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Reactive Oxygen Species Regulate Activation-Induced T Cell Apoptosis

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Summary

Reactive oxygen species (ROS) mediate apoptosis in a number of cell types. We studied the role that ROS play in activated T cell apoptosis by activating T cells in vivo and then culturing them for a short time. Activated T cells died independently of Fas and TNFα. Their death was characterized by rapid loss of mitochondrial transmembrane potential ($\Delta\psi_m$), caspase-dependent DNA fragmentation, and superoxide generation. A superoxide dismutase mimetic, Mn (III) tetrakis (5, 10, 15, 20-benzoic acid) porphyrin (MnTBAP), protected T cells from superoxide generation, caspase-dependent DNA loss, loss of $\Delta\psi_m$, and cell death. These results indicate that ROS can regulate signals involved in caspase activation and apoptosis and may contribute to peripheral T cell deletion.

Introduction

During the decline of the primary T cell response, the majority of the antigen-specific, activated T cells die (Murali-Krishna et al., 1998). Identification of death-inducing stimuli for activated T cells is important because an understanding of T cell life and death is crucial to our knowledge of how productive immune responses occur and how autoimmunity is prevented. Molecules such as Fas and TNFα have been shown to be sufficient to mediate the apoptotic death of activated T cells (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995; Zheng et al., 1995). Fas and TNFα receptors transmit their death signals through activation of the caspase family of cysteine proteases. Caspases are synthesized as inactive proenzymes and are proteolytically processed into active enzymes that cleave proteins after aspartic acid residues (Thornberry et al., 1992; Nicholson et al., 1995; Cohen, 1997). Caspases, when activated, cleave several molecules involved in cell structure and integrity. For example, caspase-3 has been shown to activate an endonuclease involved in apoptotic DNA fragmentation (Liu et al., 1997; Enari et al., 1998), while caspase-8 has been shown to cleave Bid, which can cause mitochondria to release cytochrome c (Li et al., 1998; Luo et al., 1998). Caspase inhibitors have been developed and shown to inhibit most cell death triggered by cell surface death receptors (Armstrong et al., 1996; Cohen, 1997; Kunstle et al., 1997). Thus, the presence of active caspase enzymes provides a direct link from extracellular death signals to intracellular disintegration and death.

While caspases can be activated directly through cell surface receptors such as Fas and TNFα, they can also be activated by cytochrome c released by mitochondria. During apoptosis, mitochondria can be induced to release cytochrome c, which can then activate caspase-9 (in the presence of Apaf-1 and dATP) (Liu et al., 1996; Li et al., 1997). Activated caspase-9 can process procaspase-3 into caspase-3 and thereby trigger intracellular disintegration (Li et al., 1997). Thus, caspases can be activated upstream or downstream of mitochondria dysfunction during apoptosis.

However, caspases are not the only molecules that can mediate apoptosis. Several recent reports have shown that caspase inhibition sometimes fails to block cell death and the appearance of some features of apoptosis (Xiang et al., 1996; Ink et al., 1997; McCarthey et al., 1997; Deas et al., 1998; Pastorino et al., 1998; Susin et al., 1999). For example, in vitro activation of human T cells with anti-CD2 and staurosporine leads to caspase-independent apoptotic cell death (Deas et al., 1998). Also, apoptosis inducing factor (AIF), a mitochondrial protein, can mediate chromatin condensation, mitochondrial swelling, and release of cytochrome c in the presence of caspase inhibitors (Susin et al., 1999). Further, transgenic expression of proapoptotic Bcl-2 family members Bax or Bak within transformed cells or in yeast have demonstrated a caspase-independent form of cell death (Xiang et al., 1996; Ink et al., 1997; McCarthey et al., 1997; Pastorino et al., 1998). Thus, factors other than caspases can mediate programmed cell death. One consistent feature of these caspase-independent forms of cell death is the loss of mitochondrial transmembrane potential ($\Delta\psi_m$). Therefore, mitochondria play a central role in apoptosis, because they can be involved in both caspase-dependent and -independent cell death.

ROS mediate apoptosis in several model systems, including excitotoxic neural cell death, PMA-induced death of neutrophils, and HIV-induced death of T cells (Buttke and Sandstrom, 1995; Patel et al., 1996; Dobmeyer et al., 1997; Fadeel et al., 1998). Metallic porphyrins, synthetic antioxidant compounds, have been shown to be highly effective in their antioxidant capacity and can mediate the dismutation of superoxide with a rate constant of $4 \times 10^7\ M^{-1}S^{-1}$ (Pasternack et al., 1981). Furthermore, manganese (III) tetrakis (5, 10, 15, 20-benzoic acid) porphyrin (MnTBAP) has been shown to inhibit excitotoxic-, staurosporine-, and ceramide-induced death of neural cells and paraquat-induced death of endothelial cells (Day et al., 1995, 1997; Patel et al., 1996; Patel,

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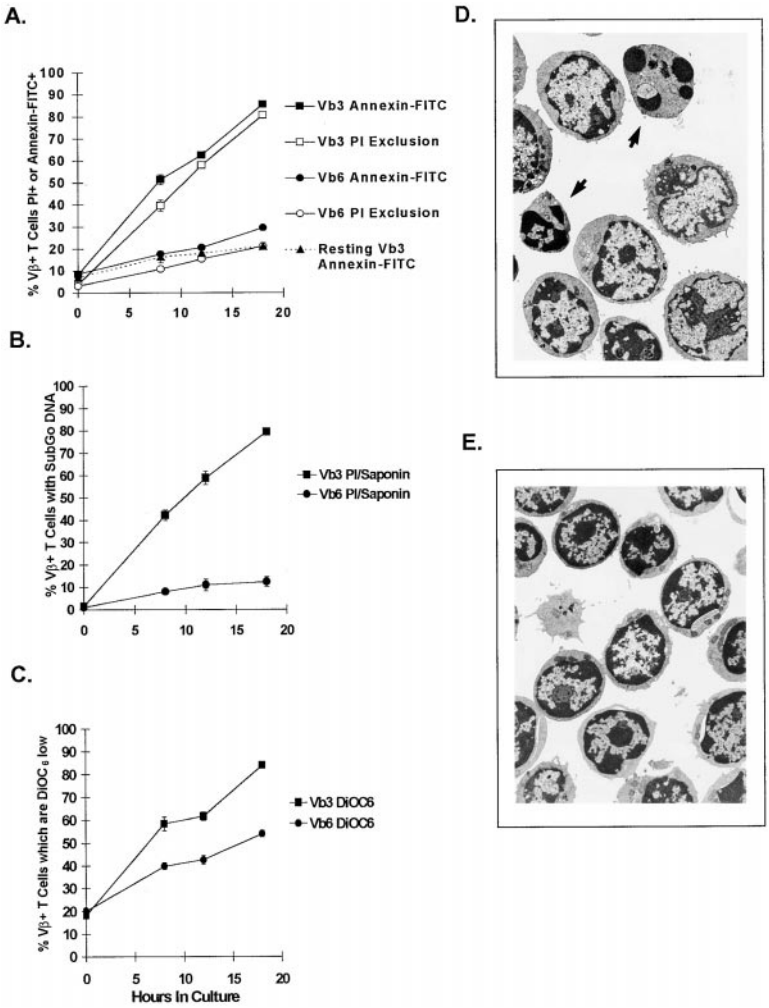

Figure 1. In Vitro Death of Activated versus Resting T Cells

(A) B10.BR mice (n = 3/group) were injected i.v. with 0.1 μg SEA or with BSS as a control. After 48 hr, LN T cells were nylon wool purified from SEA or control noninjected mice and plated separately in 96-well plates at 1.5×10^6 cells/ml. At indicated times, cells from SEA-injected mice were stained with either anti-Vβ3-PE or anti-Vβ3-FITC (squares) or anti-Vβ6-PE or anti-Vβ6-FITC (circles), while cells from control noninjected mice were stained with anti-Vβ3-PE (triangles, dashed line). Anti-Vβ-PE-stained cells were washed twice in AnnexinV binding buffer, stained with AnnexinV-FITC (closed symbols), and analyzed on a FACScan flow cytometer. Anti-Vβ-FITC-stained cells were washed twice in BSS wash buffer and resuspended in 0.5 μg/ml propidium iodide (PI) in PBS (open symbols) and analyzed by flow cytometry. Results show the percent of $V\beta3^+$ or $V\beta6^+$ cells that were either PS^+ or $PI^+ \pm$ SD. This experiment is representative of three independent experiments with similar results.

(B) At indicated times, cells from SEA-injected mice in (A) were stained with anti-Vβ3-FITC (closed squares) or anti-Vβ6-FITC (open squares), washed, and resuspended in 5 μg/ml propidium iodide in 0.03% saponin buffer. After a 20 min incubation at room temperature, cells were analyzed by flow cytometry to assess DNA content. Results show the percent of $V\beta3^+$ or $V\beta6^+$ cells having subG0 DNA content ± SD. This experiment is representative of three independent experiments with similar results.

(C) At indicated times, cells from SEA-injected mice in (A) were stained with anti-Vβ3-PE (closed squares) or anti-Vβ6-PE (closed circles), washed, resuspended in 25 nM $DiOC_6$, incubated at 37°C for 40 min, and then analyzed by flow cytometry to assess loss of $\Delta\psi_m$. Results show the percent of $V\beta3^+$ or $V\beta6^+$ cells that were also $DiOC_6^{low} \pm$ SD.

(D and E) B10.BR mice were injected i.v. with 0.1 μg SEA, and, after 48 hr, their LN T cells were purified and cultured for 4 hr in vitro. Cells were stained with anti-Vβ3-FITC monoclonal antibody and anti-CD3 PE monoclonal antibody and sorted at 4°C for $CD3^+V\beta3^+$ (D, arrows point to apoptotic cells) or $CD3^+V\beta3^-$ (E) cells. Sorted cells were fixed, embedded in paraffin, stained with osmium tetroxide, and observed under a transmission electron microscope.

1998). While activated T cells have been shown to have increased levels of ROS (Castedo et al., 1995), little has been done to examine the relationship between ROS and other apoptotic pathways within these cells.

In this paper, we investigated the relationship between ROS and the regulation of $\Delta\psi_m$, caspase activation, and cell death. We found that primary activated T cells died by Fas- and TNFα-independent apoptosis characterized by caspase-independent loss of $\Delta\psi_m$, enhanced generation of ROS, and caspase-dependent DNA loss. Interestingly, MnTBAP protected T cells from activation-induced cell death, caspase-dependent DNA loss, superoxide generation, and loss of $\Delta\psi_m$. Our results suggest a previously undescribed role for ROS within primary activated T cells as we provide evidence that ROS can regulate both caspase activation and apoptosis. ROS can, therefore, be targeted in the prevention or induction of the apoptotic pathway within activated T lymphocytes.

Results

Apoptosis of T Cells Stimulated with Antigen In Vivo

Many previous reports studying activation-induced T cell death involved in vitro T cell receptor stimulation, a system that does not necessarily mimic T cell activation in vivo. To study T cell death in a system that more closely resembles events in vivo, we injected mice with the superantigen SEA. SEA activates target T cells bearing Vβ3, 1, and 11 as part of their T cell receptor but does not react with T cells bearing other Vβs (Kotzin et al., 1993). Forty-eight hours after SEA injection, LN T cells were isolated and cultured. A greater percentage of SEA-activated, $V\beta3^+$ T cells died in culture, as defined by binding of AnnexinV to phosphatidyl serine (PS) residues or by the uptake of propidium iodide, than did $V\beta6^+$ T cells from the same SEA-injected animals or $V\beta3^+$ cells from noninjected mice (Figure 1A). $V\beta3^+$ but not $V\beta6^+$ T cells were affected, demonstrating the de-

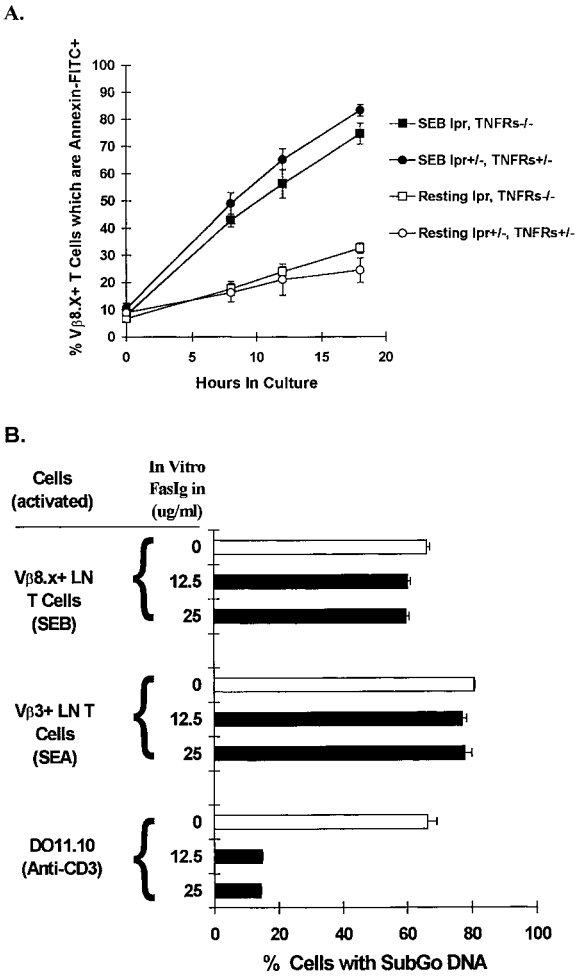

Figure 2. Death of Superantigen-Activated T Cells in the Absence of Fas and TNFRs

(A) Groups of *lpr*, p55$^{-/-}$, p75$^{-/-}$ mice (n = 3/group) (squares) or heterozygous controls (n = 3/group) (circles) were either uninjected (open symbols) or were injected i.v. with 150 μg SEB (closed symbols). Forty-eight hours after SEB injection, mice were sacrificed and LN T cells were nylon wool purified and plated separately in 96-well plates at 1.5 × 10^6 cells/ml. At indicated time points, cells were stained with anti-Vβ8.X-PE, washed twice in AnnexinV binding buffer, stained with AnnexinV-FITC, and analyzed on a FACScan flow cytometer. Results show the percent of Vβ$^+$ (PE$^+$) cells that were also PS$^+$ (FITC$^+$) ± SD. This experiment is representative of four independent experiments with similar results.

(B) B10.BR mice (n = 3) were injected with 0.2 μg SEA 48 hr before sacrifice, while C57Bl/10 mice (n = 3) were injected with 150 μg SEB 48 hr before sacrifice. LN cells were plated in triplicate in 96-well plates with the indicated concentrations of FasIg. After 15 hr in culture, cells were stained with either anti-Vβ3-FITC or anti-Vβ8.X-FITC, washed twice with BSS wash buffer, resuspended in PI/saponin buffer, and analyzed by flow cytometry. Results show percent of Vβ8$^+$ or Vβ3$^+$ cells having subG0 DNA ± SD. DO-11.10 cells were plated in triplicate in 96-well plates previously coated with anti-CD3 antibody (100 μg/ml) along with the indicated concentrations of FasIg. After 15 hr in culture, cells were washed once with BSS wash buffer, resuspended in PI/saponin buffer, and analyzed by flow cytometry. Results show percent of DO11.10 cells having subG0 DNA ± SD.

pendence of this cell death upon signals from the T cell receptor. We observed similar results using Vβ8$^+$ T cells stimulated with SEB (Figure 2) or in TCR transgenic T cells activated with peptide in vivo (data not shown). Both the activated and resting T cells underwent significant membrane permeability, a characteristic of necrosis (Figure 1A). However, these primary activated T cells were probably dying apoptotically because PS was exposed on their surfaces prior to their acquisition of membrane permeability, a characteristic of apoptotic cells (Figure 1A).

Another hallmark of apoptosis is rapid DNA fragmentation. It has been previously shown that T cells activated in vivo with superantigen exhibit significant DNA fragmentation after a few hours of culture (Kawabe and Ochi, 1991). We, therefore, assessed the kinetics of DNA degradation within activated and resting T cells. In culture, the activated Vβ3$^+$ T cells rapidly lost DNA, with 83% of the cells having subG0 DNA by 18 hr in culture compared to 13% for the resting Vβ6$^+$ T cells (Figure 1B). During apoptosis, cells also rapidly lose $\Delta\psi_m$, a process that can be measured cytofluorometrically using a cationic dye, $DiOC_6$, that accumulates in mitochondria that have an intact $\Delta\psi_m$ (Zamzami et al., 1995a, 1995b). We used $DiOC_6$ to assess the kinetics of the loss of $\Delta\psi_m$ within activated versus nonactivated T cells. Activated T cells underwent a very rapid loss of $DiOC_6$ staining compared to nonactivated T cells, with 85% of the cells having $DiOC_6^{low}$ staining by 18 hr in culture compared to 50% for the nonactivated Vβ6$^+$ T cells (Figure 1C).

Finally, to confirm that this cell death was apoptotic, Vβ3$^+$ T cells were activated in vivo, isolated, cultured for 4 hr, and then sorted into Vβ3$^+$ and Vβ3$^-$ T cell populations. Sorted T cell populations (>95% purity) were visualized by electron microscopy. 12.02% ± 2.95% of the Vβ3$^+$ T cells were apoptotic, with chromatin condensation and the beginnings of nuclear degeneration (Figure 1D, arrows point to apoptotic cells), whereas only 3.78% ± 1.65% of the sorted Vβ3$^-$ T cells had these properties (Figure 1E). This was a statistically significant difference, with $p < 0.0001$, Student's t test (Minitab 12 for Windows). Thus, these activated T cells underwent primary apoptosis that was followed by a rapid secondary necrosis.

SAg-Induced Activated T Cell Death Is Independent of Fas and TNF

Because Fas/FasL interactions have been shown by many to be involved in T cell death (Singer and Abbas, 1994; Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995), we tested whether these molecules were responsible for the cell death described above. To do this, we injected triple mutant mice, deficient in expression of Fas and both TNF receptors, with SEB, removed their T cells after 48 hr, and assessed T cell death in vitro. Vβ8$^+$ T cells from SEB-injected *lpr*, p55$^{-/-}$, p75$^{-/-}$ mice died as rapidly in vitro as Vβ8$^+$ T cells from SEB-injected heterozygous control mice (Figure 2A). Both sets of activated Vβ8$^+$ T cells died faster than resting Vβ8$^+$ T cells from uninjected control animals (Figure 2A), indicating that neither Fas nor TNF receptors were required for activated T cell death.

However, it could be that *lpr*, p55$^{-/-}$, p75$^{-/-}$ mice have developed a compensatory death pathway and that the death of activated T cells from normal mice

is dependent upon Fas. To check this, we examined activated T cell death in the presence of FasIg, a reagent that blocks Fas-mediated cell death in several model systems (Brunner et al., 1995; El-Khatib et al., 1995; Ju et al., 1995). While FasIg completely blocked the death of DO-11.10 T hybridoma cells stimulated in vitro with anti-CD3, it failed to block in vitro cell death of either SEA- or SEB-activated T cells after 48 hr stimulation in vivo (Figure 2B). Hence, results from both normal and genetically deficient mice indicate that in vitro T cell death following SAg stimulation in vivo was not dependent upon FasL or TNF signaling to the T cells.

Caspase Dependence of Different Forms of Cell Death

Caspases have been shown to be required for apoptosis in a number of experimental systems (Nicholson et al., 1995; Armstrong et al., 1996; Sarin et al., 1996; Haviv et al., 1997; McCarthy et al., 1997). We used two broad spectrum caspase inhibitors, ZVAD-fmk and BD-fmk, to test whether activated T cell death required caspases. Both BD-fmk and ZVAD-fmk, but not the control cathepsin B inhibitor ZFA-fmk, prevented DNA loss in activated T cells in a dose-dependent manner (Figure 3A). It is unclear why ZVAD-fmk was less effective than BD-fmk at inhibiting DNA loss within activated T cells. In shorter experiments (8 hr), we observed that ZVAD-fmk was more effective at inhibiting DNA loss than in these longer (14 hr) experiments (data not shown). It could be that ZVAD-fmk is less stable than BD-fmk in cell culture. Nonetheless, neither caspase inhibitor had an effect on activated T cell death as measured by Annexin staining (Figure 3B). Even when we added BD-fmk and ZVAD-fmk together, they had no effect on either the rate or amount of activated T cell death (data not shown). BD-fmk had similar effects on activated T cells from *lpr*, TNFRs$^{-/-}$ mice, that is it blocked DNA loss but not the death of these cells (data not shown). Thus, caspase activation within activated *lpr*, TNFRs$^{-/-}$ T cells does not require Fas or TNFα.

We next tested whether these caspase inhibitors could protect cells from death induced by different stimuli. We compared Fas-driven death of L1210Fas^{+} cells and growth factor withdrawal–induced death of HT-2 cells to the death of primary activated T cells. BD-fmk completely prevented the death of L1210Fas^{+} cells treated with anti-Fas (Figure 4) and HT-2 cells withdrawn from IL-2 (Figure 4) but failed to protect activated T cells from death (as assessed by the uptake of propidium iodide, Figure 4). ZFA-fmk had no effect on cell death in any of the cell types tested (Figure 4). Thus, caspases were required for cell death in some, but not all, models of apoptosis. Caspases were always required for DNA loss, but primary activated T cells died even when caspase-dependent DNA loss was blocked.

SAg-Activated T Cells Exhibit Increased Reactive Oxygen Species and Are Rescued from Death with MnTBAP, a SOD Mimetic

Reactive oxygen species (ROS) have been shown to mediate apoptosis in a number of systems (Buttke and Sandstrom, 1995; Patel et al., 1996; Dobmeyer et al., 1997; Fadeel et al., 1998). In order to test whether SAg-activated cells generate ROS, we used dihydroethidium (HE), a relatively specific indicator for the presence of superoxide anion. As shown in Figure 5, SAg-activated Vβ8.X^{+} T cells exhibit a significant increase in HE staining compared to resting Vβ6^{+} cells within the same population straight out of the animal ($p < 0.0008$, Student's t test, Minitab). We next tested whether or not the SOD mimetic, MnTBAP, could affect superoxide generation as measured by HE staining. Culture of activated T cells with MnTBAP resulted in a significant decrease in HE staining within both SAg-activated T cells and nonactivated T cells ($p < 0.0008$ and $p < 0.0004$, respectively. Student's t test, Minitab) (Figure 5).

Because increased levels of ROS within activated T cells was correlated with increased cell death, we next determined whether antioxidants could inhibit cell death. We were unable to inhibit cell death with ascorbic acid, α-tocopherol acetate, n-acetyl cysteine, or s-methyl isothiourea (data not shown). However, MnTBAP rescued activated T cell death in vitro in a dose-dependent manner as measured by PS expression (Figure 6A), loss

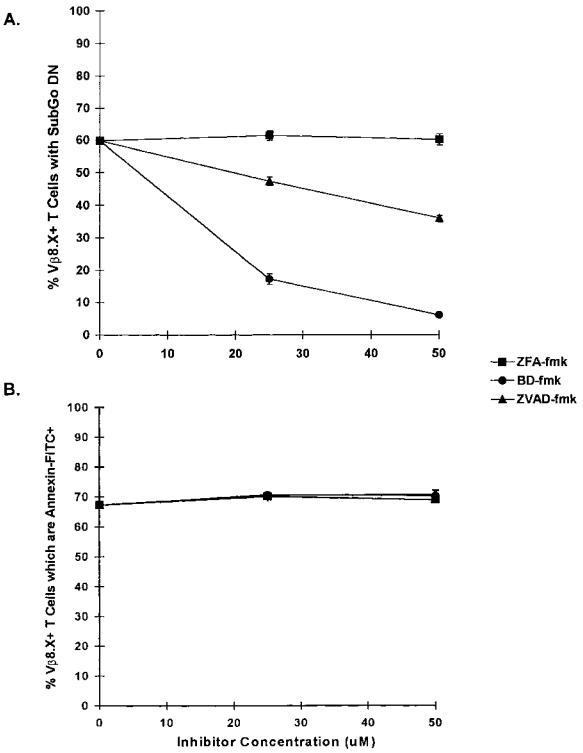

Figure 3. Caspase Inhibitors Block DNA Degradation but Not Death of SAg-Activated T Cells

C57BL/10 mice were injected i.v. with 150 μg SEB ($n = 3$). After 48 hr, purified LN T cells were plated separately in 96-well plates. Caspase (BD-fmk, ZVAD-fmk) and control (ZFA-fmk) inhibitors were added at the indicated concentrations at the beginning of culture. After 14 hr in vitro, T cells were stained with anti-Vβ8.x-FITC monoclonal antibody and were either (A) resuspended in 5 μg/ml propidium iodide in 0.03% saponin or (B) washed twice in AnnexinV binding buffer and then stained with AnnexinV-FITC and analyzed by flow cytometry. Results show the percent of Vβ8^{+} cells that were PS^{+} (FITC) or had subG0 DNA $\pm$ SD. This experiment is representative of six independent experiments with similar results.

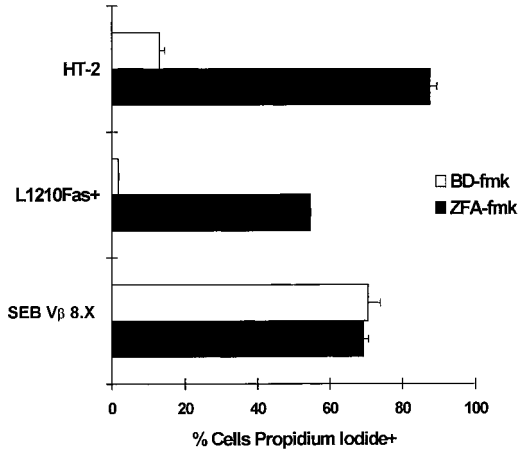

Figure 4. Caspase Inhibitors Block Fas and Growth Factor Withdrawal–Induced Death but Not Activated T Cell Death

Death-inducing stimuli were as follows: HT-2 cells were washed three times, resuspended in media containing either BD-fmk (50 μM) or ZFA-fmk (50 μM), and plated in triplicate in 96-well plates for 18 hr at 37°C. L1210Fas$^+$ cells were plated in triplicate with either BD-fmk (50 μM) or ZFA-fmk (50 μM) in 96-well plates coated with 100 μg/ml anti-Fas antibody for 15 hr at 37°C. SEB Vβ8.X$^+$ T cells were activated in C57BL/10 mice by i.v. injection of 150 μg SEB, and, 48 hr after injection, LN T cells were plated in triplicate with either BD-fmk (50 μuM) or ZFA-fmk (50 μM) in 96-well plates for 18 hr at 37°C. After culture, HT-2 and L1210Fas$^+$ cells were spun down and resuspended in media containing 0.5 μg/ml propidium iodide and analyzed by flow cytometry. After culture, LN cells were stained with anti-Vβ8.X-FITC antibody, washed twice and resuspended in media containing 0.5 μg/ml propidium iodide, and analyzed by flow cytometry. Similar results were obtained when AnnexinV-FITC was used to assess death (data not shown). Results show the percent of cells that were PI$^+$ ± SD. This experiment is representative of three independent experiments with similar results.

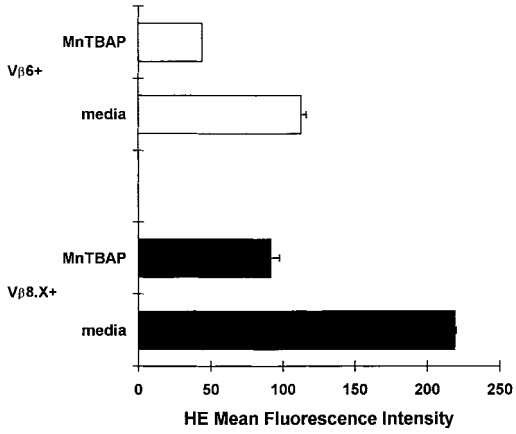

Figure 5. Increased Dihydroethidium Staining in Activated versus Resting T Cells and Decrease of Dihydroethidium Staining with MnTBAP

C57BL/10 mice (n = 3) were injected i.v. with 150 μg SEB, and, after 48 hr, LN T cells from individual mice were harvested and stained with either anti-Vβ8.X-FITC (filled bars) or anti-Vβ6-FITC (open bars). Cells were then washed and incubated with 2.5 μM dihydroethidium either with or without 100 μM MnTBAP for 40 min at 37°C. Cells were washed once, resuspended in media, and analyzed on a FacsCalibur flow cytometer. Results show the mean FL2 fluorescence intensity within Vβ$^+$ (FITC$^+$) cells ± SD. Similar results were obtained with SEA-activated Vβ3$^+$ T cells from B10.BR/SgSnJ mice (data not shown). This experiment is representative of three independent experiments with similar results.

of membrane integrity (PI uptake, data not shown), and DNA fragmentation (subGo PI staining, Figure 6A). ZnTBAP, a compound that has much less SOD activity than MnTBAP, was much less effective at rescuing activated T cells from death (data not shown). Further, MnTBAP also failed to rescue growth factor withdrawal–induced death of HT-2 cells and Fas-mediated death of L1210Fas$^+$ cells (data not shown). Taken together, these results suggest that reactive oxygen species play a significant and perhaps pivotal role in the death of activated T cells.

SAg-Activated T Cells Exhibit Caspase-Independent, ROS-Dependent Loss of Mitochondrial Transmembrane Potential

We determined whether or not the loss of mitochondrial transmembrane potential ($\Delta\psi_m$) we had observed within activated T cells was dependent upon caspases and/or ROS. We cultured LN T cells from SAg-injected mice for 12 hr in the presence of BD-fmk, ZFA-fmk, or MnTBAP and then stained them with anti-Vβ8.X antibody and $DiOC_6$. Culture with MnTBAP resulted in a significant increase in the percentage of Vβ8.X$^+$ T cells that also were positive for $DiOC_6$, compared to culture with media alone, BD-fmk, or ZFA-fmk (Figure 6B) (MnTBAP versus media $p < 0.0006$; BD-fmk versus media $p < 0.85$; Student's t test, Minitab).

These studies show that T cells can go through apoptosis by two different routes. One is driven by direct caspase activation through cell surface receptors such as Fas. In this case, mitochondrial dysfunction is caspase dependent, and death is preventable with caspase inhibitors but not antioxidants (Figure 7A). The second form involves generation of ROS, disruption of $\Delta\psi_m$, and caspase activation. In this latter case, caspase activation is needed for DNA degradation but not for cell death, whereas ROS control caspase activation, loss of $\Delta\psi_m$, and cell death (Figure 7B).

Discussion

During a typical response to an antigenic stimulus, a naive resting T cell is converted into a pool of "effector" cells through a process of activation, proliferation, and differentiation. It is generally thought that as the antigenic stimulus wanes, the majority of the responding T cells die. Elimination of these T cells is probably important for three reasons. First, there would be an increased metabolic cost to the organism to keep these cells alive. Second, failure to remove these cells would probably overwhelm the lymphoid compartment after a few infections. Third, the existence of a large number of activated, effector T cells in the animal could result in a lethal shock-like reaction were they to suddenly encounter a large bolus of antigen. Each of these situations is avoided by inducing apoptotic death in activated T cells when they are no longer needed.

Many factors have been shown to be involved in the apoptotic death of activated T cells (Lenardo, 1991; Brunner et al., 1995; Buttke and Sandstrom, 1995; Dhein

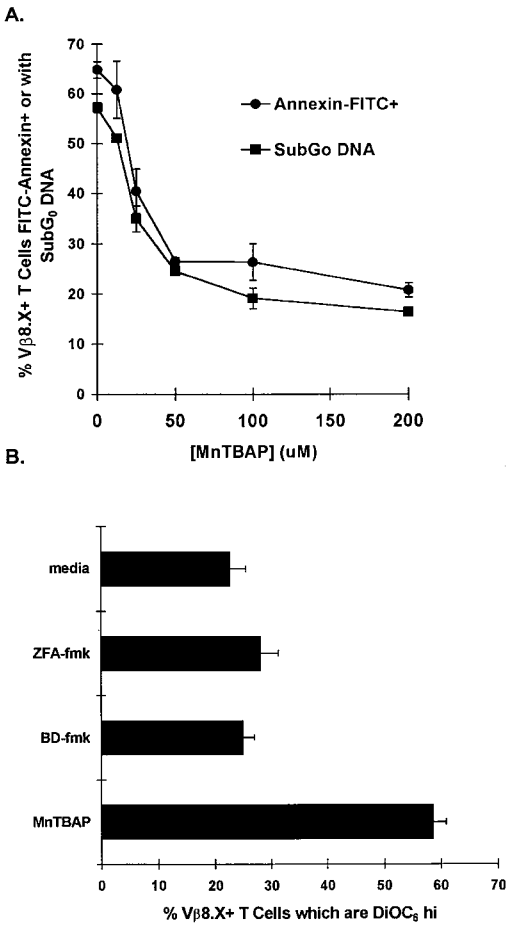

Figure 6. MnTBAP Rescues Activated T Cells from Death, Caspase-Dependent DNA Loss, and Loss of $\Delta\psi_m$

(A) C57BL/10 mice (n = 3) were injected i.v. with 150 μg SEB, and, after 48 hr, LN T cells from individual mice were harvested and cultured with the indicated concentrations of MnTBAP for 15 hr at 37° C. Cells were then stained with anti-Vβ8.X-FITC, washed twice in AnnexinV binding buffer, stained with AnnexinV-FITC (circles), or washed twice in BSS wash buffer and resuspended in 5 μg/ml propidium iodide in 0.03% saponin (squares). Results show the percent of $V\beta8.X^+$ that were also AnnexinV-$FITC^+$ or have subGo DNA ± SD. This experiment is representative of five independent experiments with similar results.

(B) C57BL/10 mice (n = 3) were injected i.v. with 150 μg SEB, and, after 48 hr, LN T cells from individual mice were harvested and cultured with either 200 μM MnTBAP, 50 μM BD-fmk, 50 μM ZFA-fmk, or media alone for 15 hr at 37°C. Cells were then stained with anti-Vβ8.X biotin and streptavidin-phycoerthyrin conjugate, washed and resuspended in 25 nM $DiOC_6$, and cultured at 37°C for 40 min. Data were acquired on a FacScan flow cytometer. Results show the percent of $V\beta8.X^+$ cells that were also $DiOC_6^{high}$ ± SD.

et al., 1995; Ju et al., 1995; Zheng et al., 1995, 1998). The involvement of these factors in all apoptotic deaths of activated T cells is controversial, however. Some papers support a role for Fas and TNF receptors, while others do not (Scott et al., 1993; Singer and Abbas, 1994; Zheng et al., 1995; Miethke et al., 1996; Van Parijs et al., 1996, 1998). We cannot be certain of the cause for this discrepancy, except that the experimental designs vary from one study to the other. Perhaps T cells in the various systems are differentially exposed to IL-2,

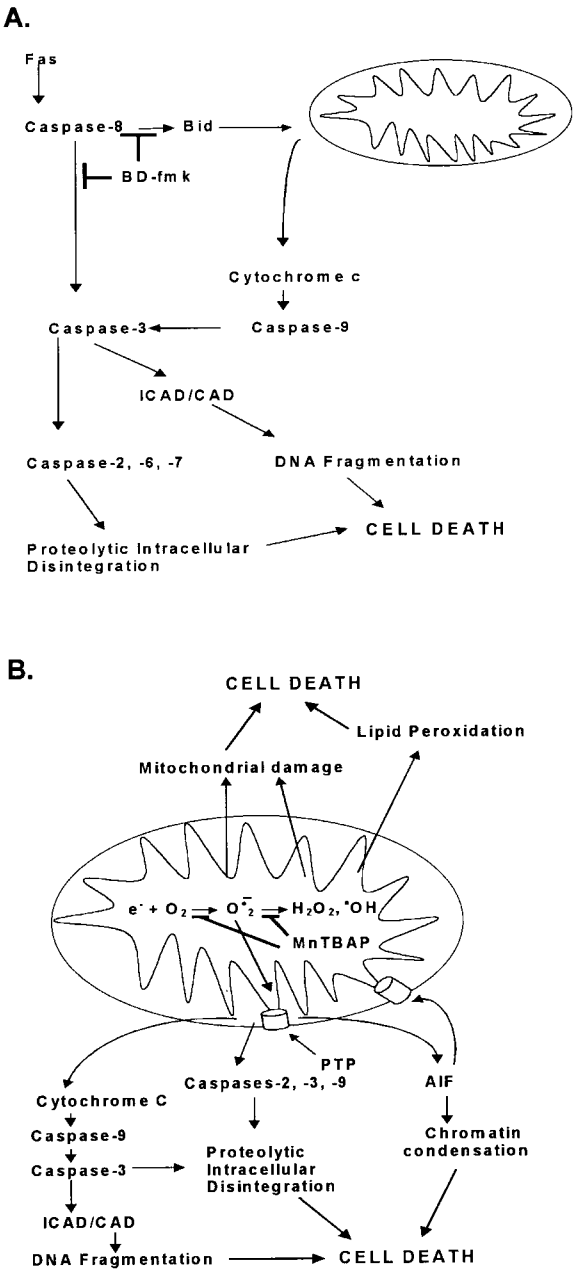

Figure 7. Comparison of Cell Surface Death Receptor–Mediated Apoptosis and ROS-Mediated Apoptosis

(A) Extracellular death receptors provide direct caspase activation and cleavage of Bid, which can lead to cytochrome c release from mitochondria and further amplification of caspase activation. It is important to note that Bid causes release of cytochrome c prior to the appearance of mitochondrial permeability transition (Luo et al., 1998). In this form of apoptosis, caspase inhibitors prevent mitochondrial damage and cell death, but MnTBAP has no effect on cell death.

(B) Superoxide-mediated apoptosis leads to caspase activation but also to the production of other toxic ROS such as H_20_2 and ·OH. Superoxide, hydrogen peroxide, and hydroxyl radical can cause damage to mitochondrial enzymes and initiate lipid peroxidation, which can lead to cell death (Halliwell and Gutteridge, 1985; Patel et al., 1996). In this form of apoptosis, caspase inhibitors do not prevent mitochondrial damage and subsequent cell death, but MnTBAP prevents both mitochondrial damage and caspase-mediated damage. PTP denotes permeability transition pore.

a factor that is known to increase the susceptibility of T cells to Fas-induced death (Lenardo, 1991; Zheng et al., 1998). Whatever the reasons for the conflicting results, in our hands, both in vivo and in vitro, the death of primary activated T cells did not require Fas or TNFα receptor expression.

In this paper, we show that activated T cells died more rapidly than did resting T cells, independently of Fas or TNFR signaling. The rapid death of these primary activated T cells probably involved superoxide, a species that is, itself, toxic for cells and can lead to the production of even more toxic species such as hydrogen peroxide, hydroxyl radical, and peroxynitrite (Halliwell and Gutteridge, 1985). Two pieces of evidence shown in this paper support this idea. First, the activated T cells contained more superoxide, as assessed by dihydroethidium staining, than their resting counterparts. This superoxide was probably produced by the increased rates of respiration within the activated cells, since superoxide is known to be a byproduct of the mitochondrial electron transport chain (Halliwell and Gutteridge, 1985). Second, we showed that activated T cell death was prevented by MnTBAP. MnTBAP has been shown to protect cells from superoxide-mediated toxicity in neural cells and endothelial cells, likely through its mimicry of superoxide dismutase and/or catalase (Day et al., 1995, 1997; Patel et al., 1996; Patel, 1998). MnTBAP has an additional activity, the ability to scavenge peroxynitrite (Zingarelli et al., 1997). However, we do not think this activity is involved in the protective effects of MnTBAP because inhibitors of nitric oxide production, such as S-methyl isothiourea, had no effect on the deaths of activated T cells (data not shown). Thus, MnTBAP probably acts by detoxifying superoxide and hydrogen peroxide into oxygen and water, thereby protecting cells from damage mediated by superoxide and hydrogen peroxide.

The detoxification of superoxide by SOD leads to the generation of more toxic ROS, like hydrogen peroxide, which are in turn detoxified by other cellular enzymes such as catalase and glutathione peroxidase. Thus, balances in the activities of these antioxidant enzymes are important in protecting cell damage from oxygen toxicity. Because MnTBAP has both superoxide dismutase and catalase activity, it can substitute for these enzymes and thus ensure the complete detoxification of superoxide and its downstream metabolites. MnTBAP may, therefore, be particularly useful in preventing cellular damage in which superoxide is the initiating ROS.

Another question that warrants addressing is whether loss of $\Delta\psi_m$ is responsible for the increase in ROS or the other way around. A previous report showed that, in dexamethasone-induced death, ROS were only generated after loss of $\Delta\psi_m$ (Zamzami et al., 1995a). However, since the mitochondrial permeability transition pore (PTP) has been shown to be sensitive to ROS (Takeyama et al., 1993; Zoratti and Szabo, 1995; Kroemer et al., 1997; Chakraborti et al., 1999), it is possible that in activated T cells ROS are generated first and the PTPs open subsequently, thus leading to loss of $\Delta\psi_m$. Indeed, others have postulated that ROS may play a dual role in apoptosis, either as an inducer of permeability transition or a consequence of permeabilty transition, depending upon the death stimulus (Zoratti and Szabo, 1995; Kroemer et al., 1997). In our experiments, the reagent that decreased superoxide levels, MnTBAP, also blocked loss of $\Delta\psi_m$. Thus, in these activated T cells, it is likely that ROS are generated and cause opening of the PTPs and the loss of $\Delta\psi_m$.

Others have previously shown that loss of $\Delta\psi_m$ is a very early event in the death of lymphocytes activated with SAg in vivo and occurs prior to DNA fragmentation (Zamzami et al., 1995b). The data in this paper confirm this observation and also show that loss of $\Delta\psi_m$ and DNA damage are differentially sensitive to caspase inhibitors. Thus, caspase inhibitors blocked DNA loss but not loss of $\Delta\psi_m$. Contrastingly, incubation with MnTBAP prevented both loss of $\Delta\psi_m$ and DNA degradation. Taken together, these results suggest that, in this type of activated T cell death, caspase activation is a secondary event, downstream of mitochondrial dysfunction, and that mitochondrial dysfunction alone is sufficient for cell death (Figure 7). Caspase activation in this system is probably caused by release of cytochrome C, which may or may not be dependent on the release of apoptosis-inducing factor from mitochondria (Susin et al., 1999). In confirmation of this idea, we have observed release of cytochrome C into the cytosol of dying activated T cells (data not shown).

The caspase-independent death described in other reports has sometimes been termed necrotic (Kroemer et al., 1997, 1998). However, in at least two reports, features of apoptosis, such as membrane blebbing, PS exposure, and partial chromatin condensation, were observed (McCarthey et al., 1997; Deas et al., 1998). In the dying activated T cells in this report, we observed rapid acquisition of cell membrane permeability, a characteristic of necrosis. On the other hand, the cells exposed PS prior to their acquisition of membrane permeability, underwent rapid DNA fragmentation, and were clearly apoptotic as viewed by electron microscopy. Therefore, it appears that these cells exhibited the primary features of apoptosis but underwent a very rapid secondary necrosis. MnTBAP inhibited both types of death within these activated T cells, suggesting that ROS can be a signal that regulates both necrotic and apoptotic cell death.

In summary, we have provided evidence for ROS as decisive contributors to the death of activated T cells. The ROS lead to cell death by at least two pathways, one mediated by caspase activation and subsequent proteolytic cellular disintegration and the other driven by ROS themselves. This explains why caspase inhibitors have no affect on the death of cells in this system despite their complete effectiveness in inhibiting caspases. These results provide a mechanism to account for several previous descriptions of caspase-independent cell death. The fact that T cells contain several pathways that lead to cell death may ensure that an animal clears itself of unneeded and perhaps damaging collections of activated antigen-specific T cells after the antigen has been eliminated.

Experimental Procedures

Mice and Injections

Female B10.BR/SgSnJ (H-2^k) and C57BL/10SnJ (H-2^b) mice, purchased from the Jackson Laboratory, were used between 8–12

weeks of age. Heterozygous breeding pairs of *lpr*$^{+/-}$, p55$^{+/-}$, p75$^{+/-}$ mutant mice, a generous gift from Dr. David Lynch (Immunex), were used to produce *lpr*$^{-/-}$, p55$^{-/-}$, p75$^{-/-}$ and heterozygous mice. Such animals were used between 4 and 5 weeks of age since the homozygous-deficient animals began to accumulate CD4$^-$, CD8$^-$, B220$^+$ T cells after 6 weeks of age (Laouar and Ezine, 1994). Mutant genotypes of *lpr*, p55$^{-/-}$, p75$^{-/-}$ mice were confirmed by PCR on genomic DNA from ear clips. All animals were kept under specific pathogen-free conditions in the Biological Resource Center at National Jewish Medical and Research Center. Mice were injected via the tail vein (i.v.) with either 0.1 μg SEA (Sigma) or 150 μg SEB (Sigma) in a volume of 0.25 ml balanced salt solution (BSS).

Cell Culture and Inhibitor Assays

Inguinal, brachial, axillary, and superficial cervical lymph nodes (LN) were harvested and single cell suspensions generated by passage through 100 micron nylon cell strainers (Falcon). T cells were purified from LN on nylon wool columns as described previously (Julius et al., 1973). In brief, 20 ml syringes were filled with washed nylon wool and sterilized by autoclaving. Cells were added to prewarmed nylon wool columns, incubated for 1½ hr at 37°C, and eluted with warm BSS containing 5% FBS. This method consistently results in the purification of greater than 90% viable T cells. Following purification, T cells were resuspended in complete tumor medium (CTM) (DMEM containing 10% FBS, sodium bicarbonate, penicillin/streptomycin, 2-mercaptoethanol, sodium pyruvate, and nonessential amino acids) at 1.5 × 10^6 cells/ml and were cultured in triplicate in 96-well flat-bottomed plates.

BD-fmk and ZFA-fmk were purchased from Enzyme Systems Products, diluted to a 50 mM working stock in DMSO, and kept at 4°C before diluting in CTM for use in blocking experiments. MnTBAP was made as described (Melov et al., 1998). In brief, H_2TBAP was dissolved in dimethylformamide (DMF), refluxed overnight at 80°C in the presence of excess manganese chloride, and bubbled with room air. The reaction product was chilled in an ice bath, and the precipitate was collected. The precipitate was washed with ice-cold water and dried in a vacuum oven. The product, termed MnTBAP in this report, was found to be >91% pure by HPLC analysis using a Microsorb MVC18 column. MnTBAP was dissolved in 0.1 M NaOH before use.

L1210 cells transfected with mouse Fas cDNA was a gift from Dr. R. Duke (University of Colorado Health Sciences Center, Denver, CO). Death of L1210Fas$^+$ cells was induced by plating cells in wells previously coated with 100 μg/ml anti-Fas (clone Jo2, PharMingen). HT-2 (an IL-2-dependent helper T cell clone) was a kind gift of Dr. James Watson (University of California, Irvine, CA). Death of HT-2 cells was induced by withdrawal from IL-2. FasIg was purified from 3T3-FasIg cells (a gift from Dr. Shr-Te Ju, Boston University School of Medicine) by passage of their supernatant over a protein A column. The eluted FasIg was tested for its ability to block the death of D0-11.10 T hybridoma cells stimulated with anti-CD3 monoclonal antibody as described (Brunner et al., 1995).

Flow Cytometry and Cell Sorting

Two-color flow cytometric staining was performed by incubating LN T cells with either anti-Vβ3-FITC (clone KJ25A) or anti-Vβ8-FITC (clone F23.1) in the presence of blocking solution (anti-Fc receptor, 2.4G2 supernatant containing 10 μg/ml human gamma globulin, and 5% normal mouse serum) for 40 min at 4°C. Cells were then washed twice with BSS wash buffer (BSS containing 2% FBS). To assess DNA content, stained cells were resuspended in 200 μl of a solution containing 0.03% saponin (Sigma), 5 μg/ml propidium iodide, 50 μg/ml RNAase, and 5 mM EDTA, and incubated at room temperature for 25 min in the dark. FITC$^+$ cells having less FL2 fluorescence than that in the 2N DNA peak were considered having subG0 DNA.

To assess phosphatidyl serine exposure, purified LN T cells were first stained with either phycoerythrin-labeled anti-Vβ3 (clone KJ25A), anti-Vβ8.X (clone F23.1), or anti-Vβ6 (clone RR4-7) in the presence of blocking solution for 40 min at 4°C. Cells were washed twice with Annexin V binding buffer (BSS containing 2.5 mM $CaCl_2$) and stained with Annexin V-FITC conjugate (R & D Systems) for 15 min at room temperature. Cells were resuspended in ice-cold binding buffer, and data on the stained cells were acquired on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). To assess membrane permeability, purified LN T cells were first stained with either FITC-labeled anti-Vβ3 (clone KJ25A), anti-Vβ8.X (clone F23.1), or anti-Vβ6 (clone RR4-7) in the presence of blocking solution for 40 min at 4°C. Stained cells were washed twice and resuspended in BSS wash buffer containing 0.5 μg/ml propidium iodide, and data on the stained cells were acquired on a FACScan flow cytometer. Data for at least 10^4 cells were analyzed using PC Lysis II software (Becton Dickinson). FITC$^+$ cells were considered dead if they were also FL2$^+$. Dihydroethidium (Molecular Probes) staining was performed by incubating T cells in CTM containing 2.5 μM dihydroethidium for 40 min at 37°C. $DiOC_6$ (Molecular Probes) staining was performed by incubating T cells in CTM containing 25 nM $DiOC_6$ for 40 min at 37°C. Anti-TCR Vβ costains with these dyes were performed at 4°C, prior to incubation with the dyes.

For cell sorting, nylon wool purified LN T cells from SEA-injected B10.Br mice were cultured for 4 hr in vitro followed by costaining with anti-Vβ3-FITC and anti-CD3-PE monoclonal antibodies. Twenty million cells were sorted using a MoFlo flow cytometer (CytoMation) into CD3$^+$ Vβ3$^+$ and CD3$^+$ Vβ3$^-$ populations and were greater than 95% pure.

Electron Microscopy

Sorted, SAg-activated CD3$^+$ Vβ3$^+$ or resting CD3$^+$ Vβ3$^-$ cells were fixed in 3% glutaraldehyde, stained with 1% osmium tetroxide, enrobed in seaplaque agarose, dehydrated with ethanol, and embedded in Epon/Araldite resin. Thin sections were cut, placed on butvar-coated 200 mesh copper grids, post stained with 3% aqueous uranyl acetate and Reynolds lead citrate, and observed in a Philips 400 transmission electron microscope. Apoptotic cells, characterized by chromatin condensation and cytoplasmic shrinkage were counted, along with intact nonapoptotic cells, in ten randomly selected fields. Percent apoptotic cells ± SD was calculated as follows: number of apoptotic cells/total cells × 100 = percent apoptotic cells.

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