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A Role for FADD in T Cell Activation and Development

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Summary

FADD is a cytoplasmic adapter molecule that links the family of death receptors to the activation of caspases during apoptosis. We have produced transgenic mice expressing a dominantly interfering mutant of FADD, lacking the caspase-dimerizing death effector domain, as well as mice overexpressing the poxvirus serpin, CrmA, an inhibitor of caspases downstream of FADD. While thymocytes from either line of mice were completely protected from CD95-dependent cytotoxicity, neither transgene afforded protection from apoptosis induced during thymocyte selection and neither led to the lymphoproliferative disorders associated with deficiencies in CD95. However, in FADD dominant negative (FADDdd) mice, early thymocyte development was retarded and peripheral lymphocyte pools were devoid of normal populations of T cells. We show that thymocytes and peripheral T cells from FADDdd display signaling anomalies, implying that FADD plays a previously uncharacterized role in T cell development and activation.

Introduction

The process of self versus nonself discrimination in the immune system occurs both in the development of T and B cells and the formation of an antigen-naive repertoire, as well as in the maturation of lymphocytes that undergo either antigen-induced activation, anergy, or cell death. These processes depend on signaling through antigen receptors and, importantly, several different coreceptors. A number of coreceptors have been characterized, and initially they appeared to have distinct functions. Some provided a survival or helper signal; some were found to be inhibitory to cellular activation; and some overtly promoted apoptosis. As work in the field has progressed, studies now indicate that these coreceptors do not provide a single function to the cell, but rather, the effect of their signaling is multifaceted and depends on cell type, activation status, and location in the cell cycle. For example, cross-linking of CD95 (Fas/Apo-1) is known to induce apoptosis under some but not all conditions (Nagata and Golstein, 1995), but it can also provide a survival signal (Rathmell et al., 1996). On the other hand, CD28 has a major role in providing a helper signal to T cells, and yet it can also facilitate the induction of apoptosis in late-stage developing thymocytes (Punt et al., 1994; Kishimoto and Sprent, 1997).

A particularly vexing problem has been identification of the molecules essential in mediating the apoptosis in the thymus that is associated with antigen-induced negative selection. In part, the problem is to design a model experimental approach that reflects physiological antigen-mediated deletion (Page et al., 1998). Depending on the experimental design, CD30 (Amakawa et al., 1996), CD40-ligand (Foy et al., 1995), or CD95 (Castro et al., 1996) can be shown to play a role in negative selection. Thus far there is no consensus concerning a coreceptor involved in negative selection.

Candidates for the receptors that could mediate thymocyte cell death include members of the tumor necrosis factor (TNF) receptor family: CD95, TNFRI, DR3, DR4, and DR5 (Tewari and Dixit, 1996; Darnay and Aggarwal, 1997; Yuan, 1997), some of which contain cytoplasmic "death domains" (Kitson et al., 1996; Bodmer et al., 1997; Pan et al., 1997). Trimerization of these molecules through TNF-family ligand binding causes the association of cytoplasmic death domain–containing proteins and assembly of the death-inducing signal complex (Kischkel et al., 1995; Boldin et al., 1996; Muzio et al., 1996; Nagata, 1997), as well as other proteins involved in signal transduction.

One of the cytoplasmic molecules that appears to be essential for apoptosis mediated by CD95, TNFRI, and DR3 is the cytoplasmic molecule FADD/MORT1, a Fas-associating protein with a novel death domain (Boldin et al., 1995; Chinnaiyan et al., 1995; Kischkel et al., 1995; Baker and Reddy, 1996; Boldin et al., 1996; Hsu et al., 1996; Varfolomeev et al., 1996; Zhang and Winoto, 1996). Overexpression of FADD can cause apoptosis, and this apoptosis can be inhibited by CrmA, the poxvirus inhibitor of the caspase-1 and caspase-8 molecules (Chinnaiyan et al., 1996). In addition to a death domain, FADD contains a death effector domain, and studies have shown that mutant FADD encoding the death domain alone can act as a dominant interfering inhibitor of death mediated by Fas ligand or TNF, whereas the death effector domain can mediate apoptosis (Chinnaiyan et al., 1996).

To determine whether signaling mediated by FADD plays a role in thymic negative selection, we expressed the FADD death domain (FADDdd) in transgenic mice under control of the proximal promoter of p56^{lck} (Chaffin et al., 1990). For comparison, we produced transgenic mice that express CrmA, the bovine poxvirus serpin-like molecule that can inhibit the activity of caspases-1 and -8. T cells and thymocytes from these mice were insensitive to killing through CD95 cross-linking, showing that both transgenes were effective, although no *lpr*-like lymphoproliferative disease was evident in mice up to 1.5 yr of age. In FADDdd but not CrmA mice, thymic development was retarded, a feature most evident by

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the small number of mature T cells in homozygous FADDdd transgenic mice. Neither FADDdd nor CrmA mice showed a defect in thymic negative selection, indicating that signaling through FADD is not essential for the induction of cell death; however, thymic T cells from FADDdd-transgenic mice were defective in activation through the T cell receptor (TCR) and CD28. These results suggest that there is a necessary signal for T cell development and activation that is transmitted through FADD or a molecule that binds to FADDdd.

Results

Transgenic Mice Expressing the Human FADDdd or Pox Virus CrmA

To inhibit the function of death domain–containing receptors that signal through FADD and caspase-8, two types of transgenic mice were produced. One set of transgenic lines was made to express the human FADDdd. This construct, which includes an AU1 epitope tag, was previously shown in transfection experiments to block apoptosis in both human and mouse cells (Chinnaiyan et al., 1996). A second set of lines was made to express the CrmA serpin-like protein encoded by bovine poxvirus. This protein serves as a competitive inhibitor of caspase-1 (Ray et al., 1992)– and caspase-8 (Muzio et al., 1996)–mediated cell death and protease activity (Komiyama et al., 1994), and it has also been shown to inhibit CD95-mediated cell death (Enari et al., 1995). During the course of these experiments transgenic mice expressing CrmA under control of the human CD2 promoter were described and shown to lack a defect in negative selection mediated by endogenous superantigens (Smith et al., 1996).

The cDNA coding sequences from the two different genes were subcloned into the p1017 vector containing the proximal promoter of p56^{lck} and the human growth hormone gene (Chaffin et al., 1990) (Figure 1A). The cloning site places the gene of interest into the 5′ untranslated region of the growth hormone gene. Founder lines were screened by Western blotting, and lines that expressed high levels of protein in the thymus were bred for testing. As depicted in Figure 1B, there was a high level of the CrmA product in thymocytes but not in splenocytes. For comparison, a stably transfected EL4 clone was tested in the same experiment. This is the expected pattern based on the specificity of the p56^{lck} proximal promoter for thymocytes, but not for mature T cells. In contrast, the FADDdd transgene product was expressed in thymocytes, but also at approximately the same levels in splenocytes and lymph node cells. Either the transgene has integrated in the genome in such a way as to extend the expression of the construct to include mature T cells, or else the transcribed message or the protein is stable for an extended period after thymic emigration. Either way, the transgenic product could be expected to affect the phenotype of both developing and mature T cells.

Inhibition of Apoptosis by FADDdd and CrmA Transgenes

To test the functional effectiveness of human FADDdd and CrmA in primary cells, we measured the ability of

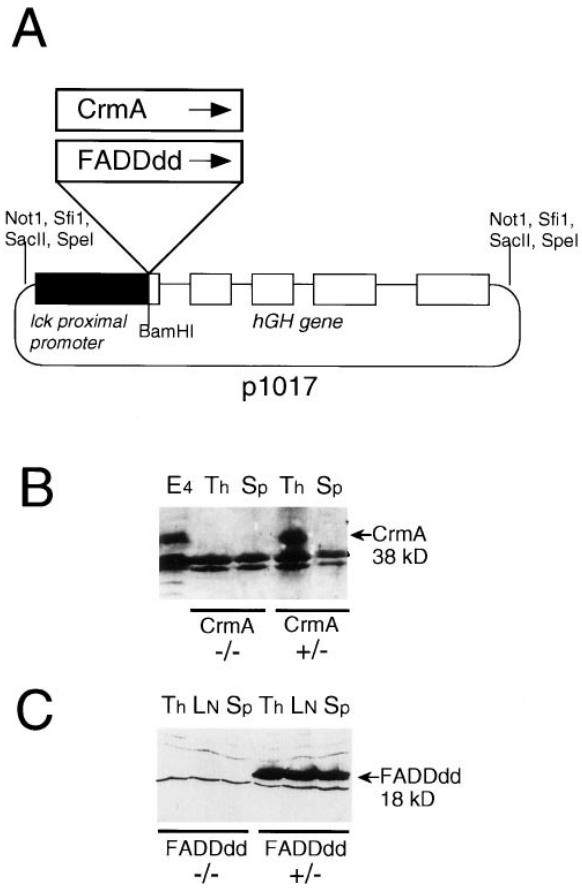

Figure 1. Production of p1017-FADDdd and p1017-CrmA Mice

(A) The FADDdd and CrmA constructs. CrmA and FADDdd inserts were cloned into the p1017 transgenic vector, flanked by the murine *lck* proximal promoter and the entire human growth hormone (*hGH*) gene.

(B) CrmA is expressed in the thymus of CrmA-transgenic mice. Western blot of thymocyte (Th) and splenocyte (Sp) lysates from CrmA-transgenic (CrmA$^{+/-}$) and wild-type (CrmA$^{-/-}$) mice using a rabbit polyclonal antibody to CrmA (38 kDa). Also included is a lysate from an EL4 murine T cell clone (E4) transfected with a CrmA mammalian expression construct (pβ-actin*neo*-CrmA).

(C) FADDdd is expressed in p1017-FADDdd–transgenic mice. Western blot of thymocyte (Th), lymph node (LN), and splenocyte (Sp) lysates from transgenic (FADDdd$^{+/-}$) and wild-type (FADDdd$^{-/-}$) mice. Two 18 kDa FADDdd bands migrating very closely during electrophoresis are evident in these types of Western blots, presumably as a result of posttranslational modification of the mutant polypeptide. Immunoprecipitation with anti-AU1 mAb revealed a similar pattern of bands in the FADDdd-transgenic mice only.

CD95 cross-linking or dexamethsone treatment to cause apoptosis. An early consequence of apoptotic commitment in thymocytes is a loss of the mitochondrial inner membrane potential $\Delta\psi_m$, a proton gradient across the inner mitochondrial membrane (Castedo et al., 1995; Petit et al., 1995; Zamzami et al., 1995a). This parameter was assayed by the lipophilic fluorochrome DiOC6(3), which fluoresces in the presence of a normal mitochondrial proton gradient. As depicted in Figure 2A, the FADDdd (top graphs) and the CrmA (bottom graphs) transgenes were effective in blocking apoptosis mediated by anti-CD95 but not that mediated by dexamethasone. The level of CD95 expression in the thymocytes

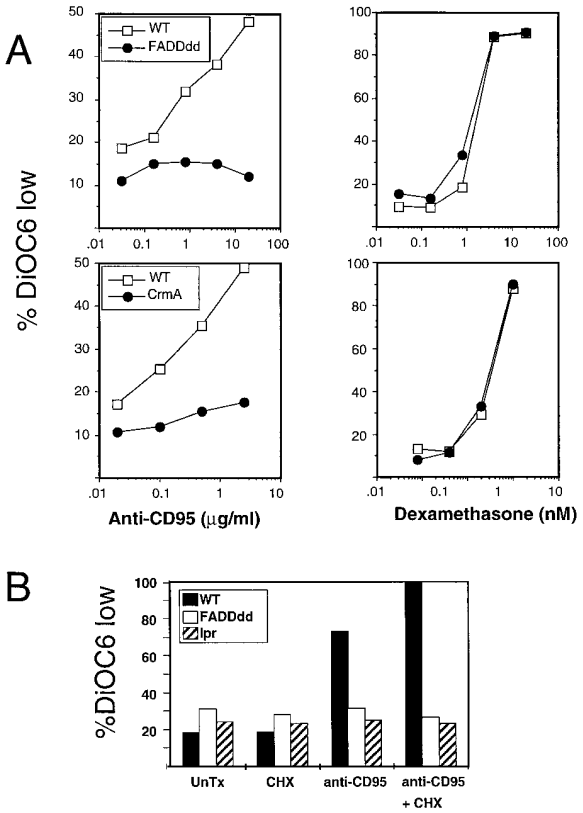

Figure 2. FADDdd and CrmA Transgenes Inhibit Anti-CD95–Mediated Apoptosis

(A) A comparison of apoptotic inhibition by FADDdd and CrmA. Apoptosis was measured by flow cytometry using DiOC6(3) to detect mitochondrial potential after 18 hr in culture. Numbers represent the percentage of thymocytes with low levels of DiOC6(3) staining relative to the entire population.

(B) Comparison of CD95-induced apoptosis in FADDdd and *lpr* mice. As in (A) except for the addition of 10 μg/ml cycloheximide (CHX) to some cultures. UnTx, untreated.

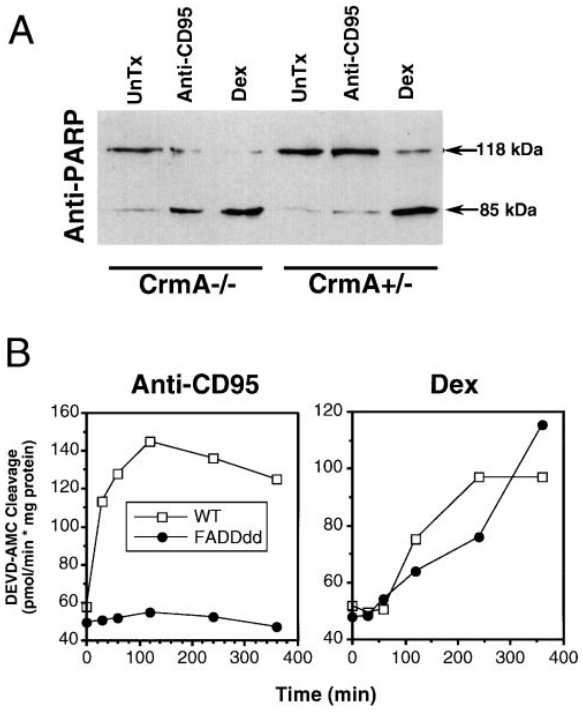

Figure 3. FADDdd and CrmA Transgenes Inhibit Caspase Activation

(A) Thymocytes from wild-type and CrmA$^{+/-}$ littermates were treated with 2 mg/ml anti-mouse CD95 or 10 nM dexamethasone (Dex) for 18 hr. Lysates from 5×10^6 cell equivalents were loaded into each lane of a 10% SDS-PAGE gel, electrophoresed, and transferred to nitrocellulose. PARP cleavage was visualized by incubation with rabbit polyclonal anti-bovine PARP antibody followed by enhanced chemiluminescence. Uncleaved PARP migrates at 118 kDa, whereas processed PARP migrates at 85 kDa. A small amount of PARP cleavage was consistently observed in untreated (UnTx) thymocyte cultures, likely a consequence of death resulting from thymocyte isolation and ex vivo culture.

(B) Time course of caspase activation in FADDdd$^{+/-}$ mice as measured by cleavage of the fluorogenic caspase substrate DEVD-AMC. Thymocytes from FADDdd and wild-type littermates were incubated for the noted times with 2 μg/ml anti-mouse CD95 or 10 nM dexamethasone; following culture, cells were harvested and lysed. Results are expressed as picomoles of AMC released (relative to a standard curve of free AMC) per minute of substrate incubation per milligram protein in the lysate. Untreated thymocytes have basal DEVDase activity for the same reason as described in (A).

of FADDdd-transgenic mice was normal as measured by flow cytometry (data not shown). Additional experiments using thapsigargan and staurosporine showed that neither FADDdd nor CrmA blocks these inducers of apoptosis. In addition, later stages of apoptosis were measured using annexin V (which measures the exposure of phosphotidylserine on the surface of the cell), hydroethidium (which fluoresces brightly in the presence of oxygen radicals produced during apoptosis) (Castedo et al., 1995; Zamzami et al., 1995b), and TUNEL (TdT-mediated dUTP-biotin nick end labeling) analysis of DNA fragmentation (Gavrieli et al., 1992). The only protection afforded by either the FADDdd or CrmA transgenes was from death induced by anti-CD95. With the addition of cycloheximide such that 100% of wild-type thymocytes were committed to apoptosis, thymocytes from FADDdd mice showed no change in mitochondrial potential over background; this level of inhibition was comparable to that afforded by the *lpr* mutation (Figure 2B). Consistent with its extended expression pattern, experiments showed that FADDdd inhibited apoptosis in activated peripheral T cells (data not shown).

Another early indication of apoptotic commitment is the activation of caspases and subsequent cleavage of a variety of cellular substrates. While CrmA can block apoptosis induction by directly interfering with caspases-1 and -8, FADDdd blocks the activation of these cascades by inhibiting the association of endogenous FADD with death-domain–containing molecules. It was of interest to determine whether these transgenes were capable of inhibiting the activation of caspase cascades by measuring the cleavage of known substrates. One such method is to assay for the cleavage of endogenous poly(ADP-ribose) polymerase (PARP), a DNA-associating nuclear protein inactivated by cleavage between its DNA-binding and catalytic domains during apoptosis; this cleavage can be detected in anti-PARP Western blots as processing of the normally 118 kDa PARP protein to an 85 kDa cleavage product (Lazebnik et al., 1994). As shown in Figure 3A, the CrmA transgene blocks PARP cleavage induced by anti-CD95 but not

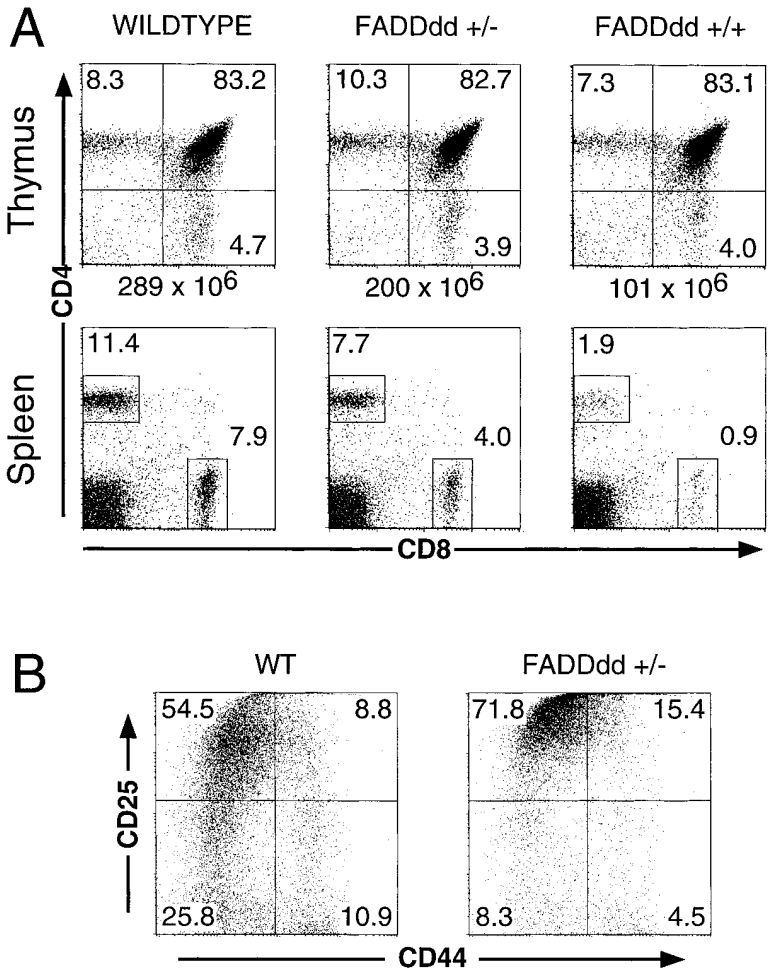

Figure 4. FADDdd Transgene Disrupts T Cell Development

(A) Thymocytes and splenocytes in FADDdd$^{+/-}$ and FADDdd$^{+/+}$ are diminished in number compared to littermates. Thymus and spleen CD4 versus CD8 populations were analyzed in 4-week-old littermates. The numbers below the dot plots indicate total thymus cellularity, whereas the numbers in the quadrants represent the percentage of cells staining in each gate.

(B) Populations defined by CD25 and CD44 expression were analyzed in DN thymocytes from FADDdd$^{+/-}$ and wild-type littermates. Thymocytes were treated with anti-CD4, anti-CD8, and complement; washed; and stained with anti-CD25 and anti-CD44 for analysis.

that induced by dexamethasone. These results demonstrate that glucocorticoid activation of apoptosis does not require caspases-1 and -8, although they do not rule out the involvement of other caspases. As a method for determining the levels of caspase activity present in apoptotic extracts, we employed a fluorogenic assay (Fernandes-Alnemri et al., 1995) that follows the release of the fluor aminomethyl coumarin (AMC) from the DEVD (aspartate-glutamate-valine-aspartate) tetrapeptide, the PARP cleavage site (see Experimental Procedures). As shown in Figure 3B, incubation of thymocytes with anti-CD95 leads to rapid activation of DEVD cleavage activity, whereas DEVD cleavage activity was not detected in similarly treated FADDdd$^{+/-}$ thymocytes. As expected, glucocorticoid-induced caspase activation occurs with slower kinetics and does not require functional FADD. Similar results were obtained with CrmA thymocytes (data not shown).

Peripheral Expression of FADDdd Does Not Lead to *lpr*-Like Lymphadenopathies

Defects in Fas signaling lead to lymphadenopathies first characterized in Fas-defective mice (Allen et al., 1990; Matsuzawa et al., 1990; Matsuzawa et al., 1991; Watanabe-Fukunaga et al., 1992) and Fas ligand–defective mice (Shirai et al., 1990; Wadsworth et al., 1990; Lynch et al., 1994; Takahashi et al., 1994). Surprisingly, despite the profound inhibition of CD95-mediated apoptosis in FADDdd mice and the expression of the transgene in peripheral T cells, we found no indication of lymphoproliferative disease, nor did we observe development of CD3$^+$B220$^+$ population in mice up to 1.5 years of age (data not shown). One explanation for this apparent enigma is that the FADDdd is not sufficiently effective to prevent physiological activation-induced cell death. A second is that activation of the cascade commencing with caspase-8 may be only part of the signaling activity mediated by CD95, and other signals are sufficient to mediate apoptosis. A third explanation is that FADDdd inhibits T cell activation, and thus, even if the cells are defective for activation-induced cell death, there is no disregulated lymphoproliferation. These possibilities are not mutually exclusive.

T Cell Development and Selection in FADDdd and CrmA Transgenic Mice

T cell development in FADDdd- and CrmA-transgenic mice was analyzed by characterizing thymocytes and splenocytes for the percentage and total number of cells in each of the subsets as defined by CD4 and CD8 expression (Figure 4A). Thymi from CrmA mice (including CrmA$^{+/+}$ mice) were consistently indistinguishable

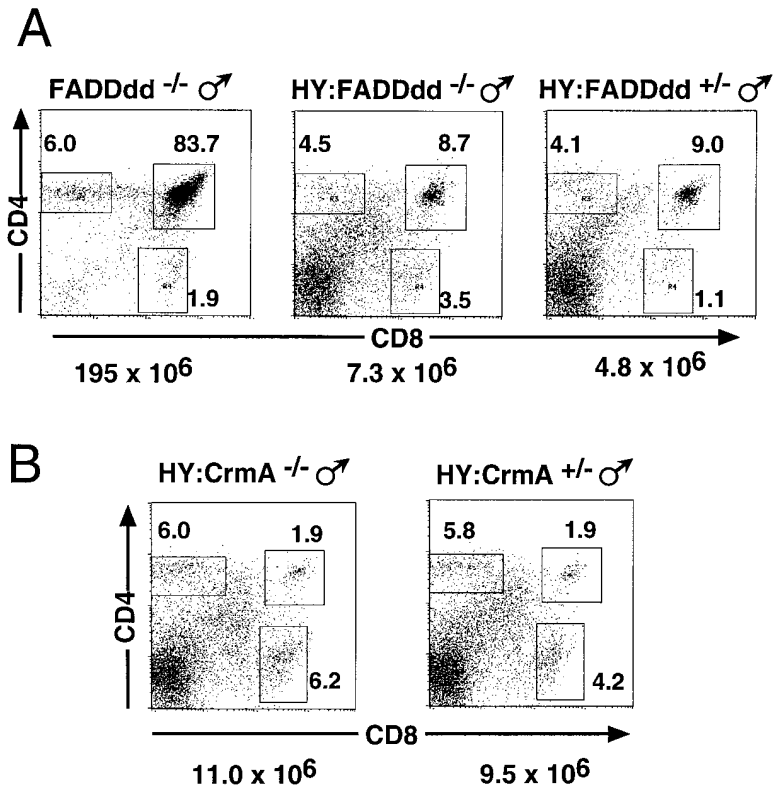

Figure 5. Negative Selection of Male $H2^b$ Thymocytes by the HY TCR Transgene Is Unperturbed by FADDdd and CrmA Transgenes

(A) $FADDdd^{+/-}$ mice were bred with a transgenic mouse carrying the male HY-specific TCR transgene, and (FADDdd × HY TCR) F_1 transgenic mice were obtained. Shown are profiles from 6-week-old littermates; the number below each dot plot is the number of thymocytes harvested from each mouse. All mice shown are MHC homozygous for $H\text{-}2^b$. Gates for CD4 and CD8 SP and DP thymocytes were drawn using thymocytes obtained from a non-TCR/non-FADDdd littermate (left dot plot); numbers next to these boxes indicate the percentages of thymocytes in each population.

(B) CrmA does not block negative selection in HY-specific TCR transgenic mice. $CrmA^{+/-}$ mice were bred with an HY TCR-transgenic mouse and the resulting double-transgenic thymocytes were harvested and analyzed by flow cytometry. The thymocytes were harvested from 7-week-old littermates.

from those of wild-type littermates, in agreement with previously published results (Smith et al., 1996). In contrast, the thymus subsets from FADDdd mice were substantially altered. Most notably, the total number of cells in homozygous transgenic mice was approximately 35% that of normal littermates. A compilation of thymus cellularity found in $FADDdd^{+/-}$ mice by week of age, assaying a total of 44 littermates and 46 $FADDdd^{+/-}$ mice, revealed a decrease consistent with the example presented in Figure 4A (data not shown). This defect was apparently more profound than revealed by the numbers in the thymus since the number of splenic T cells was dramatically reduced (as described below).

One possibility for the decreased thymus size is that signaling through the preTα–TCRβ receptor is inhibited, and this inhibition could cause both increased apoptosis and an inhibition of thymic expansion. Since we did not see an increase in the number of overtly apoptotic cells as measured by mitochrondrial potential or DNA fragmentation (TUNEL), the cells do not have the phenotype of preselection cells found in RAG-2–deficient mice or mice with severe combined immunodeficiency (Penit et al., 1995; Maraskovsky et al., 1997). A caveat is that engulfment of dead cells is extremely efficient, so a partial inhibition of signaling and the associated increased death rate may be difficult to detect (Surh and Sprent, 1994).

If there is a halt in development that prevents normal maturation, we might also expect to see abnormal DN precursor populations. The thymocytes from FADDdd-transgenic mice and their littermates were analyzed for the populations within the $CD4^-CD8^-$ (DN) subset. Thymocytes depleted of $CD4^+$ and $CD8^+$ cells were stained for CD25 and CD44. Previous work indicates that there is a progression from $CD44^+CD25^-$→$CD44^+CD25^+$→$CD25^+CD44^-$→$CD25^-CD44^-$ (Godfrey and Zlotnik, 1993; Godfrey et al., 1994). As shown in Figure 4B, $FADDdd^{+/-}$ mice have an enhanced population of $CD25^+CD44^-$ cells and a paucity of cells in the $CD25^-CD44^-$ population. This staining pattern provides evidence that there is a partial block in development at the $CD25^+CD44^-$ stage of development, a stage that corresponds to the expression of the preTCR–CD3 complex and the initiation of thymocyte proliferation (Kisielow and von Boehmer, 1995). This observation appears to account for the diminished thymus found in FADDdd mice. It is unlikely that this developmental block results from defects in the expression or presentation of the TCR complex, since normal levels of TCR chains were detected on the surface of $FADDdd^{+/+}$ mice and since TCR transgenes do not rescue this block (data not shown).

To determine whether these transgenic proteins can cause a defect in thymic negative selection, both types of transgenic mice were crossed with TCR–transgenic mice that have incorporated the HY–specific TCR. This model of negative selection is manifested by a small thymus containing almost no double-positive (DP) thymocytes (Kisielow et al., 1988). The interpretation is that deletion occurs at the DN stage or in the transition of thymocytes from DN to DP. As shown in the flow cytometry profiles presented in Figures 5A and 5B, neither FADDdd nor CrmA afforded protection against HY–mediated negative selection as is evidenced by the small number of DP thymocytes in these double-transgenic animals. In addition, the relative populations of peripheral T cells were essentially identical to wild-type (data

Table 1. Splenic T Cells in FADDdd$^{+/-}$ and Wild-Type Littermates

Age (wk)	Wild-Type			FADDdd$^{+/-}$		
	CD4 (%)	CD8 (%)	n	CD4 (%)	CD8 (%)	n
2	5.8 ± 1.6	2.2 ± 0.62	3	3.6 ± 1.6	1.3 ± 0.5[a]	4
3	11.8 ± 1.5	5.9 ± 0.5	2	6.6 ± 1.1[a]	2.0 ± 1.1[a]	2
4	8.3 ± 2.6	5.2 ± 2.2	6	6.2 ± 2.2	2.3 ± 1.1[a]	7
5	10.2 ± 2.1	4.8 ± 1.3	4	8.5 ± 2	2.4 ± 0.4[a]	4
6–9	16.2 ± 3.5	9.2 ± 2.5	4	15 ± 3.4	4.2 ± 0.7[a]	3

[a] Different from wild-type according to Student's t test ($p < 0.05$).

not shown). Previous work indicates that there is no single model of negative selection that serves as an indicator of thymic deletion, and future studies will examine the role of FADDdd in other models of negative selection.

FADDdd Mice Have a Diminished Population of Peripheral T Cells

To determine whether cells that had progressed past the CD44$^-$CD25$^+$ stage went on to complete development, the peripheral T cell population was analyzed in hemizygous and homozygous transgenic mice. The first readily apparent observation was that in 3- to 4-week-old FADDdd mice there are few splenic T cells and their number appears to depend on the number of copies of the transgene (Figure 4A, right graphs). In these mice, the number of T cells ranged from approximately 20% in wild-type mice to 3% in homozygous transgenic mice. Table 1 provides a compilation of the percentage of T cells in the spleen of FADDdd$^{+/-}$ mice by age. As shown, there was a significant reduction in the number of T cells in hemizygotes, especially CD8$^+$ T cells, but we expect that the number of CD4$^+$ T cells would become significant with a larger sample size. In older mice the numbers of CD4 T cells in the spleen and lymph nodes rise to wild-type levels, although the number of CD8 T cells remains low (Table 1 and data not shown). Although we have not examined as many FADDdd homozygous mice, there is a consistent and profound deficiency in the CD4 and CD8 T cell populations. A similar decrease in mature T cell populations was not seen in CrmA-transgenic mice (data not shown).

FADDdd but Not CrmA Inhibits Activation of T Cells through the TCR and CD28

To measure the ability of T cells to be activated through TCR and coreceptor interactions, thymocytes were stimulated with various combinations of plate-bound anti-CD3 and anti-CD28 antibodies. The responding cells in wild-type and FADDdd mice were entirely CD4$^+$ and CD8$^+$ single-positive (SP) cells as assessed by flow cytometric analysis of the blast cells after activation (data not shown). In addition, the level of CD3 and CD28 expressed on the mature T cells was identical to that of wild-type littermates (data not shown). Thymocytes from FADDdd mice were defective for proliferation when stimulated with either anti-CD3 and -CD28 or anti-CD3 and an optimal concentration of phorbol myristate acetate (PMA) (Figure 6A). The cells were competent to respond since they proliferated normally in response to PMA and ionomycin. The cause of the unresponsiveness does not seem to be readily explained by a lack of maturity, since FADDdd mice have normal numbers of CD4$^+$CD8$^-$HSA^{lo} and CD4$^-$CD8$^+$HSA^{lo} cells, a phenotype indicative of antigen responsiveness (Ramsdell et al., 1991). The defect was seen within a wide dose of anti-CD3 and superoptimal anti-CD28, as shown in the experiment presented in Figure 6B. One interpretation is that the FADDdd-transgenic protein blocks the signal transduction that is required for T cell activation. Furthermore, since CrmA does not have the same effect, the signal does not depend on caspase-8 activation, but rather it is a distinct signaling cascade that branches upstream of activation of caspases-1 and -8. Combined with the paucity of T cells in the spleens of young mice, these data indicate that newly developed T cells are defective for activation and emigration from the thymus.

The proliferative defect found in peripheral T cells was different. As presented in Figures 6C and 6D, the responsiveness to anti-CD3 plus anti-CD28 was not significantly different from that of littermate controls. However, the lymph node T cells from FADDdd mice displayed a highly abnormal phenotype with respect to other methods of activation. The cells appeared able to respond to some extent to the addition of PMA alone, whereas they were reproducibly deficient when activated in response to anti-CD3 plus PMA or even ionomycin plus PMA (Figure 6C).

The ability of the lymph node T cells from FADDdd mice to be stimulated to divide by the addition of PMA has not previously been observed, and so it was investigated further. PMA was titrated into cultures of thymus and lymph node cells from FADDdd mice and wild-type littermates. The results (Figure 6E) revealed that indeed lymph node T cells undergo proliferation in response to PMA alone, whereas neither wild-type T cells nor thymocytes from FADDdd show the same responsiveness. Clearly, there is a dramatic imbalance in the signaling circuitry in thymocytes and T cells from FADDdd mice, and this imbalance is manifested differently in thymic and lymph node T cells.

Discussion

To date, work in the field has shown that FADD is an essential adapter between the receptors that possess a death domain—CD95, TNFRI, and DR3—and caspase-8.

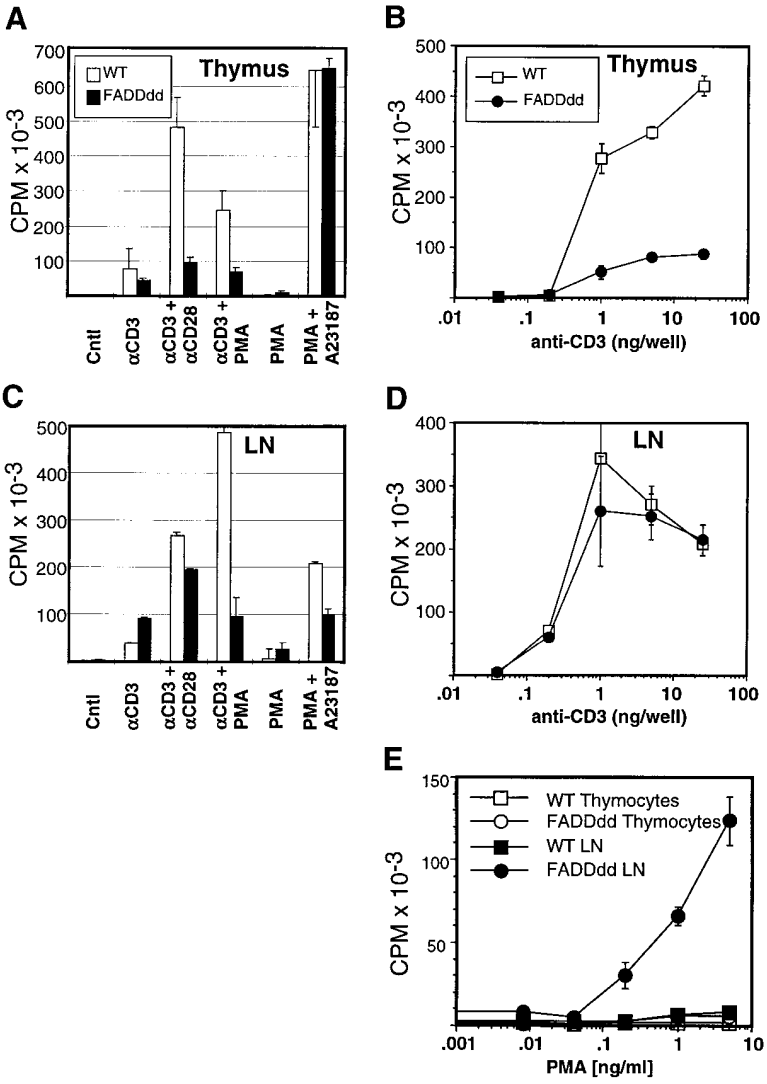

Figure 6. The FADDdd Transgene Alters the Proliferative Responses of Thymocytes and Peripheral T Cells

(A) Activation of FADDdd thymocytes. Thymocytes (equalized for 5×10^5 CD4 SP cells/well) in a 96-well plate were incubated in culture media alone (Cntl), with 5 ng/well of plate-bound anti-mouse CD3 (αCD3) without and with superoptimal anti-mouse CD28 (αCD28). In some wells, PMA was added at 1 ng/ml without or with plate-bound anti-CD3 or with 0.1 μg/ml of the calcium ionophore A23187. Each result represents the mean counts per minute from an experiment performed in triplicate; error bars represent the standard deviation from this mean.

(B) Thymocytes from FADDdd mice are defective for anti-CD3 plus anti-CD28 activation. FADDdd$^{+/-}$ thymocytes were stimulated with the indicated amount of plate-bound anti-mouse CD3 antibody and soluble anti-CD28 ascites and analyzed as described above.

(C) Lymph node cells from FADDdd$^{+/-}$ transgenic are defective in T cell activation. Lymph node (LN) cells (equalized for 5×10^5 CD4 SP T cells/well) were treated and analyzed as described in (A).

(D) Peripheral T cells from FADDdd mice do not show a defect in anti-CD3 plus anti-CD28 activation. Dose dependence of TCR ligation during activation of LN cells from wild-type and FADDdd$^{+/-}$ mice.

(E) Thymocytes and LN cells obtained from FADDdd$^{+/-}$ and wild-type mice were cultured with the indicated concentrations of PMA for 48 hr prior to an 18 hr pulse with [^{3}H]thymidine. Thymocytes and LN cells were added at a concentration normalized to 5×10^5 CD4 T cells/well. Results represent the mean ^{3}H counts per minute incorporated during triplicate experiments.

Since this cascade of signaling has been directly shown to mediate all of the hallmarks of apoptosis, FADD has justifiably been implicated as a mediator of activation-induced cell death (Vincenz and Dixit, 1997). As in all biological processes such a simplistic explanation appears to be insufficient to explain the role of death domain–containing proteins in the physiology of the immune system. In this report we show that a dominant-interfering form of FADD inhibits T cell development and activation, and these results imply that functional FADD signaling is essential for normal T cell development and T cell activation.

In FADDdd-transgenic mice there is a defect in the progression of thymocytes from the CD25$^+$CD44$^-$ to the CD25$^-$CD44$^-$ phenotype that was previously shown to be associated with preTCR expression and p56^{lck} activation (Anderson et al., 1992; Levin et al., 1993; Godfrey et al., 1994). In mice that lack the capacity for TCRβ rearrangement, there is also a block at this stage and a large number of apoptotic cells (Penit et al., 1995; Maraskovsky et al., 1997). We did not find any increase in the number of apoptotic cells, indicating that the block may occur after TCR β-chain selection. On the other hand, since preTα–TCRβ signals promote expansion, survival, and β-chain exclusion (Groettrup and von Boehmer, 1993), it is possible that effects of the FADDdd molecule are selective. There is a further defect in the ability of mature thymocytes in these mice to respond through the TCR and CD28 even though they have a normal population of mature thymocytes as assessed by TCR, CD4, CD8, and HSA expression. The implication of these latter results is that there does not seem to be a defect in positive selection and that FADD does not play a role in this aspect of signaling-mediated maturation. This hypothesis was confirmed by analyzing mice that express FADDdd together with either class I– or class II–specific TCR trangenes. In these mice there were normal proportions of CD8 or CD4 SP thymocytes, respectively. Finally, there was found to be a deficit of T cells in peripheral lymphoid organs: either mature T cells did not emigrate from the thymus efficiently; or they did not home efficiently to the spleen; or their rate of survival was decreased. Since the lack of T cells was most severe in young mice and diminished to some extent with age,

we favor the idea that the cells are inefficiently exported from thymus. We wish to consider the possibility that in the absence of FADD signaling, only those T cells that can compensate by becoming hyperresponsive to other signals would leave the thymus and populate the periphery. This hypothesis is strengthened by the finding that, unlike thymocytes, peripheral FADDdd T cells are capable of normal mitogenic stimulation by anti-TCR and anti-CD28, although they are activated by PMA alone, a signal insufficient to activate normal T cells. It is likely that the complete elimination of FADD could manifest an even more severe phenotypic deficiency.

Our understanding of the contribution of CD95 activation in immune development, function, and homeostasis is far from complete. It is clear that apoptosis mediated by CD95 contributes a complimentary cytotoxic mechanism to that afforded by perforin and granule release during cytotoxic T lymphocyte and natural killer cell cytotoxicity (Rouvier et al., 1993; Kagi et al., 1994; Kojima et al., 1994; Lowin et al., 1994; Walsh et al., 1994a, 1994b). In addition, CD95-induced apoptosis may also control access of activated T cells to immune privileged sites (Griffith et al., 1995, 1996). Yet, a putative role in transducing apoptotic signals during thymic selection is less clear. Several investigators have found no disruption in TCR transgene– and endogenous superantigen–mediated negative selection in CD95-mutant mice (Kotzin et al., 1988; Zhou et al., 1991; Herron et al., 1993; Singer and Abbas, 1994; Adachi et al., 1996; Sytwu et al., 1996). On the other hand, a model of negative selection mediated by in vivo administration of agonistic anti-TCR antibodies (Shi et al., 1991) has been shown to be blocked in *lpr* mice (Castro et al., 1996). We have also observed this effect in *lpr* mice and have noted a similar inhibition of this acute, wholesale thymocyte deletion in CrmA mice (data not shown). Since FADDdd, CrmA, and *lpr* mice manifest no defect in more physiological models of negative selection, we favor a model in which thymocyte negative selection occurs by processes independent of CD95 and the other death domain receptors.

While we set out to test the simple hypothesis that thymic negative selection depends on FADD as an adapter to an apoptosis cascade, we learned instead that FADD-binding molecules must have other functions in T cells—functions that are independent of the activation of caspase-8. The signal that is inhibited by FADDdd may or may not come from a death domain–containing receptor, although neither T cells from mice bearing the *lpr* mutation (Singer and Abbas, 1994), T cells from CD95-deficient mice (Adachi et al., 1995), T cells from TNFRI-deficient mice (Matsumoto et al., 1996; Page et al., 1998), nor T cells from CrmA mice (Smith et al., 1996) show the phenotypic characteristics of the FADDdd mice. The only other receptor known to mediate activity via FADD is DR3; however, it is certainly possible that there are other FADD-binding receptors that have yet to be discovered. It is possible that FADD mediates its effect on T cell activation by binding to a nonreceptor, cytoplasmic death domain, or even a protein without a death domain. The present results suggest that there is a novel signaling pathway, essential for activation and development of T cells, that involves FADD.

Experimental Procedures

Mice

Mice were generated, bred, and maintained at the University of California, San Diego animal facilities. HY mice are transgenic for TCR α and β chains that confer specificity for the male HY antigen in the context of MHC class I D^b (Kisielow et al., 1988).

Production of p1017-FADDdd and p1017-CrmA–Transgenic Mice

The p1017-FADDdd expression vector was generated by subcloning the AU1-tagged NFD4 truncation of human FADD (amino acids 80–208) (Chinnaiyan et al., 1995) into the BamHI cloning site of the p1017 *lck* proximal promoter murine expression vector. This BamHI site is flanked at the 5′ end by the proximal promoter region of the murine $p56^{lck}$ gene and at the 3′ end by the entire gene encoding human growth hormone (Chaffin et al., 1990; Sentman et al., 1991; Swan et al., 1995). This version of FADD is missing the first 80 amino acids corresponding to the putative death effector domain proposed to associate with target caspases FLICE (Boldin et al., 1996; Muzio et al., 1996) and FLICE-2 (Vincenz and Dixit, 1997). The p1017-CrmA transgene was produced by similarly cloning the entire 1.0 kb open reading frame of cowpox virus CrmA (Ray et al., 1992) into the p1017 BamHI cloning site. These constructs were prepared by cesium chloride banding, cut with NotI, and isolated by sucrose gradient extraction procedures. The isolated fragments were used for microinjection of (C57BL/6 $\times$ BALB/c) F_1 embryos by the University of California, San Diego Transgenic Core Facility. Transgenic founders were identified by Southern analysis of tail DNA, and the resulting founders were backcrossed to C57BL/6 females or intercrossed.

Western Analyses

CrmA Western blots were performed using thymocytes and splenocytes isolated from wild-type and $CrmA^{+/-}$ littermates. Following harvest, 2×10^7 lymphocytes from indicated organs were lysed in 70 μl of 1% Nonidet P-40 lysis buffer (50 mM Tris [pH 7.6], 120 mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg/ml leupeptin, 1 μg/ml aprotinin) followed by a 20 min high-speed spin in a microfuge at 4°C to remove debris. The supernates were boiled in SDS loading buffer and run on 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the samples were transferred to Protran nitrocellulose membranes (Schleicher and Schuell) and blocked by overnight incubation in PBST (1$\times$ phosphate-buffered saline [PBS], 0.1% Tween-20) supplemented with 5% nonfat milk protein (PriceClub brand). For primary incubation, blots were incubated in a 1:1000 dilution of anti-CrmA rabbit polyclonal serum (Tewari and Dixit, 1995) diluted in PBST + milk (PBSTM). Blots were washed three times in PBSTM followed by incubation with horseradish peroxidase–conjugated goat-anti-rabbit antibody (Southern Biotechnology Associates, Birmingham, AL) diluted 1:2500 in PBSTM. Blots were developed using enhanced chemiluminescence (Amersham).

FADD Western blots were performed in a similar fashion with lymphocytes harvested from wild-type and $FADDdd^{+/-}$ littermates. Following harvest, 2×10^7 lymphocytes from each indicated organ were lysed in 1% Triton X-100 lysis buffer (1% Triton, 10 mM Tris [pH 8.0], 150 mM NaCl, and the proteinase inhibitor cocktail listed above) and electrophoresed on 12% SDS-PAGE. Following separation, samples were transferred to nitrocellulose and blocked in PBSTM. Blots were incubated in anti-FADD rabbit polyclonal serum diluted 1:1000 in PBSTM and washed as described above.

PARP Western blots were performed using whole-cell lysates isolated from apoptotic thymocyte extracts. Following apoptotic induction, 5×10^6 thymocytes were resuspended, lysed in 1$\times$ PARP sample buffer (50 mM Tris [pH 6.8], 6 M urea, 6% β-mercaptoethanol, 3% SDS, 0.003% bromophenol blue), and boiled 5 min prior to loading and electrophoresis through a 10% SDS-PAGE gel. Samples were transferred to nitrocellulose, blocked overnight with PBSTM, and incubated with anti-bovine PARP polyclonal rabbit antiserum 422 (Lazebnik et al., 1994) (a kind gift from Guy Poirier) diluted 1:1000 in PBSTM.

Flow Cytometry, Antibodies, and CD4 and CD8 Depletions

Lymphocyte populations were harvested and washed in fluorescence-activated cell sorter (FACS) staining buffer (1$\times$ PBS, 2% fetal

calf serum, 0.1% sodium azide) prior to staining with antibodies. CD4 versus CD8 two-color dot plots were produced by staining lymphocytes with phycoerythrin-conjugated anti-CD4 and anti-CD8-Tricolor antibodies (Caltag, Burlingame, CA). Staining for CD3 was performed using fluorescein isothiocyanate (FITC)–conjugated anti-CD3 antibodies (Pharmingen, La Jolla, CA). Similarly, staining for the HY TCR was performed using FITC-conjugated anti-Vβ8 antibodies (Pharmingen).

To analyze double-negative (DN) thymocytes, thymocytes were depleted of $CD4^+$ and $CD8^+$ cells by incubation with rat antibodies to murine CD4 and CD8 (RL172.4 and 3.168.36, respectively) along with rabbit complement diluted 1:10 (Accurate Chemical and Scientific, Westbury, NY). Following a 30 min incubation with antibodies and complement at 37°C, cells were pelleted to remove debris, and aliquots analyzed by flow cytometry (Caltag) to ensure complete depletion of $CD4^+$ and $CD8^+$ cell populations. The cells were then stained with biotinylated anti-CD25 (Pharmingen), followed by streptavidin–phycoerythrin. Finally, cells were washed again and stained with FITC-conjugated anti-CD44 (Pharmingen).

Analysis of early apoptotic events was performed using the lipophilic/cationic dye DiOC6(3) as described (Castedo et al., 1995; Petit et al., 1995). DiOC6(3) (Molecular Probes, Eugene, OR) was diluted to 40 nM in FACS staining buffer (along with anti-CD4-phycoerythrin and anti-CD8-FITC). Thymocytes (1×10^6) were incubated in 100 μl of this cocktail for 15 min at 37°C, washed once in FACS buffer, and analyzed by flow cytometry. Events were collected on a FACScan flow cytometer (Becton-Dickinson, Palo Alto, CA) and analyzed using CellQuest software. All data represent events electronically gated in forward and side scatter to remove spurious events, dead cells, and nonlymphocytes.

DEVD-AMC Cleavage Assays

Caspase activity in apoptotic thymocyte extracts was measured fluorometrically using the fluorogenic caspase substrate DEVD-AMC (Alexis Pharmeceuticals, San Diego, CA) (Pennington and Thornberry, 1994). Extracts were prepared by pelleting thymocytes in microcentrifuge tubes, resuspending in 50 μl of KPM buffer (50 mM KCl, 50 mM PIPES-KOH [pH 7.0], 10 mM EDTA, 2 mM $MgCl_2$) supplemented with 1 mM dithiothreitol, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin (Martins et al., 1997) followed by three freeze–thaw lysis cycles in a dry ice–methanol bath. Lysates were pelleted for 20 min at high speed in a microcentrifuge tube to remove cellular debris, and supernates were retained. These lysates were tested for protein concentration using a standard Bradford protein assay (BioRad, Hercules, CA), and 100 μg of lysate protein in 50 μl was added to each well in 96-well microtiter plates for reaction with the fluorogenic substrate. DEVD-AMC was diluted to 20 μM in KPM buffer supplemented with 1 mM dithiothreitol, and 50 μl was added to each reaction well. As a control for background fluorescence, DEVD-AMC was added to 50 μl complete KPM buffer alone. These reactions were incubated at 37°C for 1 hr prior to analysis. In addition, a standard curve for AMC fluorescence was generated by diluting a known concentration of AMC (Molecular Probes) in KPM. The fluorescence in each well was read on a 96-well plate–reading spectrofluorimeter (PerSeptive Biosystems, Framingham, MA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Values represent the mean of triplicate reactions. The concentration of AMC released in each reaction was determined using the AMC standard curve and these values were adjusted for 60 min cleavage reactions with 0.1 mg of cellular protein to yield the units of DEVD-AMC cleavage activity indicated.

Culture Methods and Thymocyte Apoptosis Induction

Single-cell suspensions from lymphoid organs were pelleted, washed in Hanks' balanced salt solution, and then resuspended in EHAA (Click's) culture medium (Gibco, Gaithersburg, MD) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM glutamine, nonessential amino acids (Irvine Scientific), 0.11 mg/ml sodium pyruvate (Irvine Scientific), 5.5×10^{-5} M β-mercaptoethanol, and 10% fetal bovine serum (Hyclone, Logan, UT). For apoptosis assays, 2×10^6 cells in 1 ml of supplemented EHAA were added to each well in 24-well tissue culture plates (Costar, Cambridge, MA) for 18 hr incubations at 37°C in a 5% CO_2 buffered incubator. For Fas-induced apoptosis, the anti-mouse CD95 monoclonal antibody (mAb) Jo2 (Pharmingen) was added directly to the culture media at the concentrations indicated. Glucocorticoid-induced apoptosis was induced using dexamethasone (Sigma, St. Louis, MO) added to culture media at the indicated concentrations.

Proliferation Assays

Thymocytes and lymph node cells were stained using anti-CD4 and anti-CD8 antibodies to normalize the numbers of T cells in each culture. Cells equivalent to 5×10^5 $CD4^+$ cells in 200 μl of supplemented EHAA (see above) were added to each well of culture-treated flat-bottomed 96-well plates (Costar). Anti-CD3 was plate bound by adding goat-anti-hamster antibody (Southern Biotechnology Associates) in a carbonate buffer (pH 9.5) at a 1:100 dilution; following a wash to remove unbound goat-anti-hamster, the anti-mouse CD3 mAb 2C11 was added at a concentration of 5 ng/ml (unless otherwise indicated), incubated for 1 hr at 37°C, and washed to remove unbound 2C11. In some wells, soluble anti-mouse CD28 hybridoma supernate (Gross et al., 1992) was added at a dilution of $1{:}2 \times 10^5$. PMA (Calbiochem, San Diego, CA) was added to specified cultures at a final concentration of 1 ng/ml, unless indicated otherwise. The calcium ionophore A23187 (Sigma) was added to cultures at a final concentration of 0.1 μg/ml. Cultures were incubated at 37°C for 48 hr and then pulsed for 18 hr with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Wilmington, DE). Cultures were harvested and analyzed with a plate-reading scintillation counter with aqueous scintillation fluid. Counts displayed represent the mean of triplicate values for each experimental condition.

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