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Vesicles Released by Activated T Cells Induce Both Fas-Mediated RIP-Dependent Apoptotic and Fas-Independent Nonapoptotic Cell Deaths

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Activated T cells secrete Fas ligand (FasL)-containing vesicles (secreted vesicles) that induce death of target cells. We provide evidence that secreted vesicles from culture supernatants (CSup) of various origins are able to generate both Fas-dependent apoptotic and Fas-independent, nonapoptotic cell death. In the absence of Fas, the nonapoptotic, Fas-independent pathway could still induce cell death. In contrast to RIP-independent classical Fas-induced cell death triggered by cross-linked or membrane-bound FasL, CSup-derived stimuli-induced apoptosis exhibited unique molecular and enzymatic characteristics. It could be partially inhibited by blocking cathepsin D enzyme activity and required the presence of RIP. Whereas stimulation with CSup, derived from both FasL-overexpressing Jurkat cells and PBMC, could induce cell death, the requirements for Fas-associated death domain protein and caspase-9 were different between the two systems. Our study highlights an important distinction between cell contact-mediated and secreted vesicle-generated activation-induced cell death and also demonstrates that the type of the secreted vesicles can also modify the cell death route. We propose that besides cell-to-cell interaction-mediated Fas triggering, stimuli induced by secreted vesicles can mediate important additional cell death signals regulating activation-induced cell death under physiological conditions. The Journal of Immunology, 2012, 189: 000–000.
ulation and could be attenuated by anti-Fas neutralizing Ab, providing evidence for functional, preformed Fasl, secreted upon PHA activation (26–29, 32). Following CSup ultracentrifugation, the cytotoxic activity was retained in the pellet, and FasL could be visualized by electron microscopy in structures of 50–200 nm in diameter either in intracellular cellular stores or in the supernatant of the activated cells by Western blot (25, 30, 32).

A functional, soluble form of microvesicle-bound FasL could also be obtained after CD3 engagement (27, 31, 34) or upon stimulation of cells by PHA or PMA plus ionomycin (32).

Upon engagement by Fasl, Fas rapidly recruits the adapter molecule FADD and caspase-8 proenzyme via homologous death domain and death effector domain interactions, forming the death-inducing signaling complex (DISC) leading to caspase activation and ultimately apoptosis. The serine threonine kinase RIP, a death domain-containing binding partner of Fas, was demonstrated as a crucial factor of cell death receptor-induced necrosis (35, 36), and the Fas-induced apoptotic pathway was not affected in RIP-deficient cells (37, 38) unless FasL, was expressed on the membrane of Vsec (39, 40). RIP has also been shown to be important for the Fasl-independent activation of Fas-mediated apoptotic pathway through anokis (41).

The major goal of the present work was to characterize the Vsec-induced cell death pathway as compared with the classical route. In the current study, we compared the morphological appearance and the enzymatic and molecular requirements of cell death induced by anti-Fas–, cross-linked Fasl–, or CSup-mediated signaling. We provide evidence that Vsec are able to mediate both Fas-dependent and Fas-independent cell death. We also demonstrate that besides the Fas-independent nonapoptotic death pathway, vesicles of various origins are also able to induce a unique Fas-dependent apoptotic program that is at least partially FADD-independent but requires RIP. We propose that stimulation by Vsec can provide important cell death signals in physiological conditions.

Materials and Methods

Cells and cell culture

The human T cell lines (Jurkat series), human B cell line, WSU, and mouse lymphoblastoid B cell line, A20, were cultured in RPMI 1640 complete medium supplemented with 10% FCS at 37°C. 5% CO2. The Fas- and Fasl-deficient Jurkat Rapo cell line was stably transfected with pCR3. Fasl and pCR3.Fas plasmids. The Fas-transfected Jurkat Rapo cells, parental and RIP-negative SVT35 Jurkat subclones (a gift of B. Seed, Massachusetts General Hospital, Boston, MA) were recloned for matching Fas expression. The IA3 Jurkat cell line and its FADD–, caspase-8–, or caspase-9– negative subclones are gifts of J. Blenis (Harvard Medical School, Boston, MA). WSU cells were stably transfected with pCR3.Fas plasmid, as previously described (42).

Freshly separated human PBMC and fresh lymphocytes from human tonsils following centrifugation were incubated on Ficoll-Paque at 2000 rpm for 20 min. T cell blasts were obtained by stimulating PBMC for 1 d with 2 μg/ml PHA and for 6 d with 2 ng/ml IL-2 with medium changes every 48 h.

Abs and reagents

Human rFasl, human rTRAIL, and zVAD (pan-caspase inhibitor) were purchased from Alexis. PHA, propidium iodide (PI), RNase A, and the anti-Flag Abs (M2) used for Fasl-cross-linking were from Sigma-Aldrich; anti-RIP Ab was obtained from BD Biosciences; and PE-DX2 anti-Ab was from Milenium Biete. HRP-conjugated anti-rabbit and anti-mouse Abs were from Jackson ImmunoResearch Laboratories; Ab against human Fas (C20) was from Santa Cruz Biotechnology. Anti-Fas (CH11) and agonistic anti-Fas Ab were obtained from Upstate; anti–caspase-8, anti–caspase-9 (5B4), and anti-FADD were from MBL. CellTracker Green was from Invitrogen. Cathepsin D (pepstatin) and cathepsin B inhibitor (CA-074 Me) inhibitor, JNK inhibitor (JNK inhibitor II), and concanamycin A were purchased from Calbiochem; Nec-1, ETGA, poly(ADP-ribose) polymerase inhibitor (3-a minobenzoamide), and NO synthase (NOS) inhibitor (N-nitro-L-arginine) were from Sigma-Aldrich; and TNFR:Fc and neutralizing anti-TRAIL Ab were from Ag, Scientific.

SDS-PAGE and Western blot

Cell pellets were mixed with 2× Laemml buffer and boiled for 5 min at 95°C. Samples were briefly sonicated and boiled again before being subjected to gel electrophoresis, followed by a transfer onto nitrocellulose membranes and immunoblotting with the indicated Abs. Western blot analysis of proteins was performed according to standard protocols as given by the manufacturers.

Flow cytometry

Cells were stained with the PE-DX2 anti-Fas Ab for 20 min on ice. After washing, the surface expression of Fas was analyzed by flow cytometry (FACSCalibur; BD Biosciences) using CellQuest software (BD Biosciences).

Cell death induction

The sensitivity of target cells to cell death was examined after incubating with 20 ng/ml Flag-tagged Fasl plus 1 μg/ml anti-Flag Ab or with different dilutions of CH11 Ab for 24 h at 37°C. Cells were pretreated with inhibitors for 60 min.

A total of 4 × 10⁶ cells/ml Fasl-overexpressing Jurkat Rapo T cell line or PBMC was stimulated with PHA (100 μg/ml) for 5 min. Following the activation, the cells were washed three times and replenished with fresh medium. The CSup was then collected after 2 h. Target cells were triggered with the supernatant of PHA-activated T cells for 24 h, and cell death was determined. Inhibitors were added to the nonactivated cells 1 h before CSup.

Alternatively, CSup from activated T cell were ultracentrifuged. After T cell stimulation with PHA and incubation for 2 h, T cells were centrifuged at 15,000 rpm for 10 min. Supernatant fluids were ultracentrifuged at 70,000 × g for 2 h. For toxicity experiments, supernatants were recovered and 50 μl of the pellet was resuspended in 0.5 ml medium. Both fractions were added to nonactivated target cells to analyze the cytotoxicity.

Different inhibitors, concanamycin A (folimycin) (10 μM), ETGA (200 μM), PMSF (0.3 mM), RIP kinase inhibitor Nec-1 (40 μM), cathepsin B inhibitor CA-074 (50 μM), JNK inhibitor II (1 μM), NOS inhibitor N-nitro-L-arginine (5 μM), and poly(ADP-ribose) polymerase inhibitor 3-aminoobenzoamide (2 mM) were added 30 min before cell death induction.

Coculture

The FasL-overexpressing WSU B cell line (effector cell) was resuspended in CellTracker dye working solution (5 μM). Cells were incubated for 30 min under growth conditions and then were centrifuged. The dye working solution was replaced with fresh medium and incubated for another 30 min at 37°C. Subsequently, cells were washed three times and added to the target cells at the proper ratio. Both cell types were cocultured for 1 d.

After the indicated treatment, the number of dead cells in the Celltrackernegative population was determined by flow cytometry.

Cytotoxic assays

The killing ability of secreted Fasl-containing vesicles from activated T cells, rFasl-M2, CH11 anti-Fas Ab, and Fasl-overexpressing WSU cell line was determined by measuring the sub-G₁ peak and membrane integrity of dead cells by flow cytometric analysis.

Sub-G₁ measurement. DNA fragmentation due to apoptosis was quantified based on sub-G₁ measurement. Cells were fixed in ice-cold 70% ethanol, washed in 38 mM citrate buffer (pH 7.4), and incubated for 20 min in 38 mM citrate supplemented with 50 μg/ml PI and 5 mg/ml RNase A. Cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences), and the proportion of sub-G₁ particles was determined.

Membrane integrity. Total cell death was quantified based on the loss of membrane integrity and the uptake of PI. Cells were stained with PI (10 μg/ ml) before analysis by flow cytometry.

Caspase-3 activity assay. A cell-permeable fluorogenic substrate (Phiphilux) was used to monitor caspase-3 activity in intact cells, according to the manufacturer’s recommendations (Interchim). A total of 0.5 × 10⁶ cells in 0.5 ml was activated for 12 h. Following stimulation, cells were centrifuged and labeled with 40 μl caspase-3 substrate. The data were analyzed by flow cytometry.

Confocal microscopy

We examined cell death morphologically using an Olympus IX81 inverted Laser Scanning Confocal Microscope (Olympus-Europe, Hamburg, Ger-
many) following PI staining (10 μg/ml): original magnification ×60; oil immersion objective; numerical aperture, 1.25.

Statistical analysis

Statistical significance of differences was determined by unpaired Student t or Mann–Whitney tests. The p values are noted as follows: *p < 0.05, **p < 0.01, ***p < 0.001. The error bars represent the SD.

Results

Characterization of CSup-induced cytotoxic activity

To demonstrate the cell death-inducing capacity of CSup, we treated SVT35 Jurkat cells with the supernatants of FasL-overexpressing Jurkat T cells (RapoFasL), supernatants collected from freshly prepared or PHA-activated PBMC, and supernatants from human tonsil cells. Treatment of SVT35 cells with supernatants from all three FasL-carrying cells resulted in significant level of cell death determined by PI uptake and apoptosis based on sub-G1 measurements (Fig. 1A). The level of cell death induced by the supernatants of activated PBMC and RapoFasL cells was comparable, whereas PHA activation of tonsil cells, containing 80–90% lymphocytes, exhibited markedly less cytotoxic activity than PBMC. The fact that the CSup from freshly isolated primary blood cells induced cell death as efficiently as that from FasL-overexpressing cells implicates a potential physiological role of the cell death-inducing capacity of Vsec. To confirm that in our in vitro experimental system cell death was indeed induced by Vsec, as previously reported (25, 30, 32), the supernatant of activated Jurkat cells was ultracentrifuged and the resulted vesicular pellet was recovered. We found that the resuspended pellet was able to induce almost the same level of cell death as the original supernatant. The absence of cytotoxic activity of the supernatant obtained after ultracentrifugation indicated that the released vesicles induced the cell death (Fig. 1B). Next we investigated whether primary cells could also be the targets of CSup-induced cell death. Freshly separated PBMC, 6-d PHA-preactivated PBMC, and fresh tonsil cells were treated with the supernatant of PHA-activated RapoFasL cells. Our results showed that fresh PMBC were relatively resistant to CSup-induced apoptosis induction, as demonstrated by a minor sub-G1 population and limited total cell death (Fig. 1C).

Previous studies showed that preactivation of PBMC for 6 d renders the cells sensitive to anti-Fas stimuli (43). We thus preactivated PBMC for 6 d with PHA and observed that they acquired increased susceptibility to CSup-induced cell death demonstrated by the loss of membrane integrity and the increase of the sub-G1 population. Similar to the preactivated PBMC, freshly isolated human tonsil lymphocytes were also shown to be highly susceptible to RapoFasL-produced supernatants. These results indicated that predominantly quiescent circulating lymphocytes possess the capacity of producing highly cytotoxic CSup, but they are relatively resistant to CSup-induced cell death. Conversely, lymph node cells were highly susceptible to CSup-induced cell death, but had limited capacity to produce cytotoxic CSup. Interestingly, T cell blasts (6-d PHA-preactivated PBMC) showed both features of cytotoxic CSup production and CSup-induced cell death sensitivity (Fig. 1C). As primary cells can act as both effector and target cells in this process, our results underpin the possible physiological significance of CSup-generated cell death signaling. Moreover, the mouse B cell line (A20) was also sensitive to the supernatant produced by human Jurkat T cells (Fig. 1D), suggesting the interspecies cytotoxic effect of CSup.

![FIGURE 1.](http://www.jimmunol.org/) Characterization of culture supernatant-induced cell death. (A) FasL-overexpressing Jurkat rapo T cell line, freshly separated PBMC, or freshly isolated tonsil cells were activated by PHA for 5 min. Cells were replenished with fresh medium, and the supernatant was gathered in 2 h. SVT35 Jurkat cells were incubated for 24 h with the supernatant of different cells. (B) The collected supernatant of PHA-activated FasL-overexpressing Jurkat rapo T cells was ultracentrifuged (UC). Following ultracentrifugation, the pellet (UC pellet) or the supernatant (UC sup) was added to SVT35 Jurkat cells that were incubated for 24 h. A quantity amounting to 50 μl of the pellet was completed with 450 μl medium. (C) Freshly separated PBMC, T cell blasts (6-d preactivated PBMC), or freshly isolated tonsil cells were treated for 24 h with the supernatant of PHA-activated FasL-overexpressing Jurkat rapo T cell line. (D) A20 mouse B cell line was treated for 24 h with the supernatant of PHA-activated, FasL-overexpressing Jurkat rapo T cell line. Cell death was determined by measuring the sub-G1 peak (open bars) and the membrane integrity by PI staining (gray bars). Data represent the average of at least five independent experiments.
CSup induces Fas-dependent apoptosis and Fas-independent nonapoptotic cell death

CSup produced by various types of activated T cells have been shown to kill target cells through the Fas receptor, because antagonistic anti-Fas Ab attenuated the intensity of cell death observed (26–28, 31).

To investigate the requirements of Fas in CSup-induced apoptosis, we treated Fas-deficient Jurkat Rapo and Fas-re-expressing Jurkat Rapo cells with CSup collected from FasL-overexpressing Jurkat cells (Fig. 2A). As positive controls of Fas-induced cell death, cross-linked rFasL and cocultures of target cells with surface FasL-expressing human B cells were used. CSup, cross-linked FasL, and the coculture system were shown to trigger apoptosis in Fas-positive cells only, as measured by sub-G1 detection. The apoptotic character of the cell death was confirmed by demonstration of intensive caspase-3–7 activity in Fas-expressing Jurkat cells upon CSup and rFasL stimuli (data not shown). Thus, CSup-induced apoptosis took place only in the presence of Fas.

As apoptosis was absent in Fas-negative cells, comparable amounts of total cell death in Fas-negative and Fas-positive cells were observed based on the loss of membrane integrity evident only upon CSup treatment. This result indicates that besides transducing Fas-induced apoptosis, CSup generates Fas-independent nonapoptotic cell death, in contrast to FasL or coculture stimuli. The nonapoptotic, Fas-independent pathway could compensate for the lack of Fas-mediated apoptosis upon CSup triggering. Neither a TNFR1-Fc fusion protein nor a neutralizing anti-TRAIL Ab, which, respectively, was able to block TNF-α or TRAIL activity, was able to antagonize with the CSup-induced cell death (data not shown).

Stimulating the cells with the supernatant of freshly prepared PBMC resulted in a similar pattern of cell death demonstrating only nonapoptotic cell death in Fas-negative cells (Fig. 2B).

FIGURE 2. The absence of Fas abolishes the CSup-induced apoptosis, but not the disruption of membrane integrity. FasL-overexpressing Jurkat rapo T cell line (A) or freshly separated PBMC (B) were activated with PHA for 5 min. Cells were replenished with fresh medium, and the supernatants were collected after 2 h. Fas-negative Jurkat Rapo cell line and Fas-overexpressing counterparts, as target cells, were triggered with the supernatants of PHA-activated cells, with rFasL (20 ng/ml) cross-linked with M2 Ab or with surface FasL-expressing WSU B cell line. The level of cell death was determined after 24 h by measuring the sub-G1 peak (open bars) and the PI staining (gray bars). Data represent the average of at least five independent experiments. Statistically significant comparisons are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Enzymatic and molecular characterization of CSup-induced cell death pathways

To study the enzymatic and molecular background of CSup-induced apoptotic and nonapoptotic cell death, we pretreated the Fas-expressing target cells SVT35 Jurkat cell with z-Vad, a pan-caspase inhibitor. The treatment of target cells with 10 μM z-Vad resulted in a ~30% decrease of CSup-induced total cell death and a complete inhibition of CSup-induced apoptosis (Fig. 3A). Similarly, pretreatment of target cells with zVad completely inhibited FasL-triggered apoptosis while allowing nonapoptotic cell death. Concordant with studies of Holler et al. (35), these results indicate that Fas can induce both caspase-dependent and caspase-independent cell death. The difference between the molecular pathways of CSup- and cross-linked FasL-induced apoptosis was demonstrated by the pretreatment of the cells with the cathepsin D inhibitor, pepstatin, which partially but significantly attenuated CSup-induced, but not FasL-induced apoptosis. This result suggests a greater contribution of cathepsin D in CSup signaling than in cell death induced by cross-linked FasL signaling. zVad and pepstatin also exhibited similar inhibitory activity when CSup from PBMC induced cell death. The pan-caspase inhibitor fully blocked apoptosis, but only partially interfered with total cell death. Similarly, the cathepsin D inhibitor significantly decreased apoptosis but not total cell death (Fig. 3B).

In contrast to cathepsin D, the cathepsin B inhibitor did not inhibit cell death induced by either CSup or cross-linked FasL (data not shown). Similar to FasL, CSup could induce cell death in both a caspase-dependent and caspase-independent manner. However, in terms of apoptosis, our results suggest that CSup triggers an enzymatically different Fas-dependent pathway from that of cross-linked FasL, as CSup-induced apoptosis was shown to use the enzymatic activity of cathepsin D, at least in part, whereas the classical FasL-triggered cell death does not. These results underscore the notion that different Fas receptor stimuli are associated with different signaling pathways.

To further analyze the molecular requirements of CSup-induced cell death, we studied the role of the main DISC proteins, FADD, caspase-8, and the initiator caspase of the mitochondria-activated cell death pathway, caspase-9. JA3 Jurkat cells and their FADD-, caspase-8-, or caspase-9-negative subclones with comparable Fas expression were triggered with CSup produced from FasL-overexpressing cells, cross-linked FasL, or coculturing with FasL-expressing cells (Supplemental Fig. 1). In accordance with previously published results, the absence of FADD or caspase-8 abolished apoptosis upon activation by cross-linked FasL or coculture with FasL-expressing cells (Fig. 4A), whereas the absence of caspase-9 showed a weak attenuating effect (Fig. 4A). The role of caspase-9 in Fas-mediated cell death of Jurkat cells upon stimulation with cross-linked FasL has been controversial. Previous studies suggested a partial or dispensable role of caspase-9 in this process (44–46). We found distinct requirements for FADD, caspase-8, and caspase-9 in CSup-triggered cell death associated with Fas-dependent apoptosis. The absence of caspase-8 disrupted apoptosis almost completely, whereas the absence of caspase-9 leads only to a partial inhibition. Notably, FADD deficiency resulted only in a partial inhibition of CSup-generated apoptosis. Although FasL- and CSup-induced apoptosis are both Fas dependent, our results highlight the difference between the action of FasL and CSup not only in terms of distinct enzymatic pathways, but also in terms of the molecular complex involved, shown by partial dependence on FADD on CSup signaling as opposed to a complete requirement for this DISC component in FasL-mediated signaling.
In line with the results on CSup-generated nonapoptotic signals showing Fas independence, membrane integrity was only partially abrogated in the absence of caspase-8, and was maintained in FADD- and caspase-9–deficient cells upon CSup triggering (Fig. 4A, black bars). Different requirements for caspase-8 and FADD in AICD are supported also by previous data indicating less PHA triggering-induced cell death in caspase-8–deficient than in FADD-deficient cells (47). Distinct requirements for FADD (in

**FIGURE 3.** Cathepsin D inhibitor diminishes exclusively CSup-induced apoptosis. SVT35 Jurkat cells were pretreated with 10 µM z-Vad pan-caspase inhibitor or with 50 µM pepstatin, a cathepsin D inhibitor, for 30 min. Following the preincubation, the cells were treated with the supernatant of PHA-activated, FasL-overexpressing Jurkat rapo T cell line (A) or PBMC (B). Data represent the average of at least five independent experiments. Statistically significant comparisons are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 4.** The role of FADD, caspase-8, and caspase-9 in CSup-induced cell death. FasL-overexpressing Jurkat rapo T cell line (A) or PBMC (B) were activated by PHA for 5 min. Cells were replenished with fresh medium, and the supernatants were collected 2 h later. FADD–, caspase-8–, and caspase-9–negative JA3 Jurkat cell line and the positive counterparts were triggered with the supernatants, FasL-M2 (20 ng/ml), or FasL-transfected WSU B cell line expressing FasL on the cell surface. Twenty-four hours later, cell death was determined by measuring the sub-G1 peak (open bars) and the PI staining (gray bars). Data represent the average of at least five independent experiments. (C) FADD–, caspase-8–, and caspase-9–negative JA3 Jurkat cell line and their positive counterparts were triggered with the indicated supernatants or with FasL-M2 (20 ng/ml). After 12 h, the cells were loaded with caspase-3 substrate. The percentage of caspase active population was analyzed with flow cytometry. Data represent the average of three independent experiments. (D) FADD–, caspase-8–, and caspase-9–negative JA3 Jurkat cell line and positive counterparts were pretreated with 50 µM pepstatin for 30 min. Following preincubation, the cells were treated with the supernatant of PHA-activated, FasL-overexpressing Jurkat rapo T cell line. After 24 h, the level of cell death was determined by measuring the sub-G1 peak. Data represent the average of three independent experiments. Statistically significant comparisons are indicated as follows: **p < 0.01, ***p < 0.001.
sub-G₁-detecting death) and caspase-9 (in PI-detecting death) were observed also between cell death induced by CSup released from FasL-overexpressing Jurkat cells and PBMC. Similar to the inhibition of apoptosis by the supernatant of Jurkat cells, the apoptosis process induced by CSup from PBMC was also markedly blocked in both caspase-8- and caspase-9-deficient cells. However, unlike partial inhibition observed in FADD-negative cells in the presence of CSup from FasL-overexpressing cells, apoptosis induced by CSup from PBMC was completely blocked, whereas total cell death was greatly enhanced (Fig. 4B). In terms of membrane integrity, the absence of caspase-8 had no effect on PBMC-derived CSup treatment, whereas FADD deficiency led to a significant increase in cell death when compared with the FADD-positive parental cells. Unexpectedly, the membrane integrity of the target cells was almost fully preserved in caspase-9-deficient cells treated by PBMC-generated CSup (Fig. 4B), but not by CSup derived from FasL-overexpressing Jurkat cells or cross-linked FasL. This finding is of interest, because the pan-caspase inhibitor zVad could only partially reduce the loss of membrane integrity. However, these results altogether indicate the high variety of the outcome and requirements of the death pathways triggered by CSup of various origins.

To confirm that the observed Fas-dependent but FADD-independent cell death measured by sub-G₁ method represents an apoptotic process, we used a quantitative caspase-3 activity assay. Upon CSup stimulation, a small but clearly detectable effector caspase activity was visualized in FADD-negative Jurkat cells. Recombinant FasL activation and CSup generated by PBMC cells resulted in no detectable caspase activity according to the data demonstrated by sub-G₁ measurement (Fig. 4C). In agreement with this result, Fas-dependent but FADD-independent sub-G₁-marked cell death was completely blocked by zVad (data not shown).

Effector caspase activity was also demonstrated in caspase-9-negative cells following CSup or rFasL triggering, but not upon stimulation with supernatants of PBMC (Fig. 4C).

To evaluate whether cathepsin D activity was responsible for mediating FADD-independent apoptosis, we checked the effect of pepstatin in FADD-negative cells following stimulation with the supernatant of PHA-activated Jurkat cells. The inhibition of apoptosis in FADD-deficient cells following incubation by pepstatin indicates that FADD-independent apoptosis is at least partially executed by cathepsin D (Fig. 4D).

**RIP is required for CSup-induced but not for classical Fas-mediated apoptosis**

Previous studies with forced expression of FasL achieved by retroviral transduction showed that RIP is indispensable for membrane FasL-induced apoptosis (40). We investigated whether RIP was required also in CSup-induced cell death. Using RIP-negative Jurkat cells with Fas expression comparable to their parental cells (Supplemental Fig. 2), we found that the absence of RIP disabled CSup-induced apoptosis while leaving FasL-induced apoptosis unaffected (Fig. 5A). Lack of CSup-induced apoptosis in RIP-negative cells was shown to be independent of the ratio of the supernatant (1–1/8 diluted) and time (6–36 h) (data not shown). Whereas CSup could not trigger apoptosis in RIP-negative cells, CSup-induced nonapoptotic cell death was maintained, as a measurable fraction of cells lost their membrane integrity. Given that RIP deficiency did not affect apoptosis induced by cross-linked FasL, RIP emerged as another factor distinguishing CSup- and FasL-mediated cell death pathways. When CSup produced by PBMC was used to induce apoptosis, the contribution of RIP was also observed, albeit to a less extent than CSup derived from FasL-overexpressing Jurkat cells (50 versus 75% inhibition of apoptosis induced by Jurkat cell-produced CSup and 10% inhibition of total cell death with PBMC-produced CSup versus 35% with Jurkat cell-produced CSup [Fig. 5A, 5B]).

In Fas- and RIP-positive cells (Jurkat SVT35), activation by cross-linked FasL and CSup resulted in PI uptake with nuclear fragmentation, whereas in Fas-deficient cells (Jurkat Rapo), FasL treatment did not result in PI uptake, but CSup treatment resulted in PI uptake without nuclear fragmentation (Fig. 5C). This observation was consistent with necrotic morphology and confirmed nonapoptotic cell death, as presented in Fig. 2. In RIP-negative cells (Jurkat SVT35.RIP⁻/⁻), CSup induced less intense cell death with necrotic morphology characterized by nonfragmented nuclei, similar to that observed for Fas-negative cells, whereas cross-linked FasL induced cell death with nuclear fragmentation. These results demonstrate that, similar to the expression of Fas, the presence of RIP maintains the preference of apoptosis over necrosis in CSup-mediated cell death. They demonstrate the unique and essential role of RIP in CSup-induced apoptosis, which is distinct from the common role of RIP as a crucial player in necrosis or necroptosis that can occur with or without death receptor activation (48, 49).

To further clarify the role of RIP in Fas-mediated apoptosis, we also studied cell death of Jurkat cells upon treatments by various stimuli, including cross-linked FasL (Fig. 5D), agonistic anti-Fas Ab (CH11) (Supplemental Fig. 2B), and coculturing target cells with FasL-expressing human B cells (line WSU) (Supplemental Fig. 2C). Our results show that the absence of RIP did not exert significant effects on apoptosis induced by cross-linked FasL (Fig. 5D), agonistic anti-Fas, or coculturing with FasL-expressing WSU cells (Supplemental Fig. 2B, 2C). These results confirmed the dispensable role of RIP in non-CSup–induced Fas-mediated apoptosis.

**Discussion**

In this study, we present a detailed characterization of cell death pathways induced by CSup of various origins. AICD of activated and potentially autoreactive T cells can occur by suicide or by fratricidal execution (2). Besides direct cell-to-cell contact-induced killing, Vsec, called exosomes, can also transduce cytotoxic signals for target cells. Coexpression of Fas and FasL on the surface of a given cell has been shown to cause cell death and is responsible for AICD (1–3). The involvement of FasL–Fas–mediated killing has also been evident in CSup-induced cell death. Experiments using long-term (at least 18-h) cell cultures have shown that AICD of T cells may occur in both a Fas-dependent (1–3) and Fas-independent manner (4–8, 50).

By comparing the cell-to-cell contact and CSup-induced cell deaths, we have shown the following: 1) CSup are able to induce cell death through mechanisms that are substantially different from those induced by the process of death induced by cell-to-cell contact; 2) although both CSup and cell-to-cell contact-mediated stimuli may mediate Fas-dependent apoptosis, CSup-induced apoptosis differs from that induced by cell-surface FasL in the enzymatic and molecular pathways it uses; 3) CSup, but not cell surface FasL, is able to induce Fas-independent, nonapoptotic cell death; 4) our results also indicate that CSup released by different cells (PBMC or Jurkat) confer partially overlapping but also diverse effects; 5) CSup-induced, Fas-mediated apoptosis has unique characteristics that distinguish it from apoptosis triggered by cross-linked FasL, agonistic anti-Fas Ab, or the coculture of target cells with surface FasL-positive cells. CSup-generated apoptosis could be partially blocked by a cathepsin D inhibitor (Fig. 3), showing the
difference between the enzymatic pathways used. It required the presence of RIP (Fig. 5) and was only partially blocked in the absence of FADD when treated with Jurkat cell-produced supernatants, indicating that distinct molecular mechanisms were involved (Fig. 4). Based on the results obtained from experiments in which the target cells were cocultured with FasL-expressing killer cells, we demonstrated that membrane localization of FasL by itself is not sufficient to support RIP-mediated apoptosis. RIP is required only in the regulation of CSup-induced cell death. Functioning necroptosis requires the absence or inhibition of caspases, limiting its ability to be detected in vivo under normal conditions. In consequence, the necroptosis pathway seems to be a backup for apoptosis in the elimination of excess T cells (51). Besides the role of RIP in death receptor-induced necroptotic processes, our results highlight the function of RIP in Fas-mediated apoptosis, activated exclusively upon CSup-induced triggering.

Thus, our results characterized CSup-induced, Fas-mediated apoptosis in detail; however, the exact mechanism of the Fas-independent nonapoptotic cell death pathway awaits further analysis. Considering the requirement of enzymes involved in the apoptotic process, we showed that this nonapoptotic cell death pathway occurs in cells that lack Fas, FADD, caspase-8, and caspase-9 (except when induced by the PBMC-generated supernatant), and also RIP (Figs. 4, 5), caspases, and cathepsin D are not essential for this process (Fig. 3). When cell death was analyzed morphologically, we found that this process did not cause nuclear or DNA fragmentation (Fig. 5). Furthermore, the involvement of other relevant mechanisms such as the perforin-granzyme system could not be demonstrated. Indeed, concanamycin and the calcium chelator EGTA, which are both reported to inhibit perforin-based cytotoxic activity, were unable to block CSup-induced cell death (8, 52) (data not shown). The serine protease inhibitor, PMSF, also had no effect on the cytotoxic capacity of CSup-induced cell death, which excludes the role of granzymes in this nonapoptotic process. Other tested inhibitors, such as RIP kinase inhibitor, cathepsin B inhibitor, JNK inhibitor, NOS inhibitor, and poly(ADP-ribose) polymerase inhibitor, had had no blocking ability (data not shown). The absence of RIP and caspase-8 (when using supernatant from FasL-overexpressing Jurkat cells) decreased only slightly the loss of membrane integrity but did not diminish apoptosis (Figs. 4, 5).

We report in this study that different primary cells can also participate in this cell death pathway either as killer or target cells. Both freshly separated and PHA-preactivated PBMC were effective Vsec-producing cells, whereas lymphocytes separated from tonsils did not show this activity. Interestingly, freshly separated PBMC were almost completely resistant to CSup, but not to preactivated

**FIGURE 5.** The absence of RIP inhibits CSup-induced apoptosis. FasL-overexpressing Jurkat rapo T cell line (A) or freshy separated PBMC (B) were activated with PHA for 5 min. RIP-negative SVT35 Jurkat cell line and the positive counterpart were triggered with supernatants of PHA-activated T cell lines or with the rFasL (20 ng/ml) cross-linked with M2. Twenty-four hours later, cell death was determined by measuring the sub-G₁ peak (open bars) and the PI staining (gray bars). Data represent the average of at least eight independent experiments. (C) Wild-type, Fas-deficient, and RIP-negative Jurkat cells were treated with the supernatants of PHA-activated, FasL-overexpressing Jurkat cell or with FasL-M2. Upon PI staining, the fluorescence was visualized by confocal microscopy. A representative image from at least five independent experiments is shown. Original magnification ×60. (D) The cell death of RIP-negative Jurkat cell line and the positive counterpart were investigated upon stimulation with different doses of rFasL cross-linked with M2. After 24 h, cell death was determined by measuring the sub-G₁ peak. Data represent the average of six independent experiments. Statistically significant comparisons are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.
mediated Fas triggering, CSup-induced stimuli may be an im-

topectosis generated by Vsec differs at the levels of the enzymatic

and the methods by which the vesicles are produced. (40), indicating that the requirement for RIP is a general property

inhibitory effects (28, 31). In this system, the Fas-dependent

preactivation of the vesicle-producing cells observed only partial

the complete blockage of cell death by antagonistic anti-Fas Ab

and the immune system may vary extensively. Description

newly discovered cell death pathways in the fight against cancer.

is reasonable to assume that the vesicles produced by PBMC and

vesicles of different composition have already been shown (53), it

Jurkat cells and PBMC, which express comparable amounts of

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References

independent or Fas-dependent.

proposed cell death pathways in the fight against cancer. Our

preactivation of the vesicle-producing cells observed only partial


be an important cell death signal in physiological circumstances.

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Functional complementation between FADD and RIP1 in embryos and


Supp Figure 1
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Supplementary figure legends

Figure S1.
Comparable surface expression of Fas on different cell lines was verified by FACS analysis. The absence of the indicated molecules were visualized by western blotting of total cell lysates.

Figure S2.
A. Flow cytometry analysis was performed to ensure that cell surface expression of Fas was equivalent in the control and RIP-deficient cell line. RIP deficiency of the investigated cell line was verified by Western blotting of total cell lysates.

B. The cell death of RIP-negative Jurkat cell line and the positive counterpart were investigated upon stimulation with different doses of CH11 Fas antibody. After 24 hours the cell death was determined by measuring the subG1 peak. Data represent the average of six independent experiments

C. Different ratios of FasL-expressing WSU human B cell line as effectors cells were cocultured with the RIP-positive and negative cell pair as target cells. After 24 hours cell death was determined by measuring the subG1 peak. The figures show representative experiments from at least five independent experiments.