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*J Immunol* 2004; 173:6611-6618; doi: 10.4049/jimmunol.173.11.6611

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The Contraction Phase of Virus-Specific CD8\(^+\) T Cells Is Unaffected by a Pan-Caspase Inhibitor\(^1\)

Alexander K. Nussbaum and J. Lindsay Whitton\(^2\)

The effectiveness of protection conferred by CD8\(^+\) memory T cells is determined by both their quality and their quantity, which suggests that vaccine efficacy might be improved if it were possible to increase the size of the memory pool. Approximately 90% of virus-specific CD8\(^+\) T cells die during the contraction phase and, herein, we have attempted to increase the memory pool by reducing CD8\(^+\) T cell death. CD8\(^+\) T cell contraction has been attributed to apoptosis, or programmed cell death (PCD), which, classically, is dependent on caspases. Caspase-dependent PCD can be prevented by the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone (zVAD), and here we evaluate the effect of this compound on virus-specific T cell responses in mice. zVAD prevented caspase-dependent PCD of freshly isolated virus-specific T cells in tissue culture, and a fluorescent analog, FITC-VAD, entered CD8\(^+\) T cells following in vivo injection. However, despite using 11 different regimens of zVAD administration in vivo, no significant effects on CD8\(^+\) or CD4\(^+\) memory T cell numbers were observed. Furthermore, the CD8\(^+\) memory T cell responses to secondary virus infection were indistinguishable, both qualitatively and quantitatively, in zVAD-treated and normal mice. The absence of effect cannot be attributed to a technical flaw, because identical doses of zVAD were able to rescue mice from hepatocyte apoptosis and lethal intrahepatic hemorrhage, induced by inoculation of anti-Fas Ab. We conclude that the contraction phase of the virus-specific T cell response is unlikely to require caspase-dependent PCD. We propose that contraction can be mediated by an alternative, caspase-independent pathway(s). The Journal of Immunology, 2004, 173: 6611–6618.

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\(^{1}\) This work was supported by National Institutes of Health Grant R-01 AI-27028 (to J.L.W.), A.K.N. was supported by a long-term fellowship (LT00410/2001-M) from the International Human Frontier Science Program organization.

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\(^{3}\) Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; p.i., postinfection; zVAD, benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone; AICD, activation-induced cell death; ACAD, activated T cell autonomous death; PCD, programmed cell death; SIS, staurosporine; ICCS, intracellular cytokine staining.
various agents. The most widely used is the cell-permeable, irreversible pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone (zVAD). In vivo uses of zVAD include mouse models of fulminant liver destruction (34, 35), meningitis (36), amyotrophic lateral sclerosis (37), and sepsis (38, 39). In the latter, zVAD was shown to prevent sepsis-induced apoptosis of T lymphocytes, encouraging us to test it herein. Thus, in this manuscript we report the effects of zVAD on the contraction of virus-specific CD8+ T cells.

Materials and Methods

Mice

C57BL/6 (H-2b) and BALB/c (H-2d) mice were obtained from The Scripps Research Institute breeding colony, and CB6 mice (F1 offspring of BALB/c and C57BL/6 mating; H-2b) were obtained from the same source or from The Jackson Laboratory (Bar Harbor, ME). Female mice were used, at 8–12 wk of age.

Infection of mice with LCMV

Primary LCMV infections were conducted by injecting mice i.p. with 2 × 106 PFU of LCMV, Armstrong strain. For secondary infections, mice were injected i.p. with 106 PFU of the same virus.

Administration of the apoptosis inhibitor zVAD

The irreversible, cell-permeable, broad-spectrum caspase inhibitor zVAD (Enzyme Systems Products, Livermore, CA) was dissolved in DMSO and then diluted in sterile saline to a final DMSO concentration of 20%. The solution was administered i.p. or i.v., at doses indicated in the text, in a total volume of up to 200 μl. In all cases, zVAD and DMSO were tolerated without apparent detrimental effects.

Ex vivo induction of caspase-dependent apoptosis in virus-specific CD8+ T cells

Eight days after LCMV infection, a CB6 mouse was sacrificed and the spleen removed. The splenocytes were divided into seven samples, each containing 3 × 106 cells. zVAD was added to six samples, to final concentrations of 6.75, 12.5, 25, 50, 100, or 200 μM. Each of the seven samples was divided in two, generating two groups of seven paired samples; and to one of these groups, staurosporine (StS; Sigma-Aldrich, St. Louis, MO) was added, to a final concentration of 5 μM. StS has been shown to induce caspase-dependent apoptosis (40). Seven hours later, the proportion of NP118–126-specific cells showing signs of apoptosis was determined. Cells were stained with anti-CD3-Dr-Cy5 (Caltag, Burlingame, CA) and PE-labeled NP118–126-specific tetramer (obtained from the National Institutes of Health tetramer core) carrying the NP118 peptide. Staining for tetramer-binding cells was done for 30 min on ice. Samples were resuspended in PBS containing 2% formaldehyde, acquired on a FACScan flow cytometer (50,000–100,000 events acquired per sample), and analyzed with CellQuest software (Becton Dickinson, San Jose, CA).

Assessment of anti-Fas-mAb-induced liver damage

To test the efficacy of zVAD injections, liver disease was induced in CB6 mice by a single i.v. injection of 10 μg of anti-Fas mAb (clone Jo-2; Pharmingen) in 100 μl of sterile saline. For the prevention of anti-Fas-mediated death and liver destruction, a single injection (i.v. or i.p.) of 1 mg of zVAD was given 90 min before Ab inoculation. Surviving mice were sacrificed 100 h after Fas injection, at which time all mice were clinically normal. Livers were collected, fixed in 10% neutral-buffered formaldehyde (Sigma), and then paraffin-embedded. Five-micrometer tissue sections were cut, stained with H&E, and evaluated on a Zeiss Axioshot microscope.

TUNEL assay for detection of apoptotic cells in tissue sections

To identify DNA breaks in 5-μm sections of liver tissue, the ApopTag Red kit (Serologicals Corporation) was used, and sections were counterstained with DAPI (300 nM in PBS; Molecular Probes, Eugene OR).

Results

zVAD inhibits apoptosis of freshly isolated virus-specific CD8+ T cells in tissue culture

To form a foundation for this study, we wished first to determine whether zVAD could protect activated virus-specific CD8+ T cells from caspase-dependent apoptosis. Splenocytes were isolated from LCMV-infected CB6 mice on day 8 after virus infection, at the peak of the antiviral CD8+ T cell response, when ≥50% of the CD8+ T cells are specific for a single epitope (NP118–126). These cells can be readily identified by tetramer staining. Some of these freshly isolated splenocytes were incubated with 5 μM StS, to trigger apoptosis, in the presence or absence of various concentrations of zVAD (see Materials and Methods). Seven hours later, NP118–126+specific cells were identified by tetramer staining, and the proportion of those cells showing signs of apoptosis was determined by TUNEL staining. Only ~14% of the cells that had not been exposed to StS were TUNEL+, and this was true at all concentrations of zVAD (Fig. 1A, gray bar). The proportion of apoptotic cells was greatly increased by StS treatment, as expected; some 47% of cells were TUNEL+ in the absence of zVAD. Importantly, zVAD treatment dramatically reduced apoptosis in these activated virus-specific T cells. The viability of NP118–126+specific cells also was measured, using flow cytometry (Fig. 1B). Approximately 80–90% of day 8 NP118–126+specific T cells were viable in the absence of StS treatment (gray bar), and StS (in the absence of zVAD) reduced viability to ~43%. However, zVAD treatment was effective in rescuing T cell viability, which was increased by ~50% by a zVAD dose as low as ~12.5 μM; higher zVAD doses restored T cell viability to the levels seen in the absence of StS treatment. We conclude that caspase-dependent PCD in freshly
isolated, highly activated virus-specific CD8\(^+\) T cells can be prevented by zVAD.

A zVAD analog enters CD8\(^+\) T cells when inoculated during virus infection

Having shown that T cell apoptosis in tissue culture can be prevented by zVAD, we next attempted to ensure that the compound reached T cells following in vivo injection. For this purpose, we used FITC-VAD, a fluorescently labeled analog of zVAD that binds to activated caspases; activated T cells entering the death phase would, therefore, be expected to bind higher amounts of this compound. FITC-VAD was injected into BALB/c mice 8 days after LCMV infection, or into uninfected mice, and 3 h later the mice were sacrificed, their spleens were harvested, and the splenocytes were fixed and washed repeatedly before evaluation by flow cytometry. As shown in Fig. 2A, splenocytes from uninfected mice were almost uniformly FITC-VAD\(^{\text{low}}\), indicating that these cells have low levels of activated caspases. In contrast, in mice inoculated 8 days after LCMV infection, FITC-VAD\(^{\text{high}}\) cells were readily detectable, and the number of FITC-VAD\(^{\text{high}}\) CD8\(^+\) T cells (upper right quadrants) had increased by >20-fold compared with uninfected animals. To evaluate FITC-VAD uptake by virus-specific CD8\(^+\) T cells, NP\(_{118-126}\)-specific cells in a day 8 mouse were identified using the NP\(_{118-126}\) tetramer; as shown in Fig. 2B, almost 7% of these CD8\(^+\) T cells were FITC-VAD\(^{\text{high}}\). The approximate doubling of signal between un gated and tetramer-gated CD8\(^+\) T cells (Fig. 2A and B, respectively) is consistent with the fact that NP\(_{118}\)-specific T cells comprise ~50% of the CD8\(^+\) T cell population at this time point. The background flow cytometry signal from tetramer-gated cells from a day 8 mouse that had not received FITC-VAD also is shown in Fig. 2B.

These data show that an analog of zVAD can enter CD8\(^+\) T cells following in vivo injection, and that binding is increased during virus infection, consistent with some of the virus-specific cells having activated caspases at 8 days p.i. That only ~7% of NP\(_{118}\)-specific CD8\(^+\) T cells bind zVAD is, at first, surprising, because, at 8 days p.i., >50% of CD8\(^+\) T cells are specific for this epitope. However, there is no reason to believe that the virus-specific cells are precisely synchronized; some will still be expanding, while others will be entering—or will have entered—the death phase. Therefore, during the relatively brief time of in vivo FITC-VAD exposure (~3 h), only a subpopulation of virus-specific CD8\(^+\) T cells would be expected to contain activated caspases. This experiment also may underestimate the amount of zVAD that actually reaches T cells in any of the 11 administration regimens described below, for two reasons. First, a single dose of FITC-VAD was administered, whereas multiple doses were given in eight of the 11 in vivo regimens. Second, FITC-VAD uptake by T cells was evaluated after only 3 h, whereas zVAD was allowed to exert its effect over a much longer time period, as described below.

Eleven different zVAD regimens fail to significantly change T cell responses after LCMV infection of naive mice

Having shown that zVAD can rescue T cells from apoptosis, and that it binds to CD8\(^+\) T cells after in vivo injection, we next tested the hypothesis that zVAD administration might increase the number of CD8\(^+\) T cells that survived the contraction phase to enter the memory pool. We chose to test this hypothesis using LCMV infection because this virus induces an enormous, and well-characterized, expansion of CD8\(^+\) T cells; the subsequent contraction phase therefore encompasses a very large number of T cells, thereby providing us with a sizeable target population for zVAD therapy. Mice were infected with LCMV and, at various times p.i., zVAD was administered. Eleven different regimens of zVAD administration were compared, in three separate experiments denoted A, B, and C; each experiment included control mice that did not receive zVAD. The experiments and regimens are summarized in Fig. 3.

Experiment A. In the first, pilot, experiment, CB6 mice (H-2\(^b\)) received six different treatments as shown in Fig. 3, comprising one to three doses of zVAD during the contraction phase (0.5 mg i.v. per injection). On days 16 and 35 p.i. (the peak of the death phase and early in the memory phase, respectively), mice were sacrificed, and their spleens were used as a source of LCMV-specific T cells. The responses to four epitopes were analyzed, spanning the immunodominance hierarchy (Fig. 4). CD8\(^+\) T cell responses to the dominant epitope (NP\(_{118-126}\)) were high in all mice, as expected. The responses in most of the zVAD-treated
groups (A2–A7) were marginally elevated compared with the untreated control group (A1), but the difference was not statistically significant. Similar results were observed for CD8$^+$ T cells specific for the remaining three epitopes shown in Fig. 4, and also for CD4$^+$ T cells specific for the GP$_{61}$ and NP$_{309}$ epitopes (not shown). Any differences observed were not statistically significant. Not only were the proportions of responding T cells similar, but the absolute numbers also were comparable. For example, the total number of splenic CD8$^+$ T cells specific for these four epitopes was $10^7$ in untreated mice (group A1); $1.1 \times 10^7$ in group A3; and $8.7 \times 10^6$ in group A4. These data indicate that these six zVAD administration regimens had little effect on virus-specific T cells at this stage of the contraction phase. Nor did these regimens lead to significant changes in the memory phase; at 35 days p.i., for each of the four epitope-specific populations, the proportion of responding cells was similar in the untreated and zVAD-treated groups. Again, the same was true for LCMV-specific CD4$^+$ T cells (not shown).

**Experiment B.** The absence of a marked effect in the pilot experiment led us to make five changes to the experimental regimen: 1) the number of doses of zVAD was increased to four; 2) the interval between doses was reduced to 2 days; 3) the drug was delivered at two doses, 0.25 mg/injection (group B2) or 1 mg/injection (B3); 4) the experiment was extended until 500 days p.i., to determine whether zVAD had long-term effects; and 5) blood samples were drawn for analysis, which allowed us to maintain the same sample size at each time point, and to follow any changes in T cell response in individual BALB/c mice. The data for NP$_{118-126}$-specific T cells in the blood at 17, 39, and 500 days p.i. are shown in Fig. 4. To facilitate the evaluation of any effects of zVAD, at each time point the average response in untreated mice (group B1) is assigned the value 1.0, and the responses in the zVAD-treated groups are shown as a multiples of this response. The data, which are representative for experiment B, indicate that—as for experiment A—zVAD has no significant positive effect on the levels of CD8$^+$ T cell memory, even as late as 500 days p.i.

**Experiment C.** The expansion and contraction phases overlap, and caspase-3 activation in LCMV-specific CD8$^+$ T cells has been observed as early as day 5 post LCMV infection (42), so we considered it possible that the earlier administration of zVAD might have some beneficial effect on the subsequent memory pool. Therefore, 1 mg of zVAD was administered on three consecutive days to LCMV-infected CB6 mice, using three regimens beginning as early as day 4 p.i. (Fig. 3). This is early in the expansion phase, as indicated by the fact that LCMV-specific T cell responses are barely detectable at this time point in previously naive mice (43).

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**FIGURE 2.** zVAD enters CD8$^+$ T cells when inoculated during virus infection. FITC-VAD (dissolved in saline/40% DMSO) was injected into naive mice, or mice at 8 days after LCMV infection. As a control, a day 8 mouse was inoculated with carrier alone. Three hours later, splenocytes were harvested, washed, and analyzed by flow cytometry. A shows staining in splenocytes from an uninfected mouse (left) and from a day 8 mouse (right). B shows staining of cells from day 8 mice, gated on LCMV-specific CD8$^+$ T cells using the NP$_{118}$ tetramer. Left, Cells from a mouse that received only carrier. Right, Cells from a mouse that received FITC-VAD. For each sample, the percentage of CD8$^+$ T cells that was FITC$^+$ is shown in the upper right quadrant.

**FIGURE 3.** Eleven regimens of zVAD administration, divided among three experiments: A diagrammatic representation of the three experiments, encompassing 11 distinct zVAD administration regimens. Each symbol represents a single administration of zVAD. Experiment A comprised a control group (A1), and six zVAD regimens, each dose being 0.5 mg of zVAD. Experiment B had a control group (B1) and two zVAD regimens that were identical in times of administration, but differed in dose (•, 0.25 mg/dose; ⊗, 1 mg/dose). Experiment C had a control group (C1) and three zVAD regimens (1 mg/dose).
However, neither this early treatment (group C2), nor either of the other two zVAD regimens, significantly altered the responses to either dominant or subdominant epitopes at 11, 20, or 39 days p.i. (Fig. 4). Analyses of spleens and lymph nodes at 556 days p.i. also showed no significant differences among the four experimental groups (not shown). We conclude that none of the 11 zVAD regimens had any statistically significant positive effect on CD8$^+$ or CD4$^+$ T cell responses to dominant or subdominant epitopes, at any time during the contraction or memory phases. These results suggest that the contraction of virus-specific T cells can occur independently of caspases.

Secondary CD8$^+$ T cell numbers, function, and phenotype are not significantly changed in zVAD-treated mice

Having shown that there was no substantial change in size of the memory pool following zVAD treatment, we next investigated whether these memory cells could respond normally to secondary virus infection. At 83 days after the primary LCMV infection, mice were taken from each of the four groups of CB6 mice in experiment C and were re-infected with LCMV. Four days later, at the peak of the secondary T cell response, the number, function, and phenotype of the virus-specific CD8$^+$ T cells were evaluated. The numbers and proportions of LCMV-specific CD8$^+$ T cells in the spleen and lymph nodes were very similar in all four experimental groups, for two different virus epitopes (NP118-126 and NP396-404), both by ICCS (Fig. 5A) and by tetramer staining (for NP118-126, not shown). Furthermore, the production of IFN-γ in response to peptide stimulation (Fig. 5A) indicates that the responding memory cells have retained this key effector function.

Next, we analyzed the surface markers expressed by virus-specific cells during secondary infection (Fig. 5B). The lymph node homing receptors CCR7 and CD62L have been said to distinguish between functionally distinct subsets of CD8$^+$ memory T cells, the so-called central memory cells (CCR7$^+$, CD62L$^-$, lacking effector function) and effector memory cells (CCR7$^-$, CD62L$^+$, which have effector functions) (44). This classification has recently been challenged, because it is clear that CCR7$^+$ cells can synthesize cytokines immediately upon Ag contact and, therefore, should be considered effector cells (45–47); down-regulation of CCR7 on memory cells occurs soon after infection, and the majority of virus-specific cells are CCR7$^-$ by 4 days after secondary infection. We have confirmed these findings and, as shown in Fig. 5B, at 4 days after secondary infection of untreated mice, NP118-126-specific CD8$^+$ T cells in both the spleen and lymph nodes were CD62L$^+$ and CCR7$^*$. Most importantly for the present study,

**FIGURE 4.** Eleven different zVAD regimens fail to significantly alter CD8$^+$ T cell responses following LCMV infection of naive mice. Representative data from each of the three experiments, and all of the experimental groups, that are summarized in Fig. 3. Experiment A. The percentages of CD8$^+$ T cells (y-axes) that respond to each of the four indicated epitope peptides (x-axes) at days 16 and 35 following LCMV infection are shown. Each of the seven experimental groups is color coded. None of the differences were statistically significant. Experiments B and C. At each of the indicated time points p.i. (x-axes), the CD8$^+$ T cell responses to the indicated epitopes were determined. To facilitate comparison of responses in zVAD-treated and untreated mice against the various epitopes, the data are presented as follows: at each time point, for each epitope, the peptide-specific response in control mice (B1, C1) was accorded the value 1.0 (white bars, shown ± SD); then, the epitope-specific responses in the zVAD-treated mice were plotted as multiples of the untreated response (again, shown ± SD). This form of presentation shows clearly that zVAD failed to induce any marked changes, at any of the time points analyzed.
Fifteen CB6 mice were injected with 10 g of anti-Fas Ab (clone Jo-2). Ten of the mice received a single 1-mg dose of zVAD (the same dose administered up to four times in some of the failed regimens described above), and five mice were untreated. As shown in Fig. 6A, all of the mice in the untreated group succumbed within 2.5 h of the anti-Fas Ab injection. In contrast, 50% of the mice in the zVAD-treated group survived the anti-Fas injection, and the deaths of the remaining mice were substantially delayed compared with the untreated group; the difference between the treated and untreated groups was highly significant (p < 8 × 10^{-5}). Histological analysis revealed extensive intrahepatic hemorrhage in mice that had received the Ab, but no zVAD (Fig. 6B, upper row, center panel); and this hemorrhage was absent from mice that had been treated with zVAD (right panel). The liver sections also were evaluated for the presence of apoptotic cells. The sections were stained with DAPI, to identify all nuclei, and with the TUNEL reagent, which results in red nuclear staining of apoptotic cells. Numerous apoptotic cells were present in the livers of mice that had received anti-Fas without zVAD (Fig. 6B, lower row, center panel). In contrast, the livers of mice that had received zVAD, and survived, showed few apoptotic nuclei (right panel), and often were indistinguishable from the livers of normal mice (left panel). Thus, the zVAD injections used throughout this study can have dramatic biological benefits; nevertheless, they fail to significantly alter T cell responses to virus infection.

**Discussion**

Ninety percent of virus-specific CD8+ T cells disappear during the contraction phase, and it has been thought that the death of virus-specific CD8+ T cells occurs via classical, i.e., caspase-dependent, apoptosis, perhaps related to AICD (26, 49). Caspase-dependent T cell apoptosis has been reported in human T cells during sepsis (50, 51), and a mouse model of sepsis not only confirms this observation but also indicates that T cell apoptosis can be minimized, and mouse survival enhanced, by the in vivo administration of zVAD (38, 39). It was, therefore, surprising to us to find that—despite exhaustive attempts, using 11 different zVAD regimens (Fig. 3), and evaluating T cell numbers and functions over a period of >500 days—the contraction of primary LCMV-specific CD8+ T cells was unaffected by this pan-caspase inhibitor (Fig. 4). In addition, the CD8+ memory T cells that developed after zVAD administration appeared normal in number, function, and phenotype (Fig. 5). These outcomes cannot be attributed to a technical
flaw in the preparation or inoculation of zVAD, because the compound was able to rescue mice from anti-Fas-induced lethal fulminant hepatitis (Fig. 6), a caspase-dependent process that involves both the receptor-mediated and mitochondrial PCD pathways (52–55). Thus, zVAD is capable of inhibiting caspase-dependent PCD in vivo, regardless of whether the process is triggered by the intrinsic or extrinsic pathway; yet it has no detectable effect on virus-specific T cells. Could caspase-dependent death of activated T cells be resistant to zVAD? We considered this to be unlikely, but we tested the hypothesis by using StS to induce caspase-dependent PCD in these cells, and evaluating the effects of zVAD. Splenocytes harvested at the peak of the antiviral CD8⁺ T cell response were rapidly driven into apoptosis by StS, but this was markedly diminished by zVAD (Fig. 1), indicating that, if PCD in T cells were caspase-driven, it should be ameliorated by zVAD. This suggested the possibility that zVAD could not gain access to the T cells in vivo, but our studies using a fluorescent analog (Fig. 2) indicated that this was not the case. Furthermore, our observation that hepatocytes, but not activated T cells, can be rescued by zVAD may be particularly relevant because the liver has been proposed as a “graveyard” for CD8⁺ T cells (56). Although some T cells may already be moribund upon arrival in the liver, it appears that apoptosis usually is induced after the cells have been retained in that organ (57). Thus, our observation that zVAD efficiently protected hepatocytes from anti-Fas-induced apoptosis indicates that zVAD is present, and active, in the very organ where many activated CD8⁺ T cells may undergo apoptosis; nevertheless, T cells remain apparently unaffected by the caspase inhibitor. These results have two implications for the notion that T cells die in the liver. Either all T cells that die in the liver are already moribund at the time they are retained, or programmed T cell death in the liver is caspase-independent.

Taken together, our data are most consistent with the idea that the contraction phase of the virus-specific CD8⁺ T cell response is independent of caspases. Caspase-independent PCD of immature and mature T cells has been reported (58–65), but its biological impact has not been widely appreciated, and its contributions to AICD and ACAD remain poorly characterized. AICD was believed to rely on receptor-mediated, caspase-dependent pathways of PCD, but a major effect of caspase-independent pathways has recently been suggested (60, 66). Bim, a pro-apoptotic member of the Bcl-2-family, is a candidate mediator of caspase-independent T cell death (67). Bim-deficient activated T cells are resistant to PCD (31), and Bim has been implicated in both AICD (68) and ACAD (69). Recent data indicate that Bim is crucial for the contraction of herpes simplex virus-specific CD8⁺ T cells (70). Taken together, the data in this article, and from other laboratories, suggest that i) the contraction phase of the virus-specific T cell response may be largely independent of caspases; and ii) Bim may play a central role throughout the contraction phase as the key mediator of the caspase-independent branches of both AICD and ACAD.

Caspase activation does not invariably reflect imminent apoptosis. Caspasess appear to play a role in T cell proliferation and early activation (reviewed in Ref. 71). For example, zVAD diminished mouse T cell proliferation, but not activation (72), and a recent study in an asthma mouse model attributed the beneficial in vivo effects of zVAD to the inhibition of T cell activation (73). In our hands, the levels of LCMV-specific CD8⁺ T cells were similar in zVAD-treated and nontreated mice at all times, and our data do not support a major role for caspases in T cell proliferation. However, the earliest time point at which we administered zVAD was 4 days p.i. (Fig. 3, group C2); we cannot exclude the possibility that earlier administration would reduce the initial proliferation of LCMV-specific CD8⁺ T cells.

In summary, our results support the notion that virus-specific CD8⁺ (and CD4⁺) T cells can undergo contraction in a caspase-independent fashion. These results are in line with the recent identification of Bim, a possible mediator of caspase-independent PCD, as a crucial contributor to CD8⁺ T cell contraction. Our data do not provide formal proof that the contraction of virus-specific T cells is exclusively caspase-independent. Rather, during contraction, as for other cases of PCD, caspases might be sufficient, but not required. Why might the host retain two distinct pathways by which its cells can undergo programmed death? First, some viruses express proteins that inhibit caspases, presumably to prevent cell suicide, thereby maintaining the environment for sustained viral replication (reviewed in Ref. 74); the existence of a separate, caspase-independent, pathway may protect the host against runaway replication of these viruses. Second, the contraction of a highly expanded T cell population is thought to be crucial to prevent immunopathology and autoimmunity, and reliance on a single group of proteases might be detrimental to the host. In this light, caspase-independent contraction might represent a detour, “fail-safe” pathway chosen in the presence of caspase inhibition; thus, in our studies, zVAD may have inhibited caspase-dependent PCD in virus-specific T cells, but the existence of a second pathway of cell death ensured that the contraction phase proceeded normally. A more complete understanding of the paths leading to T cell death may reveal targets that would permit the manipulation of memory T cell levels, with concomitant benefits for vaccination.

Acknowledgments

We are grateful to Annette Lord for excellent secretarial support and to Stephanie Harkins for technical assistance. This is manuscript number 16677-NP from the Scripps Research Institute.

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