Rev. Immunol.* **2,** 319–333.

38. Mary, D., Aussel, C., Ferrua, B., and Fehlmann, M. (1987). Regulation of interleukin 2 synthesis by cAMP in Human T-cells. *Immunology* **139,** 1179–1184.

39. Tigges, M. A., Casey, L. S., and Koshland, M. E. (1989). Mechanism of interleukin-2 signaling: Mediation of different outcomes by a single receptor and transduction pathway. *Science* **243,** 781–786.

40. Smith, K. A. A. (1988). Interleukin-2: Inception, impact, and implications. *Science* **240,** 1169–1176.

41. Dolle, R. E., Hoyer, D., Prasad, C. V., Schmidt, S. J., Helaszek, C. T., Miller, R. E., and Ator, M. A. (1994). $P_1$ Aspartate-based peptide $\alpha$-((2,6-dichlorobenzoyl)oxy)methyl keytones as potent time dependent inhibitors of interleukin-1$\beta$-converting enzyme. *J. Med. Chem.* **37,** 563–564.

42. Posmantur, R., McGinnis, K., Nadimpalli, R., Gilbertsen, R. B., and Wang, K. K. W. (1997). Characterization of CPP32-like protease activity following apoptotic challenge in SH-SY5Y neuroblastoma cells. *J. Neurochem.* **68,** 2328–2337.

43. Posmantur, R., Wang, K. K. W., Nath, R., and Gilbertsen, R. B. (1997). A purine nucleoside phosphorylase (PNP) inhibitor induces apoptosis via caspase-3-like protease activity in MOLT-4 T cells. *Immunopharmacology* **37,** 231–244.

44. Wang, K. K. W., Nath, R., Posner, A., Raser, K., *et al.* (1996). An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. *Proc. Natl. Acad. Sci. USA* **93,** 6686–6692.

45. Zhivotovsky, B., Burgess, D. H., Schlegel, J., Porn, M. I., Vanags, D., and Orrenius, S. (1997). Proteases in fas-mediated apoptosis. *J. Cell. Biochem.* **64,** 43–49.

46. Souness, J. E., Houghton, C., Sardar, N., and Withnall, M. T. (1997). Evidence that cyclic AMP phosphodiesterase inhibitors suppress interleukin-2 release from murine splenocytes by interacting with a 'low-affinity' phosphodiesterase 4 conformer. *Br. J. Pharmacol.* **121,** 743–750.

47. Hivroz-Burgaud, C., and Cantrell, D. A. (1992). Interleukin 2 *In* "Interleukin-2" (J. Waxman and F. Balkwill, Eds.), pp. 1–11, Blackwell Sci., Oxford.

48. Gamen, S., Marzo, I., Anel, A., Pineiro, A., and Naval, J. (1996). CPP32 inhibition prevents Fas-induced ceramide generation and apoptosis in human cells. *FEBS Lett.* **390,** 232–237.

49. Wilhelm, S., Wagner, H., and Häcker, G. (1998). Activation of caspase-3-like enzymes in non-apoptotic T cells. *Eur. J. Immunol.* **28,** 891–900.

50. Zhang, J., Cado, D., Chen, A., Kabra, N. H., and Winoto, A. (1998). Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. *Nature* **392,** 296–300.

51. Widmann, C., Gibson, S., and Johnson, G. L. (1998). Caspase-dependent cleavage of signaling proteins during apoptosis. *J. Biol Chem.* **273,** 7141–7147.

52. Dumont, F. J., Staruch, M. J., Fischer, P., DaSilva, C., and Camacho, R. (1998). Inhibition of T-cell activation by pharmacologic disruption of the MEK1/ERK MAP kinase or calcineurin signaling pathways results in differential modulation of cytokine production. *J. Immunol.* **160,** 2579–2589.

53. Zenner, G., Dirk zur Hausen, J., Burn, J., and Mutelin, T. (1995). Towards unraveling the complexity of T-cell signal transduction. *BioEssays* **17,** 967–975.

54. Zhang, Y., Center, D. M., Wu, M. H., Cruikshank, W. W., Yuan, J., Andrews, D. W., and Kornfeld, H. (1998). Processing and activation of pro-interleukin-16 by caspase-3. *J. Biol. Chem.* **273,** 1144–1149.

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