Caspase Dependence of Target Cell Damage Induced by Cytotoxic Lymphocytes

Apurva Sarin, Elias K. Haddad and Pierre A. Henkart

*J Immunol* 1998; 161:2810-2816; ;
http://www.jimmunol.org/content/161/6/2810

---

**References**  
This article cites 48 articles, 25 of which you can access for free at:  
http://www.jimmunol.org/content/161/6/2810.full#ref-list-1

**Subscription**  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

**Permissions**  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
Caspase Dependence of Target Cell Damage Induced by Cytotoxic Lymphocytes

Apurva Sarin, Elias K. Haddad, and Pierre A. Henkart

Since the CTL secreted granule protease granzyme B can activate multiple target caspases, it has been proposed that this pathway is responsible for CTL-induced cytolysis of Fas-negative targets. However, target lysis via the granule exocytosis pathway is completely resistant to caspase inhibitors. To test the possibility that granzymes trigger a postcaspase cytoplasmic apoptotic pathway leading to lysis, we have examined the caspase dependence of several cytoplasmic changes associated with apoptotic death. Rapid prelytic phosphatidylserine externalization was induced in Jurkat target cells by both the Fas ligand (FasL)/Fas and the granule exocytosis effector pathways. This was specifically blocked by peptide ketone caspase inhibitors when induced by the former, but not by the latter, pathway. A rapid prelytic loss of target mitochondrial $\psi$ was also induced by both CTL effector pathways, and this was also specifically blocked by caspase inhibitors when induced by the FasL/Fas, but not by the granule exocytosis, pathway. Similarly, target membrane blebbing induced by CTL via the FasL/Fas, but not via the granule exocytosis, effector pathway was specifically blocked by caspase inhibitors. In contrast to the above nonnuclear damage, CTL-induced target staining by the lipid probe FM1–43 reflecting plasma membrane endocytosis was blocked by caspase inhibitors. Thus, when caspase activation is blocked, the granule exocytosis pathway triggers several parameters of target apoptotic damage in addition to lysis, suggesting that granzymes directly trigger a postcaspase cytoplasmic apoptotic death pathway. The Journal of Immunology, 1998, 161: 2810–2816.

Cytoxic lymphocytes rapidly kill target cells in vitro using two effector pathways that are triggered by membrane receptors recognizing target Ag. One is a secretory pathway involving receptor-triggered exocytosis of preformed secretory granules, while the other uses receptor-induced surface membrane expression of Fas ligand (FasL), which cross-links Fas on target cells (1). While CTL use both these effector pathways to kill target cells, NK cells chiefly use the granule exocytosis pathway (2), which postulates a vital role for the granule protein perforin (also known as cytolysin) in permeabilizing target membranes (3). The in vivo importance of this effector pathway has been confirmed by the development of perforin-deficient mice, which show a seriously compromised ability to resist infection by noncytopathic viruses as well as other immunologic defects (4).

Since target cells killed by cytoxic lymphocytes in vitro typically display characteristic apoptotic features (5), an appealing hypothesis has been that a common apoptotic death pathway existing in a latent form in all cells is triggered by these effector cells (6). Rapid progress has been made recently in identifying a protease family termed caspases as central molecular mediators of apoptotic cell death (7). The 10 currently identified human caspases are widely expressed in an inactive precursor form in many cells and have the hallmark specificity of cleaving protein substrates with aspartic acid residues at the P1 position. Their activation occurs as a result of proteolytic processing of procaspases at aspartic acid residues, so that caspases can autoactivate and process each other in an activation cascade (8). Despite the general consensus that caspases play a central role in apoptotic death, important questions remain unanswered. One of these is the nature of postcaspase cell death pathway leading to the various characteristic features of apoptotic cell death, including phosphatidylserine (PS) externalization and lysis.

Recent experiments have shown that both effector pathways used by cytoxic lymphocytes activate caspases. Cross-linking of target cell Fas by FasL on CTL membranes leads to activation of caspases 8 and probably 10 via the adaptor molecule FADD (9). These caspases can process and activate downstream caspases that, in turn, trigger cell destruction. We have shown that rapid target cell lysis and apoptotic nuclear damage by this CTL effector pathway are blocked by two classes of caspase inhibitors, confirming the predicted functional importance of caspases in this effector pathway (10).

For the granule exocytosis pathway, the requirement for caspases in target cell death is less clear. Caspase activation via the granule exocytosis pathway is predicted because the granule serine protease granzyme B recognizes a sequence motif compatible with caspase activation (11) and has been shown to initiate processing and activation of several caspases (12). CTL targets undergo rapid caspase-3 processing as predicted by these in vitro experiments (13), thus providing an explanation for apoptotic nuclear damage induced by granzyme B in the presence of sublytic doses of perforin (14). However, while CTL from mice lacking granzyme B induce target nuclear damage somewhat more slowly, their potency and rate of target lysis via the granule exocytosis pathway are unaffected by the loss of granzyme B (15). (Lysis by activated NK cells from mice lacking granzyme B is partially defective.
(16.) Since no other granule proteases are known to activate caspases directly, these data suggest that target lysis via CTL granule exocytosis might be independent of caspases. In support of this, we recently showed that two classes of caspase inhibitors that effectively blocked CTL granule exocytosis-induced target nuclear damage did not detectably block CTL-mediated target lysis (10).

One model to explain these results proposes that granzyme B mediates nuclear damage via caspase activation, but that target lysis occurs as a result of granzyme-induced cleavage of downstream cytoplasmic protein substrates which are also cleaved by caspases and lead to apoptotic cell destruction. This model predicts that other CTL granule exocytosis-induced cytoplasmic apoptotic damage might be caspase independent if it is part of the post-caspase death pathway. In this paper we describe experiments showing that three such nonnuclear changes induced by the granule exocytosis pathway in CTL and NK cells are not blocked by caspase inhibitors that block nuclear damage. These results suggest that granzymes activate a postcaspase apoptotic damage pathway that results in mitochondrial potential loss, PS surface exposure, membrane blebbing, and lysis.

Materials and Methods

Reagents

The protease inhibitors Chz-Val-Asp(O-methyl)-fluoromethyl ketone (ZVAD-FMK), Boc-Asp(O-methyl)-fluoromethyl ketone (BD-FMK), and Chz-Phe-Ala-fluoromethyl ketone (ZFA-FMK) were purchased from Enzyme Systems Products (Dublin, CA), made up as stock solutions of 50 mM in DMSO, and stored at −80°C. IgG anti-Fas mAb DX2 was obtained from Pharmingen (San Diego, CA). FM1–43, DiIC<sub>3</sub>(3), and DiOC<sub>4</sub>(3) were obtained from Molecular Probes (Eugene, OR) made up as 1 mM stocks in DMSO, and stored at 4°C. Annexin V–FITC was obtained from Trevigen (Gaithersburg, MD). PMA, ionomycin, and Hoechst 33342 in parallel wells in the same plate, as described in Materials and Methods.

Target and effector cells

The human lymphoblastoid cell line Jurkat and the erythromyeloid leukemia K562 were maintained in complete medium (RPMI 1640 supplemented with 10% FCS, 100 IU penicillin, and 10 μg/ml streptomycin) and used as target cells. To distinguish these cells from effector cells by flow cytometry or fluorescence microscopy, they were prelabeled by incubation with the lipid probe DiIC<sub>3</sub> (10 μM for 10 min at 37°C). When used as targets for CTL, they were subsequently surface TNP-modified by incubation with 1 mM trinitrobenzene sulfonate in PBS, pH 7.4, for 15 min at 37°C followed by washing.

The CTL hybridoma d11S was preactivated to express FasL by incubation for 3 h with FAMA (5 ng/ml) and ionomycin (3 μg/ml) before the cytotoxicity assay. Effector cells were generated from primary in vitro mixed lymphocyte cultures in which spleen cells from BALB/c (H-2<sup>b</sup>) mice were mixed with irradiated (300 Gy) spleenocytes from C57BL/6 (H-2<sup>b</sup>) mice at a ratio of 2.5:1 and incubated for 5 days in complete medium at 37°C. Further culture was conducted at 5 × 10<sup>5</sup> cells/ml in complete medium with 10 U/ml of IL-2. Effector cells were harvested by centrifugation over lymphocyte M (Accurate Chemical and Scientific Corp., Westbury, NJ). NK effector cells were obtained from human PBMC centrifuged over Ficoll-Hypaque (Sigma) and used directly or activated by overnight culture at 5 × 10<sup>5</sup> cells/ml in complete medium with 20 U/ml IL-2.

Assays of target damage

All experiments assessing the CTL granule exocytosis pathway used primary cultures of allo-CTL redirected to TNP target cells with 100 ng/ml αCD3×α-TNP heteroclonal as previously described (17). In these incubations the Fas cytotoxicity pathway was blocked by addition of 10 μg/ml of IgG anti-Fas mAb DX2, which completely blocked Jurkat lysis by d11S CTL. Incubations were conducted in flat-bottom microtiter wells containing 10<sup>4</sup> target cells, which, when indicated, were pretreated with peptide-FMK caspase inhibitors for 1 h before addition of effector cells. The indicated number of effector cells was added and centrifuged at 300 rpm. The plates were then placed in a 37°C CO<sub>2</sub> incubator at time zero. At the indicated times wells were harvested by pipetting, and target damage was assessed by flow cytometry after pooling an appropriate number of wells.

To assess nuclear morphology and formation of membrane blebs, cells were stained with 5 μg/ml Hoechst 33342, and DiIC<sub>3</sub>-positive cells were scored for apoptotic nuclear morphology or the presence of blebs by fluorescence microscopy. PS exposure was assayed by flow cytometry after staining with 200 ng/ml annexin V–FITC in HEPES-buffered saline containing 2.5 mM CaCl<sub>2</sub> for 15 min in the dark and suspending in this buffer containing 10 μg/ml PI. The DiIC<sub>3</sub>-positive, PI-negative subset of cells was analyzed for fluorescein fluorescence intensity. Mitochondrial potential was estimated using DiOC<sub>4</sub>(3, 18) by incubation of the resuspended cell pellets with 40 mM DiOC<sub>4</sub>(3) in 0.5 ml of PBS for 20 min in the dark at 37°C. After centrifugation the cells were resuspended in 500 μl of PBS containing 10 μg/ml PI, and the DiIC<sub>3</sub>-positive, PI-negative subset of cells was immediately analyzed by flow cytometry.

Plasma membrane recycling was measured by incubation of the cell suspension with 5 mM FM1–43 for 10 min and washing three times in complete medium before analysis on the flow cytometer. Because of the fluorescence properties of FM1–43, it was not possible to identify target cells with DiC<sub>16</sub>, and in these experiments target cells were selectively analyzed using a scatter gate (two-dimensional forward and side scatter) defined by running effectors and targets alone. This approach limited these experiments to modest E:T cell ratios.

Results

Target nuclear damage, but not lysis, induced by human NK cells is blocked by peptide caspase inhibitors

Human PBL were tested for their ability to lyse and induce target nuclear damage in the classic NK target K562. As shown in Figure 1A, a 4-h incubation of normal PBL with K562 target cells at an E:T cell ratio of 20 resulted in both lysis and apoptotic nuclear damage of these targets, confirming previous results with these apoptosis-resistant BCR-overexpressing target cells (19). The dependence of these cytotoxic effects on caspases was tested using the cell-permeable peptide-based caspase inhibitors ZVAD-FMK and BD-FMK, which were previously shown to block a variety of death readouts in lymphocytes treated with various apoptogenic agents (20). These caspase inhibitors caused a dose-dependent inhibition of K562 apoptotic nuclear damage, while the control reagent ZFA-FMK slightly enhanced such damage. In contrast, none

FIGURE 1. Peptide caspase inhibitors block target nuclear damage but not lysis induced by NK cells. The human leukemia K562 cell line was used as a target for cytotoxicity by human PBMC, which were used directly (A; E:T cell ratio = 20) or after overnight activation with IL-2 (B; E:T ratio = 5). Four-hour incubations were conducted in the presence of peptide-FMK reagents: ZVAD-FMK (triangles), BD-FMK (squares), or ZFA-FMK (diamonds). Target lysis was measured by 31Cr release (filled symbols) in triplicate wells, and target nuclear damage (open symbols) was assessed microscopically on DiIC<sub>3</sub>-prelabeled K562 cells using Hoechst 33342 in parallel wells in the same plate, as described in Materials and Methods.
of these peptide-FMK reagents had a significant effect on target lysis. These results are strikingly parallel to those previously obtained with mouse CTL using the granule exocytosis effector pathway (10).

NK cells activated by overnight culture with IL-2 give an enhanced cytotoxic activity, and the caspase dependence of this activity was similarly tested in the experiment shown in Figure 1B. The considerably more potent cytotoxic activity of these activated NK cells showed a similar pattern of blocking by caspase inhibitors as fresh NK cells, i.e., potent blocking of nuclear damage by ZVAD-FMK, slightly weaker blocking by BD-FMK, and no effect of ZFA-FMK, while there was no effect on target lysis. In other experiments we further confirmed these findings using adherent activated mouse spleen NK cells with mouse YAC tumor targets (data not shown).

The CTL FasL/Fas effector pathway induces a caspase-dependent target cell PS exposure before lysis

Because our objective was to examine the nonnuclear apoptotic events induced in target cells by cytotoxic lymphocytes before lysis, we have used flow cytometry to identify PI-negative target cells prelabeled with a lipid probe and followed a third marker of apoptotic damage. We have separately tested the granule exocytosis and FasL/Fas effector pathways, since these together account for rapid in vitro CTL-mediated target cell death (1). We first examined the surface exposure of PS by binding of FITC-annexin V. Figure 2A shows that the FasL-bearing effector cell d11S (21) mediates a rapid PS exposure on PI-negative Jurkat target cells at modest E:T cell ratios. Figure 2B indicates that this PS exposure as well as the induction of apoptotic nuclear morphology are specifically blocked by peptide-FMK caspase inhibitors. This result is predicted by a pathway involving Fas-induced cross-linking of caspase 8 or 10 via FADD and was expected in light of previous results showing that PS exposure induced by anti-Fas Ab is caspase dependent (22, 23).

The granule exocytosis effector pathway induces a rapid target cell PS exposure before lysis that is not blocked by peptide-FMK caspase inhibitors

Figure 3A shows that primary in vitro-generated CTL, in the presence of IgG anti-Fas to block the Fas killing pathway, mediate a rapid prelytic PS exposure in Jurkat target cells. In Figure 3B we tested the ability of ZVAD-FMK and BD-FMK to block this PS exposure and observed a dramatic contrast to the results presented in Figure 2B. In this case, peptide-FMK caspase inhibitors efficiently blocked apoptotic nuclear morphology (confirming our previous results (10)), but had no effect on PS exposure.

A similar experiment with IL-2-activated human NK cells and K562 target cells is shown in Figure 4. In this case PS exposure was extremely rapid, with >35% of target cells showing increased annexin V staining within 20 min at an E:T cell ratio of 10. However, this PS exposure was not detectably inhibited by peptide-FMK caspase inhibitors, although these inhibitors blocked apoptotic nuclear damage in the same experiment (Fig. 4B).

Figure 2. PS exposure on target cells induced by CTL FasL is caspase dependent. A, Kinetics of PS exposure on Fas-bearing Jurkat cells assessed by flow cytometry using annexin V-FITC after incubation with FasL-bearing d11S-CTL (E:T cell ratio = 5). The percentage of PI-negative, DiIC16-positive (prelabeled) Jurkat that is positive for annexin V is shown. B, Effect of peptide-FMK caspase inhibitors on d11S-mediated damage to Jurkat target cells. Incubations were performed as described in A for 2.5 h in the presence of the indicated peptide-FMKs (25 μM). PS exposure was measured as described in A using annexin V-FITC (solid bars), and nuclear morphology (hatched bars) was determined using Hoechst 33342 as described in Figure 1.

Figure 3. PS exposure on target cells induced by CTL granule exocytosis is not blocked by peptide caspase inhibitors. A, Kinetics of PS exposure on Jurkat target cells induced by CTL using the granule exocytosis pathway. Primary in vitro allogeneic murine CTL were used with Jurkat target cells at an E:T cell ratio of 20. Cytotoxicity was redirected using TNP-modified target cells and oCD3×α-TNP-heteroconjugated Ab, and soluble IgG anti-Fas was added to block Fas-mediated cytotoxicity as described in Materials and Methods. Quantitation of PS exposure using annexin V-FITC binding was performed as described in Figure 2. B, Effects of peptide-FMK caspase inhibitors on target damage by CTL granule exocytosis. Incubations were performed as described in A for 3 h in the presence of the indicated peptide-FMKs (25 μM). PS exposure was measured as described in A using annexin V-FITC (solid bars), and nuclear morphology (hatched bars) was determined using Hoechst 33342 as described in Figure 1.

Figure 4. NK cell-mediated target PS exposure is not inhibited by peptide caspase inhibitors. A, Kinetics of PS exposure on K562 target cells following incubation with activated NK cells. Incubations were performed at an E:T cell ratio of 10 as described in Figure 1. Quantitation with annexin V-FITC was performed as described in Figure 2. B, PS exposure (solid bars) and nuclear morphology (hatched bars) were assessed in DiIC16-labeled K562 target cells 2 h after incubation with activated NK cells in the presence or the absence of a 50 μM concentration of the indicated peptide-FMK.
Peptide caspase inhibitors block prelytic target mitochondrial potential loss induced by the CTL FasL/Fas pathway, but not by the granule exocytosis pathway

Dying cells have been reported to undergo a rapid loss of mitochondrial potential $\psi_m$, as measured by DiOC$_6$ fluorescence (24). To determine whether CTL targets undergo a rapid prelytic loss of mitochondrial potential, we used a similar analysis by flow cytometry. Figure 5A shows that the FasL-bearing d11S cells induce a loss of mitochondrial potential $\psi_m$ that is detectable within 1 h, and Figure 6A shows that CTL using the granule exocytosis pathway shows a generally similar activity. The ability of peptide-FMK caspase inhibitors to block this mitochondrial damage was then tested. As shown in Figure 5B, the prelytic mitochondrial $\psi_m$ loss induced by the FasL-bearing d11S CTL was dramatically and specifically blocked by these inhibitors along with nuclear damage. In contrast, loss of $\psi_m$ via the CTL granule exocytosis pathway was not detectably inhibited by the caspase inhibitors, although in the same cells apoptotic nuclear damage was effectively and specifically inhibited (Fig. 6B).

Caspase inhibitors block membrane bleb formation by the CTL FasL/Fas pathway, but not by the granule exocytosis pathway

Membrane blebbing has long been recognized as an early sign of CTL-induced target cell injury (25, 26) and is part of the classical syndrome of apoptotic death (27). We have found that the DiIC$_{16}$ lipid probe used in the above experiments to distinguish target cells from CTL affords a reliable means of visualizing target plasma membrane blebs in the fluorescent microscope. Using Jurkat target cells and the CTL effector systems described above, we observed a rapid induction of blebs by both the granule exocytosis and FasL/Fas pathways, with 40 to 50% of the target cells showing clear membrane blebs by 90 min at modest E:T cell ratios (Fig. 7). When peptide-FMK caspase inhibitors were tested for the ability to block this bleb formation, they were found to specifically block that induced by d11S, but not primary CTL using the granule exocytosis pathway.

Caspase inhibitors block the CTL-induced increase in target membrane recycling by the granule exocytosis pathway

CTL have been reported to rapidly induce an increase in staining of target cells after a pulse exposure to the lipid probe FM1–43 (28). Such increased FM1–43 staining is considered a measure of endocytic membrane internalization and is found at neuronal synapses after transmitter release (29, 30). Figure 8 shows the rapid increase in FM1–43 staining in Jurkat target cells induced by the CTL granule exocytosis pathway. Figure 8A shows that 90 min after mixing CTL with target cells under conditions of TCR engagement there was a clear subpopulation of target cells with increased FM1–43 fluorescence. This increase was inhibited by the caspase inhibitors BD-FMK and ZVAD-FMK, but not by the control compound ZFA-FMK (Fig. 8B). Thus, increased FM1–43 staining is one measure of nonnuclear damage induced by CTL granule exocytosis that is blocked by peptide-FMK caspase inhibitors and thus appears to be similar to nuclear damage in its sensitivity to caspase inhibitors.

**FIGURE 5.** Loss of target cell mitochondrial $\psi_m$ by CTL bearing FasL is caspase dependent. A, Kinetics of loss of mitochondrial $\psi_m$ assessed by DiOC$_6$ fluorescence using flow cytometry in PI-negative Jurkat cells prelabeled with DiIC$_{16}$ incubated with preactivated d11S CTL (E:T cell ratio = 5). B, Mitochondrial $\psi_m$ (solid bars) and nuclear morphology (hatched bars) were assessed in DiIC$_{16}$-labeled Jurkat target cells, as described in A, 2.5 h after incubation with d11S CTL in the presence of the indicated peptide-FMK (25 $\mu$M).

**FIGURE 6.** Loss of target cell mitochondrial $\psi_m$ by CTL granule exocytosis is not inhibited by peptide-FMK caspase inhibitors. A, Kinetics of loss of mitochondrial $\psi_m$ assessed by DiOC$_{6}$ fluorescence using flow cytometry in PI-negative Jurkat cells prelabeled with DiIC$_{16}$ using primary allo-CTL (E:T cell ratio = 20). Incubation conditions are described in Figure 3. Cytotoxicity was redirected using TNP-modified target cells and αCD3/α-TNP-heteroconjugated Ab, and IgG anti-Fas was added to block Fas-mediated cytotoxicity as described in Materials and Methods. B, Effect of peptide-FMK caspase inhibitors on target damage by CTL granule exocytosis. Incubations were performed as described in A for 3 h in the presence of the indicated peptide-FMKs (50 $\mu$M). DiOC$_{6}$ fluorescence was measured using flow cytometry (solid bars), and nuclear morphology was assessed in parallel wells (hatched bars) using Hoechst 33342.

**FIGURE 7.** Effect of peptide-FMK caspase inhibitors on target cell blebbing induced by CTL granule exocytosis and FasL-Fas pathways. Jurkat target cells were prelabeled with DiIC$_{16}$ and incubated for 1.5 h with murine allogeneic CTL (E:T cell ratio = 10; hatched bars) or d11S CTL (E:T cell ratio = 5; solid bars). Redirected primary murine allo-CTL in the presence of soluble IgG anti-Fas were used to assess the granule exocytosis pathway, and preactivated d11S CTL bearing FasL were used as described in previous figures. The number of target cells with membrane blebs was assessed by examination of DiIC$_{16}$ fluorescence in the microscope.
Discussion

The experiments described here show that both cytotoxic lymphocyte effector pathways rapidly induce target cell death, which has a full spectrum of apoptotic properties. These include membrane damage, as seen by PS externalization and blebbing, as well as the fall in electrical potential across the inner mitochondrial membrane. These findings confirm that target death by both effector pathways is apoptotic, as previously revealed by nuclear damage, and show that nonnuclear target damage induced by cytotoxic lymphocytes is rapid compared with that caused by many other agents.

The above examples of target damage induced by CTL are not surprising in light of previous demonstrations that CTL activate caspases, since caspase inhibitors block all measurable apoptotic damage by a wide range of apoptotic triggers. However, the most interesting aspect of the experiments described here is that three of the four measurements of target damage induced by the granule exocytosis pathway were not blocked by caspase inhibitors that completely blocked nuclear damage in the same experiment. This means that the nonnuclear events do not require the granzyme-induced caspase activation that leads to nuclear damage, part of which is probably attributable to the recently described caspase-activated DNase (31). Since these nonnuclear changes are typically associated with apoptotic death, the results strongly suggest that granzymes directly trigger a nonnuclear molecular death pathway that leads to mitochondrial depolarization, membrane PS externalization and blebbing, and lysis, as shown in Figure 9. The molecular nature of the postcapsule cell destruction pathway has not been defined in any system. While it is sometimes assumed that caspase-induced nuclear damage leads to cell death, this is clearly not the case for CTL, since enucleated target cells are rapidly lysed by both pathways (32). We find it plausible that granzymes and caspases have common substrates that initiate this downstream death pathway, as shown in Figure 9. While a number of nuclear substrates of caspases have been identified, the critical substrates that initiate the pathway leading to lysis remain unidentified.

The data obtained from the present experiments are not compatible with the view that target lysis via the granule exocytosis pathway occurs solely as a result of perforin-induced membrane damage while target apoptotic damage is attributable to granzyme-mediated activation of caspases. This view is sometimes expressed...
by others despite its failure to explain both the kinetics of perforin-vs CTL-induced lysis and the granzyme dependence of the cyto-
toxic activity of RBL transfectants (33). The present data showing
that some nonnuclear damage is not blocked by caspase inhibitors
argue further against this model.

One limitation of our experiments is the use of caspase inhibi-
tors whose ability to block intracellular caspase activation is
incompletely defined. For that reason, we previously showed (6, 10)
that CTL granule exocytosis-induced target lysis was not affected
by baculovirus p35, which inhibits caspases 1 to 4 at a 1:1 molar ratio
(34). We have also previously shown that the peptide FMK
inhibitors used in this paper block the DEVDase activity charac-
teristic of caspases 3 and 7 in extracts of apoptotic Jurkat cells
(20). However, it is not clear how to compare the activities of such
caspases in extracts with those in intact cells, and it is not estab-
lished which of the 10 known caspases are directly inactivated by
the reagent conditions used. Thus, it is quite possible that these
peptide-FMK inhibitors block the Fas death pathway by selec-
tively reacting with caspases 8 and 10, which may be the targets of
granzyme B (12). One can postulate that the failure of peptide-
FMK caspase inhibitors to block lysis and nonnuclear damage by
the granule exocytosis pathway is due to their failure to block the
relevant granzyme B-activated caspases. However, such an expla-
nation does not address why these inhibitors so effectively block
nuclear damage in this pathway, and one would thus need to pos-
tulate that nuclear damage requires caspases sensitive to these in-
hibitors but that lysis and other nonnuclear damage use caspases
that are resistant. Granule exocytosis-triggered lysis of T lymphoid
cells appears unique among apoptotic agents in its resistance to
inhibition by peptide-FMKs, as we have shown that peptide-FMK
caspase inhibitors effectively and specifically block lysis of Jurkat
target cells induced by anti-Fas, TNF, staurosporine, vinblastine,
and several DNA-damaging chemotherapeutic agents (10, 35). In
this respect Jurkat cells reflect the apoptotic lysis of most T lymph-
oid cells induced by a wide variety of agents (20). Thus, while we
cannot formally rule out a role for peptide-FMK-resistant caspases as
described above, our experiments show that it is very likely that
CTL-injected granzymes act by directly triggering a down-
stream apoptotic death pathway without the involvement of caspases.

While unusual, there are several other reported examples of ap-
optotic cell death which are resistant to caspase inhibitors, and it
will be of interest to determine whether these deaths share mole-
cular steps with death induced by lymphocyte granule exocytosis.
In fibroblasts, ZVAD-FMK and BD-FMK retard, but do not, prevent
apoptotic nuclear damage and cell death induced by several agents
(36), and defects in the ubiquitin-activating enzyme E1 lead to an
apparently caspase-independent death (37). Some examples of ap-
optotic death resistant to ZVAD-FMK have been reported (38, 39),
but we have found cases where the analogous caspase inhibitor
BD-FMK specifically blocks death (20).

Externalization of PS is a functionally important manifestation
of apoptotic death, since this triggers one of the recognition sys-
tems used by macrophages to phagocytose apoptotic cells (40).
This lipid is normally restricted to the inner membrane leaflet but
becomes detectable externally before cytolysis during apoptotic
death (41). For the rapid target death induced by cytotoxic lym-
phocytes it has not been previously shown that PS externalization
occurs before lysis, and our findings suggest that cells killed by
CTL in vivo are likely to be rapidly phagocytosed.

The molecular pathways leading to PS externalization during
apoptosis are not well understood. In Jurkat cells treated with anti-
Fas this process requires active caspases and calcium in the me-
dium (42, 43). A plausible model for this would be that caspase
activation results in an elevation of intracellular calcium (44), thus
activating the lipid scramblase that mediates transbilayer phospho-
lipid “flip-flop” (45). For the granule exocytosis death pathway, it
is likely that perforin pores also allow leakage of external calcium
across the target membrane, although this may be temporary due to
membrane repair processes. Thus, additional granule components
such as granzymes may also be required to trigger PS exposure,
and its calcium dependence is not readily tested, since calcium is
also required for degranulation. It is clearly important to design
future experiments to probe this issue and to establish whether
target lysis is closely linked to PS exposure.

The prelytic collapse of mitochondrial potential ψ is associated
with the mitochondrial permeability transition and may occur in
both apoptotic and nonapoptotic death (46). An associated release
of a mitochondrial apoptosis-inducing factor has been proposed to
be a critical step in apoptotic signaling (47). As shown for the
CTL-mediated Fas death pathway in Figure 5B, ZVAD-FMK
blocks the anti-Fas-induced loss of mitochondrial electron trans-
port in Jurkat cells (48), the UV B-induced loss of mitochondrial
ψ in CEM cells (49), and some mitochondrial damage in apoptotic
thymocytes (46). For death induced by the granule exocytosis
pathway, induction of mitochondrial damage is one candidate
functional role for granzemes. Granzemes could be envisioned to
trigger the release of mitochondrial cytochrome e, thus activating
procaspase 9 via Apaf-1 (50, 51), or triggering the permeability
transition and release of apoptosis-inducing factor (47). However,
both of these are proposed to trigger death via activation of down-
stream caspases, making such models unattractive in light of the
present evidence for caspase independence.

The increase in FM1–43 pulse staining induced by the CTL
granule exocytosis pathway (Fig. 8) confirms a previous report that
both CTL effector pathways rapidly induce an enhancement in
plasma membrane endocytosis into internal vesicles revealed by
the technique (28). This fluorescent lipid probe rapidly and re-
versibly partitions into the outer bilayer leaflet from the medium,
so that postpulse washing removes it from the exposed plasma
membrane but not from membrane that underwent endocytosis
during the pulse. The increased rate of plasma membrane uptake is
a property of other apoptotic death systems as well, including thym-
ocytes treated with dexamethasone and etoposide in vitro (28)
(E. K. Haddad, unpublished observations). While it seemed plau-
sible to assume that such enhanced endocytosis was part of the
apoptotic membrane dysregulation also resulting in blebbing and
PS exposure, Figure 8 shows that for CTL granule exocytosis-
induced target damage, this enhanced endocytosis is part of a
caspase-dependent pathway and hence distinct from the other
types of membrane damage. These results also show that nuclear
damage is not the only caspase-dependent damage induced by the
granule exocytosis pathway.

Further molecular definition of the postcaspase cell destruction
pathway is clearly of interest. The present results are helpful in that
regard because they suggest that identification of critical protein
substrates cleaved by both granzymes and caspases may lead to
identification of critical players triggering this pathway.

Acknowledgments
We thank Dr. Charles Zacharchuk for critical comments on the manuscript.

References
tiple effector molecules. *Immunity* 1:343.
Workshop on Cell-Mediated Cytotoxicity, Kerkdriel, The Netherlands, April 5 to 9, 1997, p. 71.