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T-cell receptor ligation by peptide/MHC induces activation of a caspase in immature thymocytes: the molecular basis of negative selection

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T-cell receptors (TCRs) are created by a stochastic gene rearrangement process during thymocyte development, generating thymocytes bearing useful, as well as unwanted, specificities. Within the latter group, autoreactive thymocytes arise which are subsequently eliminated via a thymocyte-specific apoptotic mechanism, termed negative selection. The molecular basis of this deletion is unknown. Here, we show that TCR triggering by peptide/MHC ligands activates a caspase in double-positive (DP) $CD4^+CD8^+$ thymocytes, resulting in their death. Inhibition of this enzymatic activity prevents antigen-induced death of DP thymocytes in fetal thymic organ culture (FTOC) from TCR transgenic mice as well as apoptosis induced by anti-CD3ε monoclonal antibody and corticosteroids in FTOC of normal C57BL/6 mice. Hence, a common caspase mediates immature thymocyte susceptibility to cell death.

Keywords: apoptosis/caspase/negative selection/thymus

Introduction

The T-cell repertoire is generated through a tightly regulated developmental program of selection or 'filtering' in which thymocytes bearing immunologically desirable T-cell receptor (TCR) specificities are preserved and those expressing harmful specificities are eliminated (reviewed in Fowlkes and Pardoll, 1989). T-lineage cells expressing autoreactive TCRs are deleted in the thymus via a process termed negative selection (reviewed in Nossal, 1994). Multiple lines of evidence utilizing both normal and transgenic mice show that this negative selection process occurs during a restricted stage(s) of thymic differentiation (Fowlkes *et al.*, 1988; Kisielow *et al.*, 1988; Fowlkes and Pardoll, 1989; Murphy *et al.*, 1990; Vasquez *et al.*, 1992; Sebzda *et al.*, 1994). The deletion process requires TCR recognition of antigenic peptides displayed on APCs in complex with MHC class I or class II molecules and generally involves thymocytes at the DP stage of development.

Although the mechanism of negative selection is unknown, the targeted thymocytes die via apoptosis (Murphy *et al.*, 1990), a physiologically controlled form of cell death utilized by metazoan organisms during normal development as well as for homeostasis (Vaux, 1993; Steller, 1995; Vaux and Strasser, 1996). Apoptosis occurs as a consequence of extracellular signaling events such as crosslinking of certain receptors including CD95 (Trauth *et al.*, 1989; Yonehara *et al.*, 1989; Itoh and Nagata, 1993; Alderson *et al.*, 1994; Takahashi *et al.*, 1994), TNF receptors (Tartaglia *et al.*, 1993) and a recently identified Death Receptor 3 (DR3) (Chinnaiyan *et al.*, 1996a) or as a result of growth factor withdrawal (reviewed in Yang and Korsmeyer, 1996). Upon induction of apoptosis, cells undergo a morphologically characteristic process of nuclear condensation, blebbing of cellular membranes and disintegration into small fragments which are removed by phagocytes (Vaux, 1993; Vaux and Strasser, 1996; for review see Steller, 1995).

Recent analyses of the death pathways in various systems have begun to delineate the biochemical basis of apoptosis. Particularly, activation of ICE-like cysteine proteases now termed caspases has proven to be a hallmark of apoptotic death (reviewed in Nalin, 1995; Henkart, 1996). There are presently 10 human homologues of the ced 3 cysteine protease first defined by genetic analysis of cell death in *Caenorhabditis elegans* (Alnemri *et al.*, 1996; Duan *et al.*, 1996a; Fernandes-Alnemri *et al.*, 1996; Muzio *et al.*, 1996; reviewed in Henkart, 1996). These enzymes exist as inactive proenzymes which become active when cleaved to subunits of ~17–20 kDa and ~10–12 kDa. The molecular structures of ICE (caspase-1) and Yama (caspase-3) have been determined (Walker *et al.*, 1994; Wilson *et al.*, 1994; Rotonda *et al.*, 1996). The active enzymes exist as tetramers made up of two large and two small subunits. Comparison of these structures suggests that the caspase family falls into two major groups: those that most resemble caspase-1 and those that most resemble caspase-3 (and also ced-3). The substrate binding pockets of these two groups have distinct differences pointing to potentially unique substrate specificities. The active sites contain a critical cysteine within the canonical pentapeptide, QACR/QG, and R and Q residues (Arg179, Gln283 and Arg341 for example in caspase-3) conserved among all caspase family members. A unique characteristic of these enzymes is that they cleave after an aspartic acid residue in their substrate (Sleath *et al.*, 1990; Howard *et al.*, 1991; Thornberry *et al.*, 1994). Thus, the fact that activation of these enzymes occurs by cleavage at aspartic acid residues suggests both autocatalytic capabilities and the possibility of a cascade of various cysteine proteases with one family member activating others during apoptosis.

To date, the characterization of caspase enzymatic activities has typically been investigated using transfected cell lines. Hence, the physiological roles of most of these various cysteine proteases *in vivo* are at present unclear. Three cysteine proteases, caspase-3, Mch2 (caspase-6) and ICE-LAP3 (caspase-7), have been shown to be proteolytically activated by apoptotic stimuli, implying a central role for this family of enzymes in cell death *in vivo* (Chinnaiyan *et al.*, 1996b; Duan *et al.*, 1996b; Orth *et al.*, 1996a; Schlegel *et al.*, 1996). Given that apoptosis is linked to negative selection in the thymus, we herein examine the potential role of caspase activity in antigen-triggered deletion of $CD4^+CD8^+$ double-positive (DP) fetal and adult thymocytes. Using peptide-based enzyme substrates we show that TCR ligation by peptide/MHC activates a procaspase in DP thymocytes which then causes cell death; specific enzyme inhibitors block this death process. These findings provide the molecular basis for negative selection.

Results

Caspases are irreversibly inhibited by tri- or tetrapeptide sequences (VAD, YVAD and/or DEVD) linked to a chemical moiety such as fluoromethyl ketone (fmk) or various acyloxymethyl ketones (amk) which covalently modify the enzyme, thereby inactivating its catalytic function (Chapman, 1992; Thornberry *et al.*, 1992, 1994; Rotonda *et al.*, 1996). The peptide-based inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (zVADfmk), blocks the induction of apoptosis induced by expression of REAPER protein when added to *Drosophila* Schneider cells (Pronk *et al.*, 1996); such experiments demonstrate that REAPER-induced apoptosis requires activation of a caspase.

In order to analyze the effect of inhibition of members of the caspase family of cysteine proteases on negative selection in the thymus, we have utilized a fetal thymic organ culture (FTOC) system. FTOC was chosen over either *in vivo* whole-animal studies or *in vitro* cell suspension cultures for several reasons. First, FTOC allows analysis of the effects of pharmaceutical agents on thymocyte development in a system which more closely mimics *in vivo* conditions than does thymocyte suspension culture. Second, pharmacophores which cannot be utilized in whole mice either due to systemic toxicity or limitations in their bioavailability can be assayed easily in FTOC. Furthermore, the relatively small volumes required minimize the amount of chemical required.

Caspase inhibition in FTOC prevents DP thymocyte deletion mediated by anti-CD3ε mAb

Prior studies utilizing the anti-CD3ε-specific hamster anti-mouse mAb 145-2C11 (2C11) have shown that DP thymocytes are susceptible to undergo apoptotic cell death upon exposure to 2C11 in FTOC (Smith *et al.*, 1989) or upon parenteral *in vivo* administration (Shi *et al.*, 1991). We therefore first analyzed the effect of a caspase inhibitor on anti-CD3ε-induced deletion of the $CD4^+CD8^+$ DP thymocytes using C57BL/6 animals. Fetal thymocytes were dissected on day 14.5 of pregnancy and cultured in transwells for 4 days. On day 4, additions were made to the FTOCs including 2C11 mAb in the presence or absence

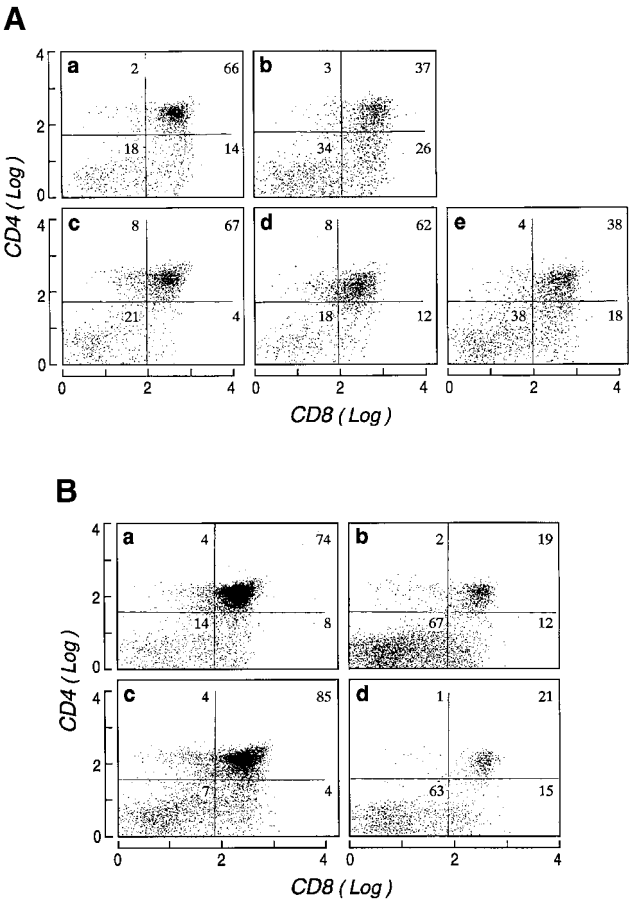

Fig. 1. A caspase inhibitor blocks deletion of DP thymocytes induced by anti-CD3ε mAb and glucocorticoids. (**A**) FACS analysis is presented for FTOC from C57BL/6 mice following 18 h of treatment with: (a) no addition; (b) 200 μg/ml 145-2C11 mAb; (c) 100 μm zVADfmk; (d) 2 h treatment with 100 μm zVADfmk followed by 18 h with 200 μg/ml 145-2C11 mAb; (e) 2 h treatment with 0.25% DMSO followed by 18 h with 200 μg/ml 145-2C11 mAb. (**B**) FACS analysis following 18 h of treatment with: (a) no addition; (b) 0.1 μM dexamethasone; (c) 2 h with 100 μM zVADfmk followed by 18 h with 0.1 μM dexamethasone; (d) 2 h pretreatment with 0.25% DMSO followed by 18 h with 0.1 μM dexamethasone. Fetal lobes (three to five per group) were cultured for 4 days, treated as indicated and harvested on day 5. Lobes were dissociated and thymocytes stained with directly conjugated anti-CD8-Red613 and anti-CD4-PE. 10 000 cells were analyzed per dot plot. The numbers within the quadrants represent the percent of cells in that quadrant.

of 100 μM zVADfmk, an irreversible inhibitor of cysteine proteases, and thymocytes harvested 18 h later. Figure 1A shows the FACS analysis of C57BL/6 fetal thymuses after such a treatment. Upon 2C11 mAb addition, the percent of DP thymocytes is reduced from 66% in the control culture to 37% in the culture treated for 18 h with 200 μg/ml 2C11 mAb (Figure 1A, compare panels a and b). Moreover, the deletion of these DP thymocytes is inhibited by a 2 h preincubation with zVADfmk (Figure 1A, panel d). Note that zVADfmk alone does not alter the percentage of DP thymocytes (Figure 1A, panel c) nor does dimethyl sulfoxide (DMSO), the additive used to solubilize zVADfmk, affect the deletion of DP thymocytes (Figure 1A, panel e). A control hamster anti-mouse mAb, H28 (Becker *et al.*, 1989), directed against a segment of the TCR α chain which is inaccessible on intact thymocytes, has no effect on the FTOC (data not shown).

zVADfmk inhibits glucocorticoid-induced DP thymocyte death in FTOC

DP thymocytes are exquisitely sensitive to pharmacological doses of glucocorticoids *in vivo* (Wyllie *et al.*, 1980). The rapid thymic involution known to result from stress is a consequence, in large part, also of endogenous release of steroids. To determine whether caspase inhibition blocks DP thymocyte apoptosis induced by corticosteroids, the effects of dexamethasone on DP thymocyte survival in FTOC were examined in the presence or absence of zVADfmk. As shown in Figure 1B, dexamethasone at 0.1 μM reduces the percentage of DP thymocytes from 74% to 19% (Figure 1B, compare panels a and b). A 2 h pretreatment with 100 μM zVADfmk protects the $CD4^+CD8^+$ population from dexamethasone-induced death (Figure 1B, panel c). The inhibition of deletion is specific to the zVADfmk reagent and not due to the DMSO solvent, as shown in Figure 1B, panel d. These results demonstrate that two well-known inducers of $CD4^+CD8^+$ DP thymocyte death, anti-CD3ε mAb and glucocorticoids, are blocked by inhibition of cysteine protease activity.

zVADfmk protects DP thymocytes from antigen-induced deletion in N15 TCR tg RAG-$2^{-/-}$ H-2^b mice

Given that negative selection of thymocytes *in vivo* is antigen-driven, we next tested the effect of zVADfmk on specific peptide-induced deletion of DP thymocytes in FTOC derived from TCR tg mice. To this end, we employed the N15 TCR tg mouse which bears a class I MHC-restricted TCR and recognizes the vesicular stomatitis virus nuclear protein octapeptide VSV8 in the context of H-2 K^b (Y.Ghendler, R.E.Hussey, T.Witte, E.Mizoguchi, L.K. Clayton, A.K.Bhan, S.Koyasu, H.-C.Chang and E.L. Reinherz, manuscript submitted). The VSV8 peptide interacts with K^b with high affinity such that a single *in vitro* or *in vivo* exposure efficiently loads the K^b molecules in the thymus of these animals. We additionally constructed the animals on a RAG-$2^{-/-}$ background (Shinkai *et al.*, 1992), thereby guaranteeing exclusive expression of this TCR on the surface of T-lineage cells. In these experiments, fetuses were dissected at day 15.5 since we observed that the development of N15 tg RAG-$2^{-/-}$ animals was -slightly slower than that of the corresponding wild-type C57BL/6 animals.

Figure 2a shows that after 5 additional days of FTOC, 60% of N15 TCR tg RAG-$2^{-/-}$ H-2^b thymocytes are DP and 24% are $CD8^+$ SP thymocytes. Addition of 10^{-5} M VSV8 peptide to the N15 FTOCs 18 h before analysis results in massive depletion of $CD4^+CD8^+$ DP thymocytes with a reduction from 60% to 2% (Figure 2b). zVADfmk completely blocks this antigen-induced depletion (Figure 2c). Importantly, zVADmk, a compound identical to zVADfmk (Figure 3) except for the absence of the fluoride atom which is required for the irreversible inhibition of caspases, has no effect on the antigen-induced depletion of DP thymocytes as shown in Figure 2d. Hence, inhibition of enzymatic function is a prerequisite for protecting DP thymocytes from depletion by specific antigen. Exposure of the FTOC to the DMSO solvent before VSV8 treatment has no effect (Figure 2e). Figure 2 also shows that the depletion of DP thymocytes is specifically induced by the VSV8 peptide: addition to the FTOC of an equivalent

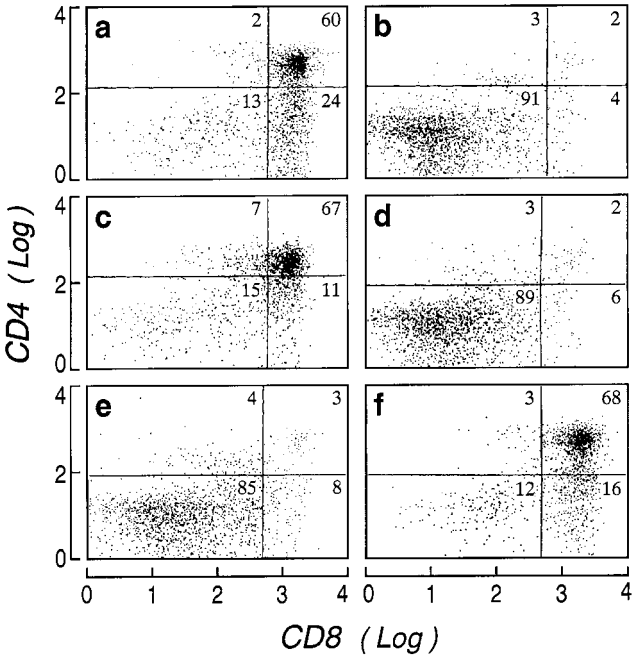

Fig. 2. A caspase inhibitor blocks peptide/MHC induced deletion of DP thymocytes. FACS analysis of FTOC from N15 TCRtg RAG-$2^{-/-}$ H-2^b mice following 18 h treatment with: (**a**) no addition; (**b**) 10 μM VSV8 peptide; (**c**) 2 h with 100 μM zVADfmk followed by 18 h with 10 μM VSV8; (**d**) 2 h with 100 μM zVADmk followed by 18 h with 10 μM VSV8; (**e**) 2 h with 0.25% DMSO followed by 18 h with 10 μM VSV8; (**f**) 18 h with 10 μM SEV9. Fetal lobes (five per group) were cultured 4 days, treated as indicated and harvested on day 5. Lobes were dissociated and stained with directly conjugated anti-CD8-Red613 and anti-CD4-PE. 10 000 cells were analyzed per dot plot. The numbers within the quadrants represent the percent of cells in that quadrant.

amount of the unrelated SEV9 Sendai virus-derived peptide, which binds to K^b with a comparable affinity with that of VSV8 but which is not recognized by the N15 TCR, does not result in thymocyte death (Figure 2, panel f).

Histological analysis of antigen-induced apoptosis in FTOC of N15 TCR tg RAG-$2^{-/-}$ H-2^b mice: blockade of cell death by zVADfmk

Negative selection results in apoptosis of DP thymocytes as judged by morphological criteria or induction of DNA fragmentation as analyzed by gel electrophoresis or TUNEL assay (Murphy *et al.*, 1990; Surh and Sprent, 1994). We confirmed that apoptosis in this TCR tg system is induced by the VSV8 peptide in N15 FTOC using a terminal deoxynucleotidyl transferase (TdT) assay on histological sections as demonstrated in Figure 4. This method exploits the fact that apoptosis results in DNA cleavage, the ends of which serve as a substrate for the TdT enzyme, allowing cells undergoing death to be labeled with biotinylated dUTP. Sections were prepared from fetal thymic lobes cultured as described above and treated for 4 h with 10^{-5} M VSV8 peptide with either no pretreatment or a 1 h pretreatment with zVADfmk or zVADmk. This early time point was chosen for the characterization as we had previously observed that, after overnight exposure to VSV8, dying DP thymocytes from adult animals were already removed by an efficient process involving the non-lymphoid stromal components of the thymus (Y.Ghendler, R.E.Hussey, T.Witte, E.Mizoguchi, L.K.Clayton, A.K.

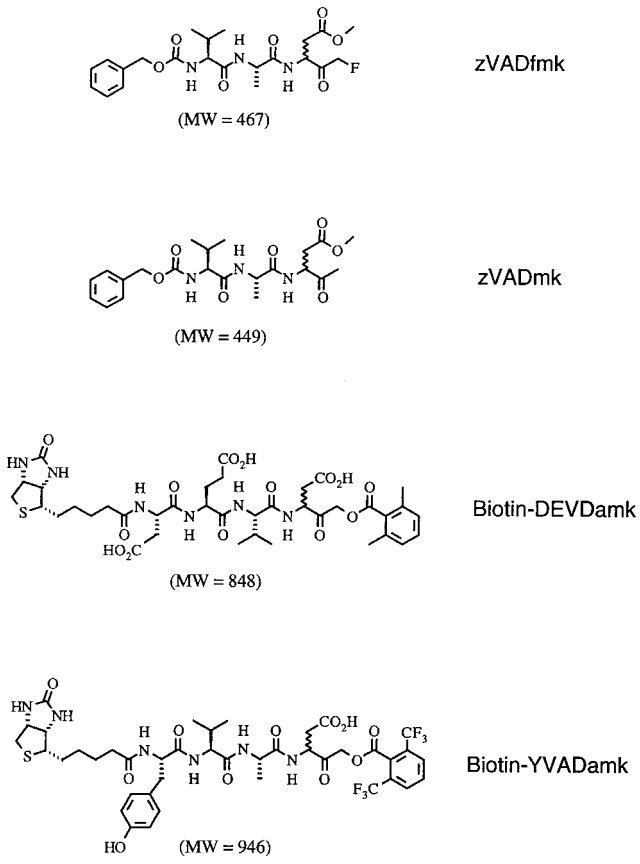

Fig. 3. Structures of the peptide-based caspase inhibitors and related compounds. The structures of the peptide-based inhibitor analogues used in these studies are shown along with their molecular weight as verified by mass spectrometry. The trivial names of the compounds are also given. Note that the cell-permeable inhibitors (zVADfmk and zVADmk) have the P_1 aspartate protected as the methyl ester. On the other hand, the P_1 aspartate of the biotinylated peptides has a free acid at the β position.

Bhan, S.Koyasu, H.-C.Chang and E.L.Reinherz, manuscript submitted). The effective removal of corpses has also been previously described in both tg and non-tg thymuses (Surh and Sprent, 1994).

As shown in Figure 4, VSV8 treatment resulted in an obvious increase in the number of TdT-positive cells as compared with the control culture which was not exposed to the VSV8 peptide. Pretreatment with zVADfmk before VSV8 addition reduces the number of TdT-positive cells to that of the control. In contrast, zVADmk-pretreated cultures which were exposed to VSV8 had levels of TdT-positive cells similar to cultures treated with VSV8 alone. Table I shows quantitative immunohistological results for three N15 tg RAG-$2^{-/-}$ H-2^b FTOC and a littermate control non-tg RAG-$2^{-/-}$ FTOC. Thus, the depletion of DP thymocytes in the N15 TCR tg RAG-$2^{-/-}$ H-2^b FTOC is blocked specifically with a cysteine protease inhibitor and this inhibition correlates with a reduction in the number of apoptotic cells as determined by TdT assays. To our knowledge, this is the first demonstration that inhibition of a caspase(s) prevents apoptotic cell death induced by antigen triggering of the TCR on immature DP thymocytes.

TCR-triggered activation of caspase(s): a biochemical analysis

Although the above results clearly demonstrate that the zVADfmk cysteine protease inhibitor can block antigen-triggered cell death, it remained to be determined whether TCR ligation specifically activates a cysteine protease. Members of the caspase family exist as proenzymes which are cleaved by an activation step to give rise to ~20 kDa and ~12 kDa subunits (reviewed in Henkart, 1996). The heterodimeric subunits then associate to form a tetramer. Because the tetramer—but not the inactive proenzyme—binds the substrate, we developed a biochemical assay to examine the state of caspases in thymuses of unstimulated or *in vivo*-VSV8-triggered N15 tg mice. To this end, we tested several possible substrates including biotin–YVADamk and biotin–DEVDamk (Figure 3). Because the biotin–DEVDamk substrate appears to have the highest affinity for the thymic caspase, only these data will be presented. The significance of this substrate selectivity will be discussed below.

N15 transgenic RAG-$2^{-/-}$ H-2^b mice were injected in the tail vein with PBS, 24 μg VSV8 or control SEV9 peptide and extracts prepared from the thymocytes of these adult mice 2 h after injection. Lysate equivalent to 2×10^6 thymocytes was incubated with or without 2 μM biotin–DEVDamk, which binds irreversibly to caspases at varying rates depending on the specific protease. Subsequently, the treated lysates were analyzed by 12.5% SDS–PAGE and blotted onto nitrocellulose. The membranes were then incubated with streptavidin horseradish peroxidase and developed by ECL. A band of ~17 kDa is induced in the 2 h VSV8-treated thymocytes (Figure 5a). This band is not found in lysates from thymuses of PBS- or SEV9-injected animals, and is not detected in thymic lysates without prior incubation with biotin–DEVDamk (Figure 5a). As expected, purified, recombinant Yama/caspase-3 and Mch2α (caspase-6) proteases bind the biotin–DEVDamk substrate in this assay (Figure 5b).

The above functional studies utilizing zVADfmk and the biochemical analysis with biotin–DEVDamk collectively show that cysteine proteases are activated during antigen-induced negative selection in the thymus. Whether the same caspase(s) interacts with both of these inhibitors remained to be determined. This possibility was tested by competitive inhibition analysis wherein the above thymic lysates were first preincubated with up to 1000-fold molar excess of zVADfmk before addition of the biotin–DEVDamk. If both substrate inhibitors bound to the same enzyme, then the non-biotin-labeled zVADfmk would block the ability of biotin–DEVDamk to bind to the activated thymic caspase and hence, eliminate detection of the ~17 kDa subunit by streptavidin horseradish peroxidase. Alternatively, if these inhibitors bound to different enzymes, then there would be no change in the appearance of the caspase band as detected by biotin–DEVDamk.

Figure 5c shows that, if the thymic lysates are incubated first with zVADfmk at molar concentrations ranging from 1000- to 100-fold that of the biotin–DEVDamk substrate, the ~17 kDa band is no longer detectable by Western blot analysis. In contrast, at lower zVADfmk:biotin–DEVDamk ratios (10:1 or 1:1), the biotin–DEVDamk substrate is bound by the TCR-triggered cysteine protease and therefore detected as a ~17 kDa biotinylated inhibitor–enzyme

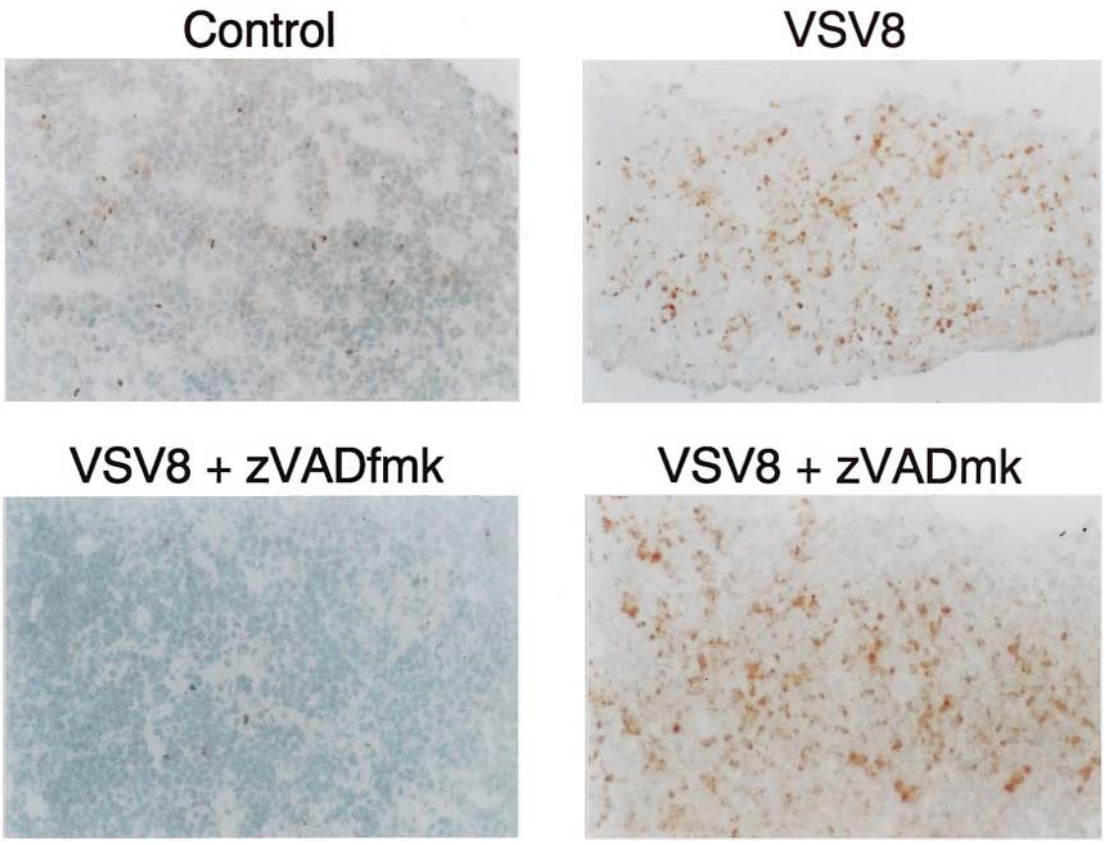

Fig. 4. Inhibition of caspase activity blocks antigen-induced apoptosis of thymocytes. TUNEL analysis was performed on histological sections of thymuses from N15 tg RAG$^{-/-}$ H-2^{b} FTOC. Thymuses were: untreated (control); treated with 10 μM VSV8 peptide for 4 h (VSV8); 1 h with 100 μM zVADfmk and 4 h with 10 μM VSV8 peptide (VSV8 + zVADfmk); 1 h with 100 μM zVADmk and 4 h with 10 μM VSV8 peptide (VSV8 + zVADmk). A 40× objective was used.

complex. Thus, the irreversible binding of zVADfmk which blocks VSV8-induced depletion of the DP thymocytes in FTOC competes with the biotin–DEVDamk substrate for binding to the same ~17 kDa band. This result links the antigen-induced negative selection of thymocytes as measured by depletion of DP thymocytes in FTOC to the appearance of an activated cysteine protease detectable in the thymuses of mice undergoing negative selection. Figure 5d shows that, while a 100-fold molar excess of zVADfmk blocks binding of the biotin–DEVDamk substrate to the antigen-activated cysteine protease, the same excess of zVADmk does not block this interaction. This finding is consistent with our observation that zVADmk fails to block antigen-induced depletion of the DP thymocytes in FTOC.

Table I. Quantitation of cell death in FTOC by TUNEL assay

Treatment	TdT-positive cells (mm^2)			
	N15tg #1	N15tg #2	N15tg #3	non-tg
Control	7[a]	28	7	1
VSV8	429	456	441	9
zVADfmk + VSV8	6	1	2	6
zVADmk +VSV8	360	525	461	ND

In the above experiments (n = 3) of which these results are representative, fetuses were offspring of N15 tg$^{+/-}$ Rag-2$^{-/-}$ H-2^{b} males and RAG-2$^{-/-}$ H-2^{b} females and derived from the same litters.
[a]Mean number of TdT-positive cells/mm^2 in six fields from Figure 4.
ND, not determined.

Localization of activated caspase to TCR-triggered cortical thymocytes in N15 tg RAG-2$^{-/-}$ H-2^{b} fetal and adult mice

To localize the activated caspase within the thymus of fetal and adult N15 tg RAG$^{-/-}$ H-2^{b} mice, we performed two-color immunohistological analysis using biotin–DEVDamk and anti-CD4 mAb. In the N15 RAG$^{-/-}$ H-2^{b} mice, CD4 staining is essentially limited to the DP thymocytes because the N15 TCR is class I MHC-restricted, resulting in positive selection of CD8^{+} SP cells exclusively. In Figure 6A, we show a control FTOC section stained with biotin–DEVDamk and anti-CD4. The majority of thymocytes demonstrate CD4 staining (red/brown), consistent with the large percentage of DP thymocytes present in the thymus of these animals. Staining with biotin–DEVDamk (blue) is negative. In contrast, 4 h after treatment with VSV8, the CD4 staining has become patchy and the morphology of the CD4 cells is altered with less discrete cell boundaries. In addition, there is widespread biotin–DEVDamk staining throughout the cortex, indicative of antigen-induced activation of a thymic caspase. Consistent with the biochemical analysis, this activation is blocked by pretreatment with zVADfmk but not with zVADmk.

The same staining pattern is evident in adult N15 tg RAG-2$^{-/-}$ H-2^{b} mice as shown in Figure 6B. Thymic sections of N15 tg RAG-2$^{-/-}$ H-2^{b} animals are positive

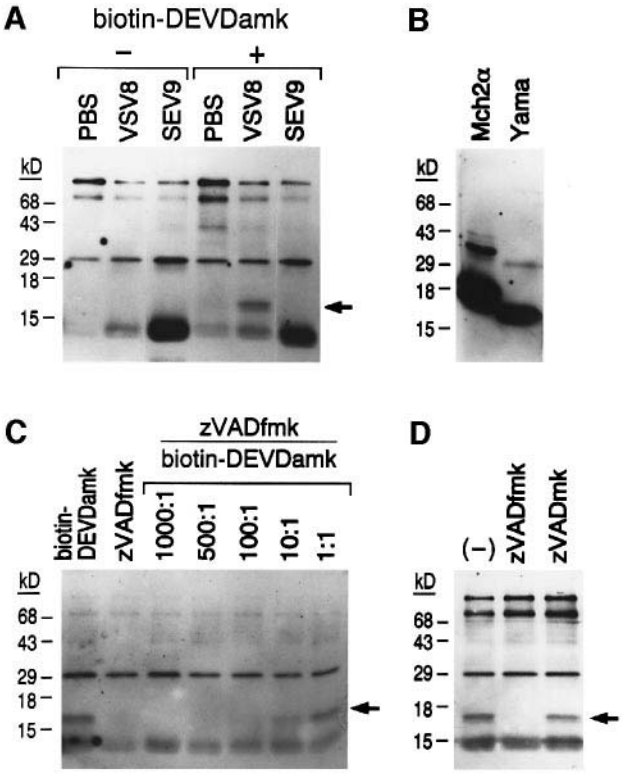

Fig. 5. Western blot analysis reveals antigen-induced activation of a thymic caspase. (**A**) Thymic extracts prepared from PBS, VSV8 or SEV9 injected N15 tg RAG-$2^{-/-}$ H-2^b mice were incubated 15 min with (+) or without (–) 2 μM biotin-DEVDamk, run on 12.5% SDS–PAGE under non-reducing conditions and blotted onto nitrocellulose. The membrane was incubated with horseradish peroxidase-conjugated streptavidin and developed by ECL. Each lane contained thymic extract equivalent to 2×10^6 thymocytes. VSV8 peptide injections were performed 1.5 h before. (**B**) 140 ng of purified, recombinant Mch2α and 3.5 ng of purified, recombinant Yama were incubated 15 min with 2 μM biotin–DEVDamk and analyzed as described in (a). (**C**) Thymic extracts prepared from mice 2 h after VSV8 peptide injection were incubated 15 min with 2 μM biotin–DEVDamk alone, 2 μM zVADfmk alone, or preincubated 15 min with an excess of 1000×, 500×, 100×, 10× or an equal concentration of zVADfmk relative to biotin–DEVDamk. Biotin–DEVDamk was then added to 2 μM and the incubation continued for 15 min. Lysates were then run on 12.5% SDS–PAGE and analyzed as described in (a). (**D**) Thymic extracts isolated from mice 2 h after VSV8 peptide injection were incubated 15 min with 2 μM biotin–DEVDamk alone (–), or, alternatively, incubated 15 min with 200 μM zVADfmk or zVADmk and then biotin–DEVDamk added to 2 μM for another 15 min. Lysates were then analyzed as described in (a). Arrows indicate the position of the activated thymic caspase subunit which binds the biotin–DEVDamk.

for anti-CD4 reactivity and negative for that of biotin–DEVDamk. However, 4 h following tail vein injection of VSV8 peptide, the thymic section demonstrates widespread biotin–DEVDamk staining and the same alterations in anti-CD4 reactivity as seen in the VSV8-treated FTOCs. PBS injection does not induce damage to the $CD4^+$ thymocytes or detectable activation of the cysteine protease. Thus, deletion of DP thymocytes occurs through antigen-stimulated activation of a cysteine protease in both FTOC and adult mice, and this activity localizes to the thymocytes themselves.

Discussion

We have demonstrated that peptide/MHC-induced negative selection of DP thymocytes involves the activation of a thymic caspase. Double staining with anti-CD4 mAb and biotin–DEVDamk of thymic sections from both fetal and adult N15 TCRtg RAG-$2^{-/-}$ H-2^b mice demonstrates that the caspase is activated in the DP thymocyte subpopulation within a time frame of ⩽2 h. Subsequently, at 4 h, we observed a tremendous increase in the number of apoptotic thymocytes determined by TUNEL assays of histological sections of thymuses. At 18 h, by FACS analysis and immunohistological analysis, >90% of the DP thymocyte population is deleted. Both the disappearance of DP thymocytes and the increase in numbers of apoptotic cells in histological sections are blocked by treatment of FTOC with zVADfmk, a peptide-based inhibitor of cysteine proteases.

Biochemical analysis of thymic lysates prepared from adult N15 TCRtg RAG-$2^{-/-}$ H-2^b mice using a biotinylated substrate of cysteine proteases, biotin–DEVDamk, confirms the induction of an active caspase within 2 h after tail vein injection of VSV8 antigen. This enzyme activation is specifically induced as it is not activated in control (PBS) or irrelevant peptide (SEV9) -treated animals. Pretreatment of the thymic lysates with an excess of the irreversible inhibitor zVADfmk prevents binding of the biotin–DEVDamk to the caspase while the chemically related control compound zVADmk does not. zVADmk also has no effect on antigen-induced depletion of DP thymocytes in FTOC or the appearance of TUNEL-positive cells in histological sections upon VSV8 peptide treatment of FTOC. Thus, the zVADfmk which blocks depletion of DP thymocytes in FTOC also blocks binding of the biotin–DEVDamk to its substrate. We conclude that the caspase functionally inhibited by zVADfmk in FTOC is the same as that detected by biotin–DEVDamk by Western blot analysis. Activation of this caspase is the molecular basis for negative selection of DP thymocytes.

Our interpretation of the above data is that the TCR of the double-positive thymocyte binds the peptide/MHC on an antigen-presenting stromal cell with the restricted association of the CD4 or CD8 co-receptor; in the case of the N15 TCR transgenic mouse, the CD8 co-receptor is involved. If the affinity of the TCR/peptide/MHC interaction is of sufficient magnitude, as is true for the N15 TCR and VSV8/K^b complex, the thymocyte is triggered to undergo negative selection. Upon this triggering, a procaspase is cleaved to form an active tetramer which catabolizes yet to be identified cellular substrates, resulting in apoptosis of the DP thymocyte. It is reasonable to assume that a similar mechanism is operative via B-cell receptors in early B-cell development.

What is the identity of the activated thymic caspase? Analysis of the Western blots demonstrates that there is likely only one enzyme activated given that there is a single predominant band by gel analysis. The less intense band migrating slightly faster than the major band may be a degradation product or post-translational modification. We cannot exclude the possibility, however, that a second caspase might be activated. If the latter is the case, then both enzymes must be blocked by the zVADfmk inhibitor. Studies to date defining substrates and inhibitors of known caspases offer additional insights into the possible identity of the thymic caspase. Peptide-based inhibitors serve to characterize different substrate specificities among the caspase family members. The tetrapeptide aldehyde Ac-

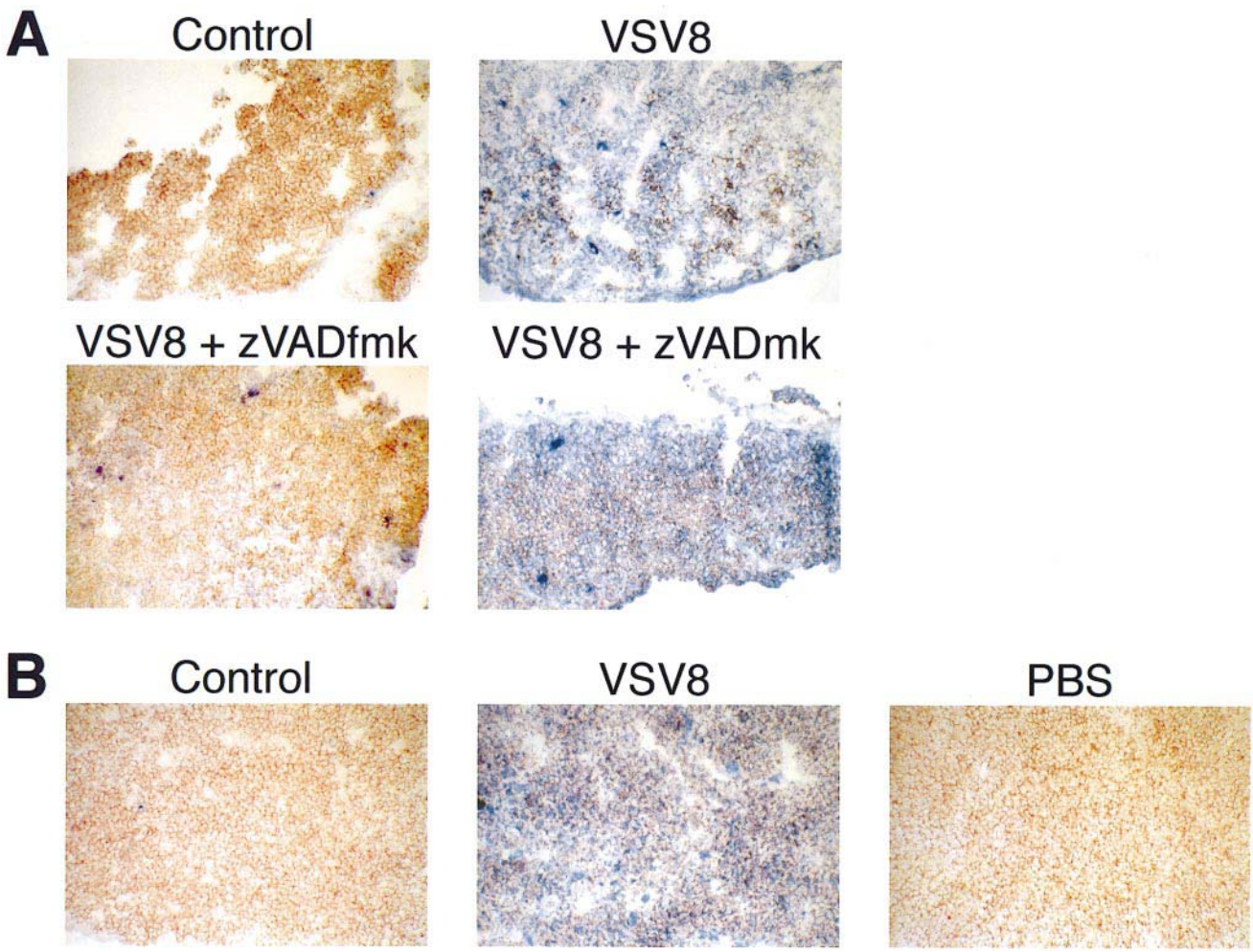

Fig. 6. Histological analysis of thymic caspase expression in fetal and adult thymuses. (**A**) Sections of thymic lobes were obtained from control N15 TCR tg RAG-2$^{-/-}$ H-2^{b} FTOC or FTOC treated for 4 h with 10 μM VSV8 peptide, 1 h with 100 μM zVADfmk followed by 4 h with 10 μM VSV8 peptide or 1 h with 100 μM zVADmk followed by 4 h with 10 μM VSV8 peptide and then double-stained with anti-CD4 mAb (red/brown) and biotin–DEVDamk (blue). (**B**) Histological analysis of thymic sections from adult N15 TCRtg RAG-2$^{-/-}$ H-2^{b} animals uninjected (control) or injected 4 h previously with 24 μg VSV8 peptide or PBS. Sections were double-stained with biotin–DEVDamk and anti-CD4 as in (A). A 25× objective was used.

YVAD-CHO inhibits caspase-1 at ~10 000-fold lower concentration than that required to inhibit caspase-3. In contrast, Ac-DEVD-CHO inhibits caspase-3 activity 49-fold better than caspase-1 (Rotonda *et al.*, 1996). Utilizing thymic lysates from VSV8-injected N15 TCRtg RAG$^{-/-}$ H-2^{b} mice, biotin–YVADamk failed to detect the caspase readily revealed by biotin–DEVDamk. The recombinant caspase-3 protein used as a positive control was detected by biotin–YVADamk only when 100-fold more of the enzyme was used (data not shown). The crystal co-complex structures of caspase-1 and -3 and their inhibitors clarify the observed substrate specificities (Walker *et al.*, 1994; Wilson *et al.*, 1994; Rotonda *et al.*, 1996): the S4 subsite in caspase-1 is a hydrophobic depression into which a tyrosine residue fits nicely as in the ac-YVAD-CHO peptide aldehyde inhibitor. In contrast, the comparable S4 subsite in caspase-3 forms a narrow pocket fitting tightly around an aspartic acid residue as in the ac-DEVD-CHO peptide aldehyde inhibitor. This substrate difference suggests that the thymic caspase should be grouped with the caspase-3-like cysteine proteases rather than the caspase-1-like group.

Studies with proteins which inhibit their functional enzymatic activity further serve to categorize the caspase family members. Bcl-2, first identified as a proto-oncogene in B-cell follicular lymphomas, inhibits some forms of apoptotic cell death including death induced by ICH-1 (caspase-2) (reviewed in Yang and Korsmeyer, 1996). The cytokine response modifier, crmA, derived from the cowpox virus inhibits caspase-1 but not caspase-2. At high concentrations, however, crmA also inhibits caspase-3 (Nicholson *et al.*, 1995; Tewari *et al.*, 1995). Negative selection is unaffected in Bcl-2 (Sentman *et al.*, 1991) and caspase-1 (Kuida *et al.*, 1995; Li *et al.*, 1995) knockout animals. The recent crmA transgenic mouse in which crmA is directed to the T-cell compartment via a CD2 enhancer demonstrates a phenotype in which CD95-induced peripheral T-cell apoptosis is inhibited, but negative selection in the thymus appears to be unaffected (Smith *et al.*, 1996). The inhibitors Bcl-2 and crmA have complementary effects in that the crmA transgene does not affect γ-irradiation or dexamethasone-induced apoptosis, while Bcl-2 does just the opposite and inhibits both. Neither Bcl-2 nor crmA transgenes, however, affect negative selection. Thus, while negative selec-

tion appears to proceed via an apoptotic pathway, the components of this process are undefined. One would expect that the thymic caspase described here would not be inhibited by crmA or by Bcl-2.

Analysis of apoptotic pathways triggered by different stimuli in cells transfected with Bcl-2 and crmA have been used to suggest molecular ordering of particular caspases within death pathways and further imply the presence of a cascade of caspases with one member activating others. Studies suggest a molecular ordering in which Bcl-2 and Bcl-x_L, a homologue of Bcl-2, function upstream of caspase-3, -6 and -7 (Chinnaiyan *et al.*, 1996c; Orth *et al.*, 1996a); crmA also appears to function by inhibiting a protease upstream of caspase-3, -6 and -7 but in a different pathway from that inhibited by Bcl-2 (Chinnaiyan *et al.*, 1996c; Orth *et al.*, 1996a).

Granzyme B is a serine protease which cleaves at an aspartic acid in its substrate (Murphy *et al.*, 1988; Odake *et al.*, 1991; Vaux *et al.*, 1992; Caputo *et al.*, 1994) and is activated only in cytolytic T-lymphocytes (Hanson and Ley, 1990; Hanson *et al.*, 1991; Heusel *et al.*, 1991). Post-activation, granzyme B is transferred into target cells where it initiates caspase cleavage, notably of caspase 3 (Darmon *et al.*, 1995; Quan *et al.*, 1996). Thus, proteases other than caspases may activate these enzymes initiating the cascade of caspase functional programs leading to cell death. ICH-3 has also been shown to be activated by granzyme B (Wang *et al.*, 1996). Ordering of ICH-3 activity places it upstream of caspase-1 in the granzyme B pathway and also upstream of caspase-3.

Certain cysteine proteases have been analyzed by targeted gene disruption. Caspase-1 knockout animals do not have a generalized death defect, suggesting that this enzyme may not be central to all death pathways (Kuida *et al.*, 1995; Li *et al.*, 1995). The CD95-induced death pathway is affected in these animals, but negative selection is not altered in *lpr* mice bearing the spontaneous CD95 mutation (reviewed by Rozzo *et al.*, 1994). Furthermore, we have not observed cleavage of poly(ADP-ribose) polymerase (PARP) in thymic lysates of C57BL/6 animals injected intraperitoneally with anti-CD3ε mAb (data not shown). Since PARP is a substrate of caspase-3, this suggests that caspase-3 is also not responsible for the observed thymic apoptosis. Assuming that the crmA levels were sufficiently high in the crmA transgenic mice described above (Smith *et al.*, 1996), then caspase-3 would have been inhibited in those mice, and yet negative selection was unaffected. The absence of an effect on thymopoiesis in caspase-3 knockout mice is also consistent with this notion (Kuida *et al.*, 1996). These studies suggest that the thymic caspase is not caspase-1 or caspase-3; however, the possibility remains that these molecules are involved in the process of negative selection but that redundancy with other proteases obscures a phenotype in knockout animals.

The anti-CD3ε mAb-induced deletion of DP thymocytes was also blocked by zVADfmk in FTOC, suggesting that the same caspase involved in peptide/MHC-triggered death is active in this apoptotic pathway. However, crosslinking with this antibody did not induce deletion of DP thymocytes to the same extent as did peptide/MHC (Figures 1a and 2). One might expect that crosslinking with anti-CD3ε mAb would be more extensive than that induced by peptide/MHC, since there are two CD3ε molecules per TCR and only one binding site for peptide/MHC. Furthermore, the mAb is bivalent, unlike the peptide/MHC which is monovalent. The most likely explanation for this apparent paradox is that peptide/MHC-induced triggering may recruit other molecules which synergize to provide a more potent death signal than that exclusively provided by crosslinking of TCR molecules by mAb. Furthermore, other signaling molecules have been shown to affect negative selection as discussed below, although their roles in activation of the caspase remain to be determined. Certainly, there is precedent for the role of dual signaling within the T-cell lineage: activation of resting peripheral T-cells requires TCR crosslinking as well as co-stimulation through CD28 and its ligands, B7-1 and B7-2 (reviewed in Bluestone, 1995). Apoptosis of peripheral T-cells involves second signals through Fas (CD95) and Fas–ligand, the expression of which are regulated by TCR signaling (Crispe, 1994). Such dual signaling may also play a role in thymic selection; a second signal derived from thymic stroma along with the specificity-determining TCR/peptide/MHC interaction may be required for negative selection (Page *et al.*, 1993; Degermann *et al.*, 1994; Punt *et al.*, 1994; Lerner, *et al.*, 1996). Targeted disruption of CD30, another member of the TNF receptor family, produces a phenotype suggestive of a defect in negative selection (Amakawa *et al.*, 1996). Thus, receptor–ligand interactions between thymocytes and stromal cells may induce a second signal(s) from the stroma which is important in the modulation of negative selection.

Glucocorticoids induce deletion of DP thymocytes in FTOC of C57BL/6 mice and this deletion is blocked by zVADfmk. Rat and mouse thymocytes in suspension also undergo glucocorticoid-induced death which is blocked by zVADfmk (Fearnhead *et al.*, 1995; Sarin *et al.*, 1996). This death pathway is inhibited by Bcl-2, in contrast to that of negative selection (reviewed in Yang and Korsmeyer, 1996). Since both forms of DP thymocyte depletion are blocked by zVADfmk while Bcl-2 selectively affects glucocorticoid-induced deletion but does not alter negative selection, it appears that the glucocorticoid and antigen/MHC pathways share some—but not all—components of the death pathway. In addition, it has recently been shown that zVADfmk also blocks *in vitro* apoptotic death in mouse thymocytes triggered by etoposide, radiation, anti-Fas and anti-CD3 (Sarin *et al.*, 1996). It thus appears that a number of independent agents trigger death pathways which may converge to a common death effector mechanism involving a thymic caspase.

It is a long-standing enigma as to how events which induce negative selection and death in immature thymocytes induce activation and accompanying proliferation in mature T-cells (Kappler *et al.*, 1987; Ramarli *et al.*, 1987). Hence, the developmental stage of the T-cell determines its response to a particular stimulus. In TCR transgenic mice, antigenic peptides which activate peripheral T-cells to proliferate trigger depletion of the immature thymocytes bearing the identical TCR (Fowlkes and Pardoll, 1989). Most TCR transgenic models in which deletion by peptide antigen has been examined demonstrate that the deletion involves the immature DP thymocytes (Murphy *et al.*, 1990; Vasquez *et al.*, 1992; Ashton-Rickardt *et al.*, 1994; Hogquist *et al.*, 1994; Sebzda *et al.*, 1994). The presence

of the peptide/MHC-activated caspase in the DP thymocyte subset further identifies this population as the one in which negative selection is operative.

Recently, using N15 TCRtg RAG-2$^{-/-}$ H-2^{b} mice, we identified a population of DP TCR^{high} thymocytes which is resistant to deletion by peptide antigen (Y.Ghendler, R.E.Hussey, T.Witte, E.Mizoguchi, L.K.Clayton, A.K. Bhan, S.Koyasu, H.-C.Chang and E.L.Reinherz, manuscript submitted). Presumably, these thymocytes have already been positively selected by an endogenous peptide antigen (APL) before VSV8 injection and are on their way to becoming mature CD8 SP T-cells. By what basis are they resistant to negative selection? If the activation of a caspase is the mechanism by which negative selection operates, then these cells must be unable to activate the thymic caspase upon TCR engagement with VSV8. It may be that the caspase mRNA is expressed only at a limited stage of development and these cells no longer express the caspase gene. The fact that zVADfmk and the related compound Boc-Asp(OMe)-fluoromethylketone have different blocking effects when tested in mouse thymocytes versus mouse T-cell blasts (Sarin *et al.*, 1996) supports the notion that death pathways in T-cells at different stages of development may use different caspase family members when death is triggered by the same input pathway. Alternatively, the DP TCR^{high} thymocytes may produce an inhibitor of the thymic caspase such as a Bcl-2-like molecule which inactivates the thymic caspase. In addition, a molecule which provides a second signal(s) necessary for thymic selection as discussed above may no longer be expressed on the DP TCR^{high} thymocytes. Once the genetic and biochemical details of the negative selection pathway are defined, it should be possible to determine which of these mechanisms accounts for the escape of the DP TCR^{high} thymocytes from peptide/MHC-induced death.

Materials and methods

Animals

All animals were maintained at the animal facility of Dana-Farber Cancer Institute. N15 TCR tg RAG-2$^{-/-}$ H-2^{b} animals were constructed as previously described (Y.Ghendler, R.E.Hussey, T.Witte, E.Mizoguchi, L.K.Clayton, A.K.Bhan, S.Koyasu, H.-C.Chang and E.L.Reinherz, manuscript submitted). These animals express α and β TCR chains isolated from the N15 CTL clone (Chang *et al.*, 1994). The constructs were engineered using TCR shuttle vectors kindly provided by M.Davis (Patten *et al.*, 1993). N15 tg lines were maintained and bred under sterile barrier standard conditions. C57BL/6 mice were purchased from Taconic. For injection of 145-2C11, the anti-CD3ε mAb was purified by standard procedures (Coligan *et al.*, 1994) and 200 μg injected intraperitoneally in PBS. For injection of peptide, 24 μg of VSV8 (RGYVYQGL) or SEV9 (FAPGNYPAL) was injected in 100 μl PBS into the tail vein of mice.

FTOC

Fetuses of C57BL/6 mice were dissected at day 14.5 of pregnancy, with the day of the vaginal plug counted as day 1. Fetuses of N15 TCRtg RAG-2$^{-/-}$ H-2^{b} mice were dissected at day 15.5. Fetal lobes were removed and cultured in 6-well Transwell dishes (Costar, Kennebunk, ME) using 1.6 ml of complete DMEM-10 as described by Coligan *et al.* (1994). The samples were incubated in a humidified atmosphere with 5% CO_2 for 4–5 days at 37°C and treated with specific reagents as described in the text. For harvesting, lobes were ground between frosted glass slides in PBS–1% BSA, washed and used for FACS analysis.

Flow cytometric analysis

Monoclonal antibodies used were R-phycoerythrin (PE) labeled anti-CD4 (H129.19) and Red613 labeled anti-CD8 (53-6.7) (Gibco-BRL, Grand Island, NY). Harvested thymocytes were washed and stained for 30 min with anti-CD4 and anti-CD8 mAbs. Flow cytometric analysis was performed on a FACScan (Becton Dickinson, San Jose, CA). Samples were gated on live cells based on forward and side scatter parameters. Data on 10 000 events per sample were collected in list mode using FACScan Research software and analyzed using LYSYSII software (Becton Dickinson).

Synthesis of peptide-based inhibitors and related compounds

N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, β-methyl ester (zVADfmk) was from Enzyme System Products (Dublin, CA). Biotin–YVADamk was purchased from Amersham Life Science (Arlington Heights, IL).

N-[biotinylaspartyl(β-*t*-butyl ester)]glutamyl(γ-*t*-butyl ester)valine was prepared by solid phase synthesis on a 2-chlorotrityl resin (Barlos *et al.*, 1991) using an HOAt/HATU coupling protocol (Carpino *et al.*, 1994). Acyloxyketone, *N*-Z-aspartic acid[(2,6-dimethylbenzoyl)oxy]-methyl ketone, β-*t*-butyl ester, was prepared according to the method of Krantz *et al.* (1991) from the corresponding bromomethyl ketone (Dolle *et al.*, 1994a) and deprotected by hydrogenolysis (Dolle *et al.*, 1994b) to afford aspartic acid[(2,6-dimethylbenzoyl)oxy]methyl ketone, β-*t*-butyl ester, as the HCl salt. Coupling of this amine with *N*-[biotinylaspartyl(β-*t*-butyl ester)glutamyl](γ-*t*-butyl ester)valine was carried out by HOAt/HATU-mediated solution phase synthesis (Carpino, 1993) as follows.

N-(biotinylaspartylglutamylvalinyl)aspartic acid [(2,6-dimethylbenzoyl)-oxy]methyl ketone (biotin–DEVDamk). To an ice-cooled solution of *N*-[biotinylaspartyl(β-*t*-butyl ester)glutamyl](γ-*t*-butyl ester)valine (150 mg, 0.215 mmol), aspartic acid[(2,6-dimethylbenzoyl)oxy]methyl ketone, β-*t*-butyl ester hydrochloride (80 mg, 0.215 mmol), HOAt (30 mg, 0.215 mmol) and HATU (125 mg, 0.33 mmol) in DMF (1 ml) was added diisopropylethyl amine (110 μl, 0.645 mmol) and the resulting solution was allowed to warm to room temperature. After stirring overnight, the mixture was concentrated under reduced pressure, and the residue was chromatographed on a silica gel column eluting with chloroform followed by chloroform:methanol (20:1). The material was further purified by a second silica gel column eluting with chloroform followed by a gradient of chloroform:acetone:methanol (4:1:0)–(2:1:0)–(2:1:1%) to afford 60 mg of the coupled product as a colorless solid. The *tert*-butyl esters were removed by treating this material with a mixture of TFA:DCM (3:2, 2.5 ml) at room temperature for 3 h. Concentration under reduced pressure followed by repeated treatment (3×) with toluene (0.5 ml) and concentration afforded 50 mg of the desired product as a light-yellow solid. Recrystallization from methanol gave a pure product which was shown by reverse-phase HPLC (C18) analysis to consist of a 1:1 mixture of diastereoisomers. Similar to the observation of Thornberry *et al.* (1994), ^{1}H NMR analysis indicated the diastereomeric center to be at the P_1 aspartic acid α-carbon. ES mass spectrum (M^-) m/e 847 [M-H], 869 [M-H+Na]; ES mass spectrum (M^+) m/e 849 [M+H], 871 [M+Na].

N-(Z-valinylalanyl)aspartic acid methyl ketone, β-*methyl ester (z-VADmk).* This was prepared as a diastereomeric mixture (racemic at aspartyl α-carbon) from *N*-(Z-valinylalanyl)aspartic acid, β-methyl ester (standard solid phase coupling) by a Dakin-West reaction with acetic anhydride, triethylamine and DMAP at 50°C (Steglich and Hofle, 1969). FAB mass spectrum (M^+) m/e 450 [M+H].

Immunohistological analysis and TUNEL assay

Fetal thymuses were embedded in O.C.T. compound (Miles, Elkhart, IN) on dry ice. 4 μm sections were prepared and subsequently stored at –80°C. Immunohistochemical studies were performed as previously described (Mombaerts *et al.*, 1993). For the TUNEL assay, frozen tissue sections were fixed in 3% buffered formalin for 10 min at room temperature. After washing with PBS, sections were fixed again in ethanol:acetic acid (2:1) for 10 min at –20°C. Endogenous peroxidase activity was blocked by covering the sections with 0.5% H_2O_2 for 20 min at room temperature. The sections were rinsed with PBS and immersed in TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). The sections were incubated with 2–4 mM biotinylated dUTP (Boehringer-Mannheim, Germany) and 10 U TdT (Promega, Madison, WI) in 25 μl TdT buffer in a humid atmosphere at 37°C for 2 h. The reaction was terminated by transferring the slides to TB buffer (300 mM NaCl, 30 mM Na citrate) for 15 min. After a PBS rinse, sections were incubated with 2% BSA for 10 min, rinsed in PBS for 5 min, incubated with avidin–biotinylated peroxidase complex (Dako, Santa Barbara, CA) diluted 1:100 in PBS for 30 min, washed in

PBS and developed with 3-amino-9-ethylcarbazole (Aldrich, Milwaukee, WI). Sections were lightly counterstained with Meyer's hematoxin (Gavrieli *et al.*, 1992).

Two-color immunohistochemical analysis was performed as previously described (Mizoguchi *et al.*, 1996). Briefly, 4-μm thick specimens were fixed in acetone for 7 min, air-dried, and incubated with purified anti-CD4 antibody (clone L3T4, PharMingen, San Diego, CA) for 1 h at room temperature. Specimens were incubated for 30 min with 0.3% H_2O_2 in PBS to block endogenous peroxidase activity. Endogenous biotin was blocked by sequential incubations with avidin-D (Vector, Burlingame, CA) and d-biotin (Sigma, St Louis, MO). For detection, biotinylated rabbit anti-rat Ig (Vector) was used, followed by an optimal dilution of avidin-biotinylated–peroxidase complex (Dako). The specimens were developed in a solution of 3-amino-9-ethylcarbazole (Aldrich) and the reaction stopped by dipping in distilled water for 5 min and washing in PBS, pH 7.4, for 10 min. The specimens were then incubated with biotin–DEVDamk peptide at room temperature for 1 h, followed by incubation with ABC-alkaline phosphatase reagent (Vector) for 30 min. After 15 min development with alkaline phosphatase substrate Kit III (Vector) and 1 mM levamisole (Sigma), the specimens were post-fixed with 2% paraformaldehyde and mounted with glycergel (Dako).

Preparation of thymic lysates and Western blot analysis

Thymocyte suspensions were prepared as described above for FACS. Cells were washed three times in PBS–1% BSA and lysates prepared as previously described (Thornberry *et al.*, 1994). Briefly, thymocytes were resuspended at 1×10^8/ml in hypotonic buffer containing 25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin. After 20 min on ice, cells were broken with 20 strokes in a tight-fitting Dounce homogenizer. Cell lysates were clarified by centrifugation at 2000 r.p.m. in an Eppendorf microfuge for 10 min followed by an 8 min spin in a Beckman airfuge at 100 000 *g*. The lysates were dialyzed overnight at 4°C against 20 mM Tris, pH 7.8, 2 mM DTT and 0.1% CHAPS. Cell lysates were aliquoted and stored at –80°C. Recombinant Yama and Mch2α were prepared as described (Orth *et al.*, 1996b).

For Western blot analysis, cell lysate equivalent to 2×10^6 cells was incubated with the indicated additions for 15 min at 25°C. Non-reducing sample buffer was added, samples boiled for 7 min and run on 12.5% SDS–PAGE under non-reducing conditions. Gels were blotted onto nitrocellulose and the membranes blocked overnight in PBS, 3% BSA, 0.1% Tween-20. The filter was washed twice for 10 min in PBS, 0.1% BSA, 0.1% Tween-20 and incubated 2 h at room temperature with a 1:10 000 dilution of streptavidin-conjugated horseradish peroxidase (ICN, Costa Mesa, CA) in PBS, 0.1% BSA, 0.1% Tween-20. The filter was washed 6×10 min with 50 mM Tris–HCl, pH 7.5, 0.25% gelatin, 0.05% Tween-20, 150 mM NaCl, 5 mM EDTA, then washed once in PBS and developed by ECL (Renaissance, NEN, Boston, MA).

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