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T Cell Receptor Ligation Triggers Novel Nonapoptotic Cell Death Pathways That Are Fas-Independent or Fas-Dependent 1

Wendy F. Davidson, 2*‡ Christian Haudenschild, † Jaeyul Kwon,* and Mark S. Williams*‡

Short-term culture of activated T cells with IL-2 renders them highly susceptible to apoptotic death triggered by TCR cross-linking. Activation-induced apoptosis is contingent upon caspase activation and this is mediated primarily by Fas/Fas ligand (FasL) interactions that, in turn, are optimized by p38 mitogen-activated protein kinase (MAPK)-regulated signals. Although T cells from mice bearing mutations in Fas (lpr) or FasL (gld) are more resistant to activation-induced cell death (AICD) than normal T cells, a significant proportion of CD8+ T cells and to a lesser extent CD4+ T cells from mutant mice die after TCR religation. Little is known about this Fas-independent death process. In this study, we demonstrate that AICD in lpr with 18 U.S.C. Section 1734 solely to indicate this fact.

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In some situations, nonaccidental, receptor-induced death may occur by a process that differs from apoptosis and involves early loss of membrane integrity, membrane blebbing, and cell swelling rather than cell shrinkage. Nuclei initially are preserved and later undergo karyolysis rather than karyorhexis. The DNA breaks down by a process that is caspase-independent and does not involve internucleosomal fragmentation. The terms oncosis, primary necrosis, and paraptosis have been coined to describe death with all or most of these features (10, 18–21). Importantly, in the presence of broad-spectrum caspase inhibitors or when effector caspases are inherently absent, death in lymphoid and nonlymphoid cells switches from apoptosis to a process with features of oncosis/primary necrosis (22–28). Thus, oncosis/primary necrosis may be a rudimentary death process available in all cells that under normal circumstances is overridden by death programs that lead to caspase activation and apoptotic death (29). Because onotic death can culminate in the release of intracellular contents, it can be proinflammatory and potentially autostimulatory especially if the cellular corpses are not efficiently phagocytosed.

It is firmly established that TCR-triggered AICD in CD4+ and CD8+ T cells is mediated in large part by Fas/Fas ligand (FasL)-mediated signals (1, 2). The preeminence of the Fas death pathway in immune homeostasis is illustrated by the massive accumulation of T cells and the development of systemic autoimmunity in mice and humans deficient in Fas or FasL (30–32). Predictably, activated CD4+ and CD8+ T cells from Fas-mutant (lpr) and FasL-mutant (gld) mice are more resistant to AICD than equivalent wild-type (WT) populations. Although reduced, death is still evident, particularly among CD8+ T cells (33, 34). The timing of propriocidal cell death and the nature of the death signals in lpr and gld CD8+ T cells are controversial. In one report, lpr and gld CD8+ T cells exhibited delayed TNF-α-dependent AICD 48 h post restimulation, similar to WT CD8+ T cells (33). In another study, a significant proportion of lpr CD8+ T cells died after overnight restimulation, suggesting that Fas- and TNF-independent...
AICD pathways may be triggered in hyperstimulated lpr and gld T cells in vitro (34). The mode of death (apoptosis vs oncasis/ne- crosis) was not determined in either report. In the present study, we further investigated the kinetics, signal requirements, and manner of propionic death in lpr and gld CD4+ and CD8+ T cells. Using short-term cultures of isolated CD8+ and CD4+ T cells from lpr and gld mice and overnight restimulation, we established that early, TNF-independent death occurs in both subsets with CD8+ T cells showing greater sensitivity. Importantly, the majority of lpr and gld CD4+ and CD8+ T cells reproducibly died by a process different from classical apoptosis and with morphologic features of oncasis/primary necrosis. We further characterized this novel death process and showed that it is caspase- and p38 mito- 

We checked by FACS and generally was >95%. Cells were cultured in complete RPMI 1640 containing 10% FCS (35).

Generation of T cell blasts

Plates (24-well) were coated with 15 μg/ml anti-TCRαβ mAb (H57-597) in PBS for 3 h at 37°C and then washed twice with PBS. In some experiments, plates were coated with 15 μg/ml anti-TCRαβ mAb and 10 μg/ml anti-CD28 mAb (37,51). Unfractionated LN cells, CD4+ and CD8+ T cell populations (2 x 10^5/well in 2 ml) were added to coated wells and cultured in a CO2 incubator for 48 h. The activated T cells were harvested and the populations were expanded for 2-4 days in medium containing 10 U/ml rIL-2 (BD PharMingen, San Diego, CA) or a 1:30 dilution of medium from an IL-2-transfected mycoplasma free cell line (a gift of Dr. D. Scott, Department of Immunology, Holland Laboratory, American Red Cross). Both sources of IL-2 supported T cell growth equally and gave comparable results in AICD assays.

AICD assay

For AICD assays, activated T cells cultured short-term in IL-2 were adjusted to 5 x 10^6/ml in IL-2-containing medium and restimulated for various times in 24- or 48-well plates coated with 15 μg/ml anti-TCRαβ mAb alone or in combination with 10 μg/ml anti-CD28 mAb. Unstimulated controls were cultured at the same cell concentration in IL-2 in uncoated wells. Various inhibitors were added to AICD assays at the indicated concentrations at the time of setup.

**TNF-α cytotoxicity assay**

L-929 cells (a gift of Dr. T. Torrey, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) were preincubated (2.5 x 10^5/well) in 24-well plates at 37°C in a CO2 incubator for 1 h in complete medium containing 2 μg/ml actinomycin D (Sigma-Aldrich, St. Louis, MO). An equal volume of medium, medium plus rTNF-α (BD Pharamingen) or medium with TNF-α and anti-TNF-α Ab (recombinant or polyclonal) was added and the cells were incubated overnight. The concentration of actinomycin D was maintained at 2 μg/ml throughout the assay. TNF-α and anti-TNF-α Ab were titrated and mixtures of TNF-α and anti-TNF-α Ab were precultured on ice for 1 h before addition to the cells. After overnight culture, the nonadherent cells were harvested and the adherent cells were removed by trypsin treatment. Pooled adherent and nonadherent cells were assayed by propidium iodide (PI) staining and FACS for the proportions of dead cells.

**Inhibitors**

Inhibitors included the broad spectrum caspase inhibitors BOC-Asp(OMe)-FMK (BD-fmk) and Z-VAD(OMe)-FMK (zVAD-fmk) and the negative control inhibitor Z-Phe-Ala-fmk (Enzyme Systems Products, Livermore, CA), the superoxide dismutase mimetic and peroxynitrite scavenger, Murild tetraakis(4-hydroxyphenyl)chloro (MurTAP; Calbiochem, San Diego, CA), the serine protease inhibitor 4-(2-aminoethyl)benzene-sulfonylfluoride (AEBSF), HCl (Calbiochem), and the p38 MAPK inhibitor, SB203580 (Calbiochem). All inhibitors were dissolved according to the manufacturer’s instructions and were titrated before use to determine the range of concentrations yielding maximal inhibition and minimal cytotoxicity. In all experiments, inhibitors were added to control and restimulated cell cultures. Other inhibitors included neutralizing anti-TNF-α mAb (BD Pharamingen), neutralizing goat anti-mouse TNF-α Ab (R&D Systems, Minneapolis, MN), blocking anti-FasL mAb (MFL3), (BD Pharamingen), cross-linked recombinant FasFc (Alexis, San Diego, CA), N-acetyl-L-cysteine (NAC; Sigma-Aldrich) and butylated hydroxyanisole (BHA; Sigma-Aldrich).

Cell death assays

**FACS.** Following restimulation, T cells were harvested and washed once in FACS buffer (1x balanced salt solution containing 0.2% BSA and 0.05% sodium azide). For enumeration of PI-positive cells, cell pellets were resuspended in FACS buffer containing 5 μg/ml PI (Sigma-Aldrich) and were analyzed on a FACSCalibur (BD Immunocytometry Systems, San Jose, CA). For enumeration of hypodiploid cells, cell pellets were resuspended in 0.25 ml of PBS containing 20 μg/ml PI, 0.3% saponin (Sigma-Aldrich), 5 mM EDTA, and 50 μg/ml RNase A (Sigma-Aldrich), incubated at room temperature for 30 min then analyzed by FACS for relative DNA content.

**UV microscopy.** The proportions of apoptotic and oncocytic/necrotic cells were determined by UV microscopy (36). Cell pellets were resuspended in 0.5 ml of FACS buffer containing 5 μg/ml Hoechst no. 33342 (Sigma-Aldrich) and were incubated at 37°C in a CO2 incubator for 10 min. Ten microliters of a 1 mg/ml solution of PI were added to each tube and the cells were pelleted after 1-2 min. All of the supernatant was removed and 15 μl of FACS buffer was added. Tubes were stored on ice protected from light and pellets were resuspended before counting. Nuclei from a minimum of 300 cells in a number of fields were examined using a 100x oil immersion objective and filters for Hoechst dye, and scored as viable, apoptotic, or oncocytic/necrotic based on their nuclear color and morphology. Cells were scored as viable if the nuclei were bright blue and showed no evidence of chromatin condensation. Cells with nuclei showing evidence of shrinkage, chromatin condensation, or fragmentation were scored as apoptotic. Both blue (PI-') and red (PI+) apoptotic nuclei were included in the total count. Cells were classified as oncocytic/necrotic if the nuclei were PI- and morphologically similar to the nuclei in viable cells. The classification of cells as oncocytic was verified by switching from UV to phase microscopy. In contrast to viable cells, oncocytic cells exhibited characteristic cytoplasmic swelling and blebbing of the plasma membrane. A third type of death process termed atypical apoptosis was observed in some reactivated CHL- 

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Data analysis

All AIDC death data are presented as mean net values for three or more experiments. Δ percent dead cells (% dead cells in restimulated culture) = (% dead cells in untreated control culture). In control cultures of all populations studied, spontaneous apoptotic death was generally <10% and oncosic death was <5%. In experiments with inhibitors, the percentage of dead cells in unstimulated control cells cultured in the presence of inhibitor was subtracted instead of background death.

Measurement of caspase activity

BALB-+/+, CD4+, and CD8+ T cell blasts were generated as described above and restimulated on anti-TCRαβ mAb-coated plates in the presence of IL-2 with or without 60 μM BD-fmk. Unstimulated cells cultured in medium with IL-2 + 60 μM BD-fmk served as controls. After 2, 4, 6, and 8 h of culture, the cells were harvested and lysed in buffer containing 100 mM HEPES, 10% sucrose, 0.1% Triton X-100, 10 mM DTT, and 1× protease inhibitor mixture (Sigma-Aldrich) (100 μl of buffer/1 × 10^6 cells) for 20 min. Lysates were cleared by centrifugation at 14,000 × g for 10 min. Caspase 3 activity was determined using a caspase 3 assay kit (BD Pharmingen) according to the manufacturer’s protocol. Lysates were titrated in a final volume of 250 μl of lysis buffer in opaque multwell plates. The amount of AMC released from the Ac-DEVD-AMC substrate was assayed and then incubated with anti-CD3 mAb (10^7/ml) on ice for 30 min in the presence or absence of the p38 MAPK inhibitor SB203580 (10 μM). For cells treated with SB203580, the inhibitor was present throughout the protocol. Cells were washed and resuspended at 1 × 10^6/ml in cold serum-free medium. At various intervals, aliquots of cells were added into tubes maintained at 37°C containing medium with 5 μg/ml rabbit anti-hamster (to cross-link anti-CD3) with or without SB203580 (10 μM). Incubation was terminated by 10-fold dilution with cold PBS containing 2 mM EGTA. Cell pellets were lysed in lysis buffer (20 mM HEPES, 10% sucrose, 0.1% Triton X-100, 10 mM DTT, 1 mM NaVO_4, 10 μg/ml aprotinin and leupeptin, and 1 mM AEBSF) for 20 min on ice. Lysates were cleared by centrifugation (14,000 × g for 10 min). Lysates were boiled in 5× Laemmli reducing sample buffer, resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBS containing 0.1% Tween 20, probed with anti-phospho-p38 MAPK or pan-anti-p38 MAPK (Cell Signaling Technology, Beverly, MA) Ab followed by HRP-conjugated secondary Ab and developed by ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

Electron microscopy

Cells were fixed, processed, and sectioned as described in detail previously (37). Briefly, −1 × 10^7 cells were resuspended rapidly in 10 ml of buffered 4% formaldehyde/1% glutaraldehyde fixative at room temperature. The cells were centrifuged and the pellets postfixed with 1% aqueous osmium tetroxide, stained en bloc with 0.5% uranyl acetate, dehydrated through graded alcohols and embedded in Epon 812. Toluen blue-stained sections perpendicular to the surface of the pellet were taken for light microscopic selection of the most representative fields for subsequent electron microscopy. Adjacent ultra thin sections were stained with uranyl acetate and lead citrate and examined with a Philips C12 transmission electron microscope (Eindhoven, The Netherlands).

Results

The majority of reactivated lpr and gld T cells die by a nonapoptotic process

In preliminary studies, AIDC was examined in unfractionated LN cells from 4- to 20-wk-old C3H-WT, C3H-lpr, C3H-gld, BALB-WT, and BALB-gld mice. Data for 8- and 20-wk-old mice summarized in Fig. 1 show that WT T cells died primarily by classical apoptosis whereas lpr and gld T cells died either by atypical apoptosis or more frequently by a mechanism with the morphologic features of oncisis/primary necrosis (henceforth abbreviated to oncisis). The ratios of atypical apoptotic-oncotic cells in C3H-lpr and -gld T cells ranged from 1:1 to 1:2 whereas BALB-gld cells died almost exclusively by oncisis (Fig. 1, A, C, and D, and data not shown). This switch in death mode was not an age-related phenomenon as similar results were obtained with 2- and 5-mo-old mice (Fig. 1). The induction of oncotic rather than apoptotic death in reactivated lpr and gld T blasts could not be explained simply by deficits in TCR signal strength because the death process was not altered by the addition of anti-CD28 mAb at the priming or restimulation stages of the AIDC assays or by priming with PMA and ionomycin (data not shown). The length of time that the T blasts were maintained in IL-2 also was not a factor in determining the mode of death as lpr and gld T cell populations expanded in culture for 2 up to 7 days were equally susceptible to oncotic death following restimulation (data not shown).

To determine the relative sensitivity of WT, lpr, and gld CD4+ and CD8+ T cells to death by oncisis, isolated subsets were primed and reactivated as described for the LN cells. Data for BALB-WT and BALB-gld T cells are summarized in Fig. 2. Consistent with previous reports, WT CD4+ T cells were highly susceptible to AIDC and died almost exclusively by apoptosis (Fig. 2, A and C). Although gld CD4+ T cells were highly resistant to activation-induced apoptosis, a significant proportion (15-20%) consistently died by oncisis (Fig. 2D). As shown previously (34, 38), WT CD8+ T cells were more resistant to AIDC than WT CD4+ T cells (Fig. 2B). Notably, in contrast to CD4+ T cells, death in CD8+ T cells was not exclusively apoptotic. A small but reproducible proportion (5-10%) of WT CD8+ T cells died by a process involving minimal visible changes in nuclear morphology and cytoplasmic changes consistent with oncisis (Fig. 2D). This was not a strain-related phenomenon as a similar proportion of oncotic cells was observed in reactivated C3H-WT CD8+ T cells (data not shown). BALB-gld CD8+ T cells also were susceptible...
primed and restimulated as described in Fig. 1.

A

Without the addition of fresh medium, the proportions of cultures were supplemented with fresh IL-2-containing medium. gld T cells, CD8<sup>+</sup> cells were more resistant to AICD than CD4<sup>+</sup> (data not shown).

In contrast to apoptotic cells, oncotic cells were TUNEL-negative. Proportions of oncotic/necrotic cells. Data represent the mean ± SEM for net cell death values for 14–18 separate experiments.

FIGURE 2. gld CD8<sup>+</sup> T cells are more susceptible to activation-induced oncosis/necrosis than gld CD4<sup>+</sup> T cells. Isolated BALB-WT CD4<sup>+</sup> and CD8<sup>+</sup> T cells (■) and BALB-gld CD4<sup>+</sup> and CD8<sup>+</sup> T cells (■) were primed and restimulated as described in Fig. 1. A, Proportions of hypodiploid, apoptotic cells. B, Proportions of total dead (PI<sup>+</sup>) cells determined by FACS. C, Proportions of apoptotic cells determined by UV microscopy. D, Proportions of oncosis/necrotic cells. Data represent the mean ± SEM for net cell death values for 14–18 separate experiments.

to AICD although less so than WT CD8<sup>+</sup> T cells and they died almost exclusively by oncosis (Fig. 2). Among BALB-gld, C3H-gld, and C3H-lpr T cells, CD8<sup>+</sup> T cells were consistently more susceptible to AICD than CD4<sup>+</sup> T cells (Fig. 2 and data not shown).

In contrast to apoptotic cells, oncotic cells were TUNEL-negative (data not shown).

Notably, although lpr and gld CD4<sup>+</sup> and CD8<sup>+</sup> T cells were resistant to activation-induced apoptosis, the machinery for undergoing apoptotic death was intact in these cells as they died by classical apoptosis in response to heat shock and a range of doses of gamma-irradiation and were equally as sensitive to these insults as WT T cells (data not shown). Our results with irradiated lpr lymphocytes are consistent with a report from Newton and Strasser (39) but differ from studies by Reap et al. (40) who observed that lpr lymphocytes were more resistant to heat shock and irradiation than WT cells. These disparities in susceptibility to stress may result from variations among mouse strains or differences in methodologies.

Death by oncosis is delayed and TNF-independent

Time course studies of cell death in AICD assays with BALB WT and BALB-gld CD8<sup>+</sup> T cells revealed that oncotic death occurred more slowly than apoptotic death. Oncotic death in gld cells was first detectable microscopically and by FACS at 6–8 h and peaked between 14 and 16 h post restimulation whereas apoptotic death in WT cells was detectable at 2 h and was mostly complete by 12 h (W. F. Davidson, unpublished observation). Extension of death assays to 48 h post restimulation did not significantly increase the proportion of PI<sup>+</sup> or oncosis WT or gld T cells provided that the cultures were supplemented with fresh IL-2-containing medium (1:1). Without the addition of fresh medium, the proportions of apoptotic cells increased in the control and restimulated WT and gld cultures presumably as a result of depletion of nutrients and IL-2 as well as pH changes. The WT and gld CD8<sup>+</sup> T cells surviving overnight or 48 h restimulation continued to proliferate vigorously in the presence of IL-2 indicating that most were not subject to activation-induced growth arrest or delayed death. FACS analyses of nuclear DNA content also showed no evidence of G1/G0 arrest.

To determine whether oncotic death was induced by TNF-α, two neutralizing anti-TNF-α mAb (one monoclonal and the other polyclonal) were titrated into AICD assays. Neither oncotic death in lpr and gld CD4<sup>+</sup> and CD8<sup>+</sup> T cells nor apoptotic death in WT CD4<sup>+</sup> and CD8<sup>+</sup> T cells was inhibited significantly by either Ab. Fig. 3A shows representative data for WT and gld CD8<sup>+</sup> T cells and anti-TNF-α mAb. In contrast, over the same concentration range, anti-FasL mAb significantly inhibited apoptosis in WT CD8<sup>+</sup> T cells (Fig. 3A). To ensure that both anti-TNF-α Ab were biologically active, each was titrated into cultures of L-929 cells treated with various amounts of TNF-α. As shown in Fig. 3B, 5 μg/ml anti-TNF-α mAb significantly inhibited death over the complete range of concentrations of TNF-α (50–1000 pg/ml). Similar results were obtained with the polyclonal Ab (data not shown). Because TNF-α-induced death in L-929 cells is oncotic (41), both Abs clearly are able to efficiently inhibit oncosis mediated by TNF-α. In unpublished studies, we observed that the amount of TNF-α secreted by activated BALB-gld CD4<sup>+</sup> and CD8<sup>+</sup> T cells ranged from 200–800 pg/ml (mean 428 ± 101) and therefore was well within the range efficiently neutralized by 5 μg/ml anti-TNF-α mAb.

Electron microscopy studies confirm an alternative death process in gld CD8<sup>+</sup> T blasts

Fig. 4 illustrates the morphologic differences between apoptotic and oncotic CD8<sup>+</sup> T cells. Similar data were obtained for CD4<sup>+</sup> T cells. The cells shown are representative of the phenotypes seen.
most frequently in a light microscopic field containing a cross-section of all cells of the respective experiment. Control WT and gld CD8⁺ T cell blasts cycling in IL-2 were indistinguishable and consistently showed an intact plasma membrane with slender extrusions and a moderately electron dense cytoplasm containing normal organelles and no vacuoles (Fig. 4A). Their nuclei were large, with intact, slightly wrinkled membranes and a highly structured chromatin pattern displaying small dense specks evenly dispersed in flocculent light chromatin, and a thin rim of membrane-associated dense chromatin. Nucleoli were prominent and showed a typical distinct internal structure. Classical apoptotic cells resulting from restimulation of wild-type cells (Fig. 4B) showed the expected features including shrinkage, plasma membrane blebbing, cytoplasmic condensation, and condensation and fragmentation of the chromatin into rounded apoptotic bodies without an internal structure. The strikingly different morphology of activation-induced oncotic death in restimulated FasL-deficient (gld) T cells is shown in Fig. 4, C (early stage) and D (later stage). In sharp contrast to classical apoptotic cells, gld CD8⁺ T cells were swollen and contained cytoplasmic vacuoles and swollen mitochondria, while the nuclei showed only slight deviations from those of viable cells (Fig. 4A). This near normal nuclear morphology persisted even in more advanced stages of the oncotic death process, when the swelling was extreme and the cytoplasm appeared almost dissolved (Fig. 4D). Nuclei that were totally devoid of cytoplasm, but with a similarly preserved ultrastructure, also were observed in these preparations. We confirmed that the dense areas within the nucleus were nucleoli and not condensed DNA chromatin by light microscopic examination of cells stained for RNA with methyl green-pyronin (data not shown).

Oncosis in lpr and gld T cells is caspase-independent

To determine the role of caspases in oncotic death, two broad-spectrum caspase inhibitors, zVAD-fmk and BD-fmk were added to AICD assays with WT, lpr, and gld CD4⁺ and CD8⁺ T cells. Over a range of nontoxic concentrations (7.5–60 μM), neither inhibitor caused a significant reduction in the death of the lpr or gld CD4⁺ or CD8⁺ T cells (Fig. 5, A–C, and data not shown). By comparison, both zVAD-fmk and BD-fmk had a significant dose-dependent effect on the level and mode of AICD in BALB-WT CD4⁺ and CD8⁺ T cells. Fig. 6 summarizes data obtained with BD-fmk at 60 μM, a maximally effective but nontoxic dose. Caspase inhibitors completely inhibited apoptosis in both CD4⁺ and CD8⁺ T cell populations (Fig. 6, A and C). Depending on the method of measurement (FACS vs microscopy), BD-fmk rescued, on average, 50–63% of CD4⁺ T cells and 33–50% of CD8⁺ T cells from death (Fig. 6, B and D). These data imply that activation-induced death is more dependent on caspase activation in WT CD4⁺ T cells than in CD8⁺ T cells and that in both subsets, death can be signaled upstream of caspase activation in some cells. Death was not merely delayed as the surviving CD4⁺ and CD8⁺ T cells continued to proliferate vigorously for days in IL-2-containing medium (data not shown). To ensure that 60 μM BD-fmk was efficiently inhibiting effector caspases, caspase 3 activity was tested in lysates from cells restimulated for 4, 6, and 8 h. At all time points, effector caspase activity was completely inhibited in CD4⁺ and CD8⁺ T cells. Representative data for lysates at the 4-h time point are shown in Fig. 7A. In addition, blocking of initiator caspase activity in cells by BD-fmk was confirmed by FACS using an intracellular fluorescent tag for activated initiator caspases (data

FIGURE 4. Ultrastructure of apoptotic and oncotic cell death in CD8⁺ T cells from BALB WT and BALB-gld mice. Cells were primed, rested, and restimulated as described in Fig. 1, A. The primed, but not restimulated, WT T cell shows the typical appearance of an intact lymphoblast (see text). B, The restimulated WT T cells display the classical features of apoptotic cell death such as total fragmentation and condensation of chromatin, membrane blebbing, and cell shrinkage. C and D, Early and late stages, respectively, of caspase-independent oncotic death in restimulated gld T cells. Note the rounded nuclear shape with preserved preservation of the chromatin pattern and nucleolus, and the marked progressive cytoplasmic and mitochondrial swelling with appearance of vacuoles. E and F, Early and late stages, respectively, of the different variation of oncotic death seen in WT cells restimulated in the presence of the caspase inhibitor BD-fmk. Although the cytoplasmic and mitochondrial swelling as well as the persistence of a distinct nucleolus are similar to what is seen in C and D, the chromatin shows a greater degree of clumping, but without the appearance of multiple dense chromatin bodies as in B. (Magnification, ×1800)
Protease inhibitor, AEBSF, did not inhibit oncotic death (data not shown). In other studies, we showed that the inhibitor, Z-Phe-Ala-fmk, that inhibits cathepsin B but not caspases, had no effect on oncotic death in lpr, gld, or WT T cells. Similarly, the serine protease inhibitor, AEBSF, did not inhibit oncotic death (data not shown).

In WT CD4^+ and CD8^+ T cells, AICD induced in the presence of BD-fmk was nonapoptotic and shared a number of features with oncotic death pathways in gld T cells but are not essential for CID in WT T cells.

FIGURE 5. Oncotic/necrotic death in gld CD4^+ and CD8^+ T cells is caspase-independent. AICD assays were performed in the presence or absence of the broad-spectrum caspase inhibitor, BD-fmk. A. Proportions of oncotic gld CD8^+ T cells following treatment with a range of concentrations of BD-fmk. B and C. The proportions of PI^+ and oncotic gld CD8^+ and CD4^+ T cells after treatment with 60 μM BD-fmk. Values represent mean ± SEM for three experiments.

FIGURE 6. Broad-spectrum caspase inhibitors rescue a proportion of WT CD4^+ and CD8^+ T cells from AICD and induce a switch from apoptotic to oncotic death in the remainder. BALB-WT CD4^+ and CD8^+ T cell blasts were restimulated overnight with immobilized anti-TCRα mAb in the presence or absence of 60 μM BD-fmk. A. Proportions of hypodiploid, apoptotic cells. B. Proportions of total dead (PI^+) cells. C and D. Proportions of apoptotic cells and oncotic cells. Values represent the mean ± SEM for net cell death data from 10 separate experiments.

Consistent with our findings with lpr and gld T cells, caspase-independent oncotic death in WT CD4^+ and CD8^+ T cells is not dependent on TNF-α. This was shown using neutralizing anti-TNF-α Ab and also by performing AICD assays with CD4^+ and CD8^+ T cells from TNFR1-deficient mice. As shown in Fig. 3C, reactivated TNFR1-deficient CD4^+ and CD8^+ T cells switched from apoptotic to oncotic death with the same efficiency as WT T cells in the presence of BD-fmk.

Reactive oxygen species (ROS) contribute to activation-induced oncrosis in gld, but not WT, T cells

There is evidence that ROS can be a contributing factor in death receptor-mediated and superantigen-induced death (42–45). To investigate the role of ROS in oncotic death, the antioxidants NAC, BHA, and the superoxide dismutase mimetic, Mn-TBAP, were titrated into overnight AICD assays. Over a range of concentrations, NAC and BHA had no effect on cell death in WT, lpr, or gld CD4^+ or CD8^+ T cells (data not shown). At high, but noncytotoxic concentrations (200–400 μM), Mn-TBAP consistently caused a significant decrease in both the proportions of PI^+ (not shown) and oncotic gld CD4^+ T cells (Fig. 8). Less efficient, but significant, inhibition of oncotic death also was observed in gld CD8^+ T cells with 400 μM MnTBAP (Fig. 8). By comparison, Mn-TBAP did not protect WT T cells from activation-induced apoptosis and also had no significant effect on activation-induced oncrosis in WT CD8^+ T cells or caspase inhibitor-induced non-apoptotic death in WT CD4^+ or CD8^+ T cells (Fig. 8 and data not shown). These data suggest that O_2^- /H_2O_2 may contribute to oncotic death pathways in gld T cells but are not essential for CID in WT T cells.
Caspase activity in lysates from restimulated T cells. B, Proportions of oncotic cells were determined. Values represent the mean ± SEM for net cell death values from three experiments.

FIGURE 7. Evidence that inhibitors of caspase and p38 MAPK activation are biologically active. A, Caspase 3 activity in cell lysates from WT CD4+/T cells and CD8+/T cells was determined as described in Fig. 1. Data represent the mean ± SEM for net cell death values from three experiments. B, Immunoblot showing the efficient inhibition of phosphorylation of p38 MAPK by SB203580 in WT CD8+ T blasts before and after restimulation by TCR cross-linking. The corresponding levels of total p38 are shown underneath.

Oncosis in gld T cells and WT CD8+ T cells is p38 MAPK-independent

Signal transduction by p38 MAPK plays a key role in activation-induced death in T hybridomas and normal splenic T cells reportedly by up-regulation of FasL expression and Fas-dependent caspase activation (46, 47). To determine whether p38 MAPK also contributes to the induction of oncotic death, AICD assays were performed in the presence of various, noncytotoxic concentrations of the p38 MAPK-specific inhibitor, SB203580. In preliminary experiments with WT CD4+ and CD8+ T cells, SB203580 very efficiently inhibited constitutive and induced p38 MAPK phosphorylation (Fig. 7B, data not shown). At 25 μM, a concentration established to significantly inhibit apoptosis in WT CD8+ T cells, SB203580 had no detectable effect on oncosis induction in gld CD4+ or CD8+ T cells implying that p38 MAPK signals are not essential for sensitizing cells to this death process (Figs. 9 and 10A). Consistent with published data from unfractionated splenic T cells (46, 47), SB203850 significantly inhibited activation-induced apoptosis in WT CD4+ and CD8+ T cells. Data are shown for percent apoptotic cells enumerated by UV microscopy (Fig. 10C). Identical results were obtained by FACS analysis for hypodiploid cells (data not shown). Inhibition of apoptosis was consistently greater in the CD8+ T cells than the CD4+ T cells although the net decrease in the proportions of PI+ cells was equivalent in both populations. The difference between the degree of inhibition of apoptotic vs total death in the CD8+ T cells resulted from a >2-fold, and highly significant, increase in the proportions of oncotic cells (Fig. 10D). In the absence of SB203580, the average ratio of apoptotic:constrictive CD8+ T cells following restimulation was ~5:1 whereas in the presence of the inhibitor the ratio changed to ~1:1. The shift to oncotic death was not the result of toxicity because 25 μM SB203580 did not appreciably affect the viability of control CD4+ or CD8+ T cells (data not shown). In contrast to its effects on CD8+ T cells, SB203580 induced an apoptosis to oncosis switch in only a small proportion (~5%) of restimulated CD4+ T cells and apoptosis remained the primary mode of death. These findings with WT cells indicate that p38 MAPK signals are necessary for efficient activation of caspases and apoptotic death in both CD4+ and CD8+ T cells. They also provide further evidence that when Fas/FasL interactions are blocked or inefficient, TCR ligation triggers nonapoptotic death more readily in CD8+ T cells than CD4+ T cells.

To determine whether p38 MAPK-dependent signals are necessary for CID in WT cells, CD4+ and CD8+ T blasts were restimulated in the presence of SB203580 and BD-fmk. As shown in Fig. 10, B and D, 25 μM SB203580 significantly inhibited CID in CD4+ T cells as illustrated by the decrease in the proportions of
both PI<sup>+</sup> cells and oncotic cells. Thus, p38 MAPK is an important amplifier of both apoptotic and CID pathways in CD4<sup>+</sup> T cells. In contrast, 25 μM SB203580 significantly inhibited apoptosis in WT CD8<sup>+</sup> T cells (Fig. 10C), but had no effect on caspase-independent, nonapoptotic death in restimulated cells (Fig. 10, B and D). These data with WT CD8<sup>+</sup> T cells, together with our findings with lpr and gld CD8<sup>+</sup> T cells, suggest that TCR ligation can trigger Fas-independent CID pathways in CD8<sup>+</sup> T cells that are not contingent on p38 MAPK activation.

**Evidence for Fas-triggered and Fas-independent CID pathways in WT CD4<sup>+</sup> T cells**

Our observation that SB203580 reduced caspase-independent AICD in WT CD4<sup>+</sup> T cells (Fig. 10D) implied that this death mechanism

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**FIGURE 10.** Variation between WT CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the requirement for p38 MAPK activation for caspase-independent AICD. A, Representative dose response data for WT CD4<sup>+</sup> (●) and CD8<sup>+</sup> T (■) restimulated overnight with varying concentrations of SB203580. Data represent the proportions of PI<sup>+</sup> cells (●) and hypodiploid apoptotic cells (■) determined by FACS analysis. B–D, Data for WT CD4<sup>+</sup> and CD8<sup>+</sup> T cells restimulated in the presence of medium (■), 60 μM BD-fmk (□), 25 μM SB203580 (△) or a combination of 60 μM BD-fmk plus 25 μM SB203580 (●). B, Percent of PI<sup>+</sup> cells. C, Percent of apoptotic cells. D, Percent of oncotic cells. Values in B–D represent mean ± SEM for net death data from five experiments.

**FIGURE 11.** Evidence for Fas-triggered and Fas-independent caspase-independent cell death pathways in WT T cells. WT CD4<sup>+</sup> and CD8<sup>+</sup> T cells were restimulated overnight in the presence of medium alone (■), 5 μg/ml anti-Fasl mAb (□), 5 μg/ml anti-Fasl mAb plus 25 μM SB203580 (△), 60 μM BD-fmk (□), or 60 μM BD-fmk plus 5 μg/ml anti-Fasl mAb (■). A–C, Data for CD4<sup>+</sup> T cells. D–F, Data for CD8<sup>+</sup> T cells. A and D, Proportions of PI<sup>+</sup> cells. B and D, Proportions of cells with apoptotic nuclei. C and F, Proportions of oncotic cells. Data represent mean net cell death values ± SEM for data from five experiments.
process may be triggered by Fas-dependent and Fas-independent pathways. To investigate this possibility, AICD assays were performed with isolated WT CD4+ T cells and previously determined optimally inhibitory but noncytotoxic concentrations of anti-FasL mAb, BD-fmk, and SB203580 alone or in various combinations. As shown in Fig. 11, A and C, in combination with 60 μM BD-fmk, anti-FasL reproducibly inhibited CID −50% indicating that Fas can induce CID as well as classical apoptosis. The combination of BD-fmk, anti-FasL, and 25 μM SB203580 caused only a slight further reduction in CID suggesting that the inhibition of the Fas-dependent pathway by anti-FasL was close to maximal and that the residual death was likely to be Fas-independent (data not shown). The existence of a minor Fas-, p38 MAPK- and caspase-independent AICD pathway in WT CD4+ T cells was confirmed by efficiently blocking apoptosis with a combination of anti-FasL mAb and SB203580. As shown in Fig. 11, A–C, under these conditions, apoptosis was reduced to background levels and on average 83% of the cells were rescued from death. Among those cells not rescued from death, the majority (~16% of the total population) died by a nonapoptotic process resembling oncrosis (Fig. 11C). Thus, the highly efficient activation of caspases and rapid induction of apoptosis induced by TCR ligation in WT CD4+ T cells effectively masks two minor oncocytic death pathways, one Fas-dependent and the other Fas-independent.

**CID in CD8+ T cells is largely Fas-independent**

To further investigate the role of Fas in the induction of CID in WT CD8+ T cells, these cells were restimulated in the presence of anti-FasL mAb alone or in combination with SB 203580 or BD-fmk. As shown in Fig. 11, D and E, at 5 μg/ml anti-FasL, mAb almost completely blocked activation-induced apoptosis but at most caused only a 50% decrease in cell death. Microscopically, dead cells exhibited morphologic features consistent with oncotic/primary necrosis (Fig. 11F). Similar results were obtained with chimeric Fas-Fc (data not shown). The switch from apoptotic to oncocytic death was not prevented by SB203580 (Fig. 11F). Thus, a significant Fas- and p38 MAPK-independent, nonapoptotic AICD pathway is revealed in CD8+ T cells by loss of function mutations in Fas or FasL or by efficient blocking of Fas/FasL interactions. Our observation that SB203580 does not efficiently block caspase-independent, nonapoptotic death in WT CD8+ T cells (Fig. 10D) suggested that Fas ligation might not trigger nonapoptotic death in this population. To further address this issue, WT CD8+ T cells were restimulated in the presence of anti-FasL and BD-fmk with or without SB203580. As observed previously, BD-fmk rescued ~50% of cells from death and converted the mode of death from apoptosis to oncrosis in the remainder. Caspase-independent oncocytic death was not significantly inhibited by anti-FasL mAb alone or in combination with SB203580 (Fig. 11D, data not shown). These results demonstrate that the caspase-independent, nonapoptotic AICD pathway in WT CD8+ T cells is not dependent on Fas signaling.

**Discussion**

It is well-established that activation-induced, propriocidal death in T lymphocytes occurs predominantly by apoptosis, a process associated with distinct nuclear changes including intranucleosomal DNA cleavage, chromatin condensation, nuclear fragmentation, and the generation of apoptotic bodies (1, 2, 8, 9). These nuclear hallmarks of apoptosis are effector caspase-dependent and, in T cell AICD, the caspase cascade is activated most efficiently by Fas ligation (12–14). Although effector caspases are essential for classical apoptosis, they are not an absolute requirement for T cell death. T cells deficient in effector caspases or exposed to broad-spectrum caspase inhibitors die in AICD assays by a process that is morphologically distinguishable from apoptosis and similar in many respects to oncosis (22–25). CID is associated with cell swelling, large-scale cleavage of DNA into ~50- to 300-kb fragments, and perinuclear chromatin condensation and culminates in nuclear karyolysis rather than pyknosis and karyorrhexis (22–25, 48, 49). Although it is established that activation-induced CID occurs in T lineage cells, little is known about how the process is triggered or the relative susceptibility of CD4+ and CD8+ T cells to the process. Furthermore, it has not been established whether there are single or multiple CID pathways or whether TCR-induced CID is contingent on death receptor-triggered signals. In this study, we first establish that caspase inhibitors are not the only mechanism for inducing caspase-independent AICD. We present evidence that AICD switches from apoptosis to CID with the features of oncosis when CD4+ and CD8+ T cells are inherently deficient in Fas or FasL expression or when Fas/FasL interactions are blocked efficiently in WT T cells by neutralizing anti-FasL mAb or Fas-Fc. Under both circumstances, CD8+ T cells are more susceptible to CID than CD4+ T cells. Consistent with these observations, inhibition of p38 MAPK activation in WT T cells that putatively interferes with FasL expression (46) also caused a switch from apoptosis to oncosis that was most pronounced in CD8+ T cells. Further, CID induced by TCR ligation in the absence of Fas signaling is independent of p38 MAPK activation in WT, lpr, and gld CD4+ and CD8+ T cells. In analogous studies in which CID was induced by TCR cross-linking in the presence of broad spectrum caspase inhibitors, we showed that oncocytic death in WT CD8+ T cells occurred independently of p38 MAPK activation and Fas signaling. In contrast, under the same conditions, two pathways leading to CID were identified in WT CD4+ T cells, one independent of and the other dependent on Fas and p38 MAPK signaling. CID in WT CD4+ and CD8+ T cells was not dependent on TNFR1 signaling and was not blocked by neutralizing anti-TNF-α Ab. Ultrastructural differences in nuclear morphology and dependence on ROS between oncocytic gld T cells and caspase inhibitor-treated WT T cells suggest additional branching or modulation of oncotic pathways. In general, both CD4+ and CD8+ T cells were less susceptible to CID induced by blockage of Fas or p38 MAPK signaling than to classical caspase-dependent apoptosis suggesting that caspases play an important role in augmenting AICD as well as ensuring that death is predominantly apoptotic. Thus, Fas is an important regulator of both the extent and mode of death in activated T cells. The proposed death pathways triggered in CD4+ and CD8+ T cells following TCR ligation are illustrated in Fig. 12.

**Nonapoptotic AICD in lpr and gld T cells**

In this study, we showed for the first time that AICD induced in lpr and gld CD4+ and CD8+ T cell subsets in the first 24 h post restimulation was predominantly caspase-, p38 MAPK-, and TNF-independent and did not meet the criteria for classical apoptosis. The early membrane blebbing, cytoplasmic swelling and vacuolization, and demise of cytoplasmic organelles combined with prolonged preservation of the nucleus are features more consistent with death by oncrosis or paraptosis (10, 19, 21, 25, 48). Many of the dead lpr and gld T cells with morphologically normal nuclei also exhibited cytoplasmic vacuolization, a trait reported previously in some examples of CID and death by paraptosis (21, 25, 26, 50, 51). Because it is not known whether paraptosis is a variant form of oncrosis or an independent death process, we have chosen to classify all nonapoptotic death in lpr, gld, and WT T cells as oncrosis.
The induction of AICD within 16 h of TCR ligation observed in our study is consistent with data on lpr CD8\(^+\) T cells published by Teh et al. (34) but differs from data reported by Zheng et al. (33). In the latter report, AICD was observed in lpr and gld CD8\(^+\) T cells but not CD4\(^+\) T cells, was delayed until 48 h post restimulation, and was dependent on TNF-\(\alpha\) signaling presumably via TNFR2 (33). In both studies, death was assumed, but not confirmed, to be apoptotic (33, 34). In another study, TNFR2-induced death in CD8\(^+\) T cells was shown to be apoptotic and caspase-dependent (52). Our findings, combined with the published data, imply that in the absence of the Fas pathway, activated T cells may die by at least two pathways, one rapid, oncotic, and TNF-\(\alpha\)-independent and the other delayed by several days, apoptotic, and TNF-\(\alpha\)-mediated. Which pathway is activated may depend on the strength of the TCR signal and the availability of IL-2. In support of signal strength, Alexander-Miller et al. (52) showed that in CD8\(^+\) T cells from TCR transgenic mice, high peptide-MHC determinant density favors TNF-\(\alpha\)/TNFR2-induced apoptosis. In two other studies, TNF-\(\alpha\)-mediated apoptotic death in CD8\(^+\) T cell blasts was inhibited by IL-2 (53, 54). This effect of IL-2, alone, may explain why we did not observe delayed onset apoptotic death in our AICD assays as always were performed in the presence of IL-2. It also is possible that genetic differences between mouse strains may influence the sensitivity to the two pathways.

The cytoplasmic swelling observed in oncotic has been attributed to primary changes in the plasma membrane resulting from altered ionic fluxes and loss of cell volume regulation (10, 55). What triggers these events in nonapoptotic AICD in lpr and gld T cells is not known. One possibility is that TCR signaling is coupled to the up-regulation of death receptors or ligands that can signal CID. It already is established that Fas, TNFR, and TRAILR can trigger CID when caspase activation is pharmacologically inhibited (26, 27, 41). Conceivably, TRAILR, CD30 (56), or other death receptors may signal CID in lpr and gld cells and this possibility is under investigation.

Although our studies with a variety of antioxidants indicate that ROS are not essential for CID in WT T cells, the inhibitory effects of MnTBAP on oncotic death in lpr and gld T cells imply that \(\text{O}_2^-/\text{H}_2\text{O}_2\) may contribute directly or indirectly to cell damage in mutant populations. There are several possible explanations for the difference in sensitivity to ROS between WT and mutant T cells. Compared with normal T cells, some mutant T cells may be more dependent on \(\text{O}_2^-/\text{H}_2\text{O}_2\) for triggering or amplifying death signals or they may have reduced antioxidant defenses and consequently a greater sensitivity to ROS-associated cell damage. Preliminary studies with the oxidant sensitive dye, CM-DCHF, provide some support for the latter possibility. Alternatively, \(\text{O}_2^-/\text{H}_2\text{O}_2\)-dependent oncotic cell death pathways may exist in minor subsets of T cells that are selectively enriched in lpr and gld mice. Although such pathways have not been described previously, MnTBAP was reported to inhibit superantigen-induced apoptotic death in normal T cells (42).

Comparisons between nuclei from BALB-gld and BALB-WT T cells undergoing activation-induced CID revealed differences in morphology with caspase inhibitor-treated WT cells consistently showing a different pattern and greater degree of chromatin clumping. Death stimulus-induced peripheral chromatin condensation and large scale DNA fragmentation have been described in cells inherently deficient in effector caspses or Apaf-1 and in caspase inhibitor-treated cells and have been attributed to the activity of apoptosis-inducing factor (AIF) (22–29, 50, 51, 57). Therefore, the variation in nuclear morphology between oncotic gld and WT T cells may reflect differences in the efficiency of translocation of AIF from mitochondria to the nucleus. Alternatively, oncotic death in BALB-gld T cells may be AIF-independent. Death pathways
that are independent of caspases and AIF have been reported previously (58).

Triggering of a nonapoptotic death pathway in WT CD8+ T cells

Our finding that apoptotic death in reactivated WT CD8+ T cells was completely inhibited by anti-FasL mAb but was unaffected by anti-TNF-α Ab implies that effector caspase activation is predominantly Fas-mediated in this population. As discussed above, the fact that we did not detect delayed, TNF-α-mediated apoptotic AICD in WT CD8+ T cells as reported by others (33, 52) may be explained by the inhibitory effects of the IL-2 in our AICD assay on TNF-α-induced death and/or an insufficiently strong TCR signal to render the cells sensitive to endogenous TNF-α.

Although anti-FasL mAb completely inhibited apoptosis, it only protected ~50% of AICD-susceptible CD8+ T cells from death with the remainder dying by oncosis. Based on these results and the predominant use of an oncotic death pathway in gld CD8+ T cells, we propose that at least two independent death pathways are activated in individual WT CD8+ T cells by TCR ligation; one Fas-dependent and apoptotic and the other Fas-independent and oncotic (Fig. 12). Under normal circumstances, the oncotic pathway will be masked by Fas-dependent activation of effector caspases. The fact that SB203580 also induced a switch from apoptosis to oncrosis in reactivated WT CD8+ T cells suggests that p38 MAPK may be an important determinant in the balance between apoptotic vs oncotic AICD. One way that p38 MAPK may steer cells toward apoptosis is by up-regulation of FasL expression, as previously reported (46, 47). Early up-regulation of FasL expression may be important for both priming cells to die and activating the execution machinery (46). There appears to be some heterogeneity among WT CD8+ T cells in their ability to activate the two death pathways. The consistent appearance of a small proportion of oncotic cells in AICD assays with WT CD8+ T cells suggests that a subset of cells can selectively activate the oncotic pathway. In addition, the fact that a significant proportion of cells are rescued from death by anti-FasL mAb or SB203580 suggests that not all cells can activate the oncotic pathway or that Fas signals are required to signal oncrosis in some cells. The latter possibility seems unlikely because anti-FasL mAb had no significant effect on the level of oncotic death observed in normal CD8+ T cells reactivated in the presence of caspase inhibitors. Thus, it can be concluded that, on average, at least half of the WT CD8+ T cells susceptible to AICD trigger a Fas- and p38 MAPK-dependent death pathway that in the absence of activated effector caspases will trigger death by oncrosis (Fig. 12).

Evidence for two caspase-independent AICD pathways in CD4+ T cells

Our studies confirm earlier reports that the majority of CD4+ T cells, but only ~50% of CD8+ T cells are vulnerable to AICD (33, 34, 59). This inherent difference in susceptibility to AICD complicates comparisons of the relative sensitivity of the two populations to different modes of death. Nevertheless, if the total CD4+ T cell population is compared with the subpopulation of CD8+ T cells sensitive to AICD, several novel differences are observed. First, CD4+ T cells are more resistant to CID than CD8+ T cells. This implies that CD4+ T cells generally are more dependent on caspase activation for the execution of AICD pathways than CD8+ T cells and are less likely to be shunted down nonapoptotic pathways. Second, TCR ligation can activate two separate CID pathways in CD4+ T cells, one dependent on Fas and p38 MAPK signals and the other independent of these activities (Fig. 12). The demonstration of a Fas-dependent CID pathway in CD4+ T cells is consistent with recent reports that ligation of Fas in the presence of caspase inhibitors results in a switch from apoptotic to non-apoptotic death in lymphoid and nonlymphoid cells (26, 27). Future studies will determine whether the TCR-triggered Fas-dependent CID death pathway described in the present study also is dependent on Fas-associated death domain and receptor-interacting protein kinase for its activation (26, 27). The dependence on Fas signaling for a significant proportion of CID in CD4+ T cells may be indicative of a safety device retained to guarantee that controlled elimination of activated CD4+ T cells can proceed in the event that activation of initiator or effector caspases is impaired. Blockage of the Fas-dependent CID pathway may also explain why lpr and gld CD4+ T cells are more resistant to AICD than mutant CD8+ T cells.

So far, the Fas-independent CID pathways in CD4+ and CD8+ T cells are indistinguishable at the biochemical level and induce similar nuclear and cytoplasmic changes suggesting that they are analogous. The fact that only a subset of CD4+ and CD8+ T cells are susceptible to Fas-independent CID implies that this pathway either is retained only in certain subsets of T cells or is triggered only under particular physiological conditions (e.g., cell cycle stage or death receptor expression). If this CID pathway proves to be dependent on AIF, it may represent an ancestral death mechanism as AIF homologs are present in plants and fungi as well as animals (58, 60).

Implications for a switch in vivo from apoptotic to oncotic/necrotic death

If the CID pathways that we identified in Fas- and FasL-deficient T cells in vitro are also activated in vivo, it is possible that elimination of excess activated T cells in lpr and gld mice may occur predominantly by oncrosis rather than apoptosis. Potentially, this could have significant ramifications in terms of autosensitization. Because oncrosis is associated with early cell swelling and plasma membrane damage, cells dying by this process may be at greater risk of earlier rupture than apoptotic cells. Release of proinflammatory intracellular contents and autoantigens may result in the activation of B cells reactive with cytoplasmic and nuclear Ags. The extent to which rupture occurs in oncotic cells will depend on the efficiency with which dying cells are phagocytosed. Consistent with a previous report (61), we observed the exposure of phosphatidylserine on the outer cell membranes of oncotic cells (W. F. Davidson, unpublished observation). Studies are in progress to determine whether lpr and gld dendritic cells (DC) and macrophages show evidence of defects in the phagocytosis of oncotic cells.

Under normal circumstances, the removal of intact apoptotic cells or apoptotic bodies by immature DC is a silent process that results in the presentation of processed autoantigens in a tolerogenic rather than immunogenic form (62–64). However, if DC receive maturation signals after ingestion, responses can be elicited to Ags derived from apoptotic cells (62–67). Similarly, macrophages are poor presenters of processed autoantigens derived from apoptotic cells (66, 68). Primarily, this is because uptake of apoptotic cells induces macrophages to produce the immunosuppressive agents IL-10, PGE2, and TGF-β and to down-regulate MHC class II expression and IL-6 and TNF-α production (62, 66, 68). In contrast, uptake of necrotic cells can augment macrophage activation and increase the secretion of the proinflammatory cytokines, IL-6 and TNF-α (68). Interestingly, when both apoptotic and necrotic cells are present, the immunosuppressive effects of apoptotic cells are dominant (68). Conceivably, a wholesale switch from apoptotic to necrotic death may signal immunologic “danger” and tip the balance from tolerance to sensitization of autoreactive Th cells (69). It will be interesting to determine whether oncotic T

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cells also can augment macrophage activation and whether there are differences in responses induced by oncotic WT T cells vs lpr and gld T cells.

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