The EMBO Journal Vol.17 No.3 pp.706–718, 1998

A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes

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Members of the tumour necrosis factor receptor family that contain a death domain have pleiotropic activities. They induce apoptosis via interaction with intracellular FADD/MORT1 and trigger cell growth or differentiation via TRADD and TRAF molecules. The impact of FADD/MORT1-transduced signals on T lymphocyte development was investigated in transgenic mice expressing a dominant negative mutant protein, FADD-DN. Unexpectedly, FADD-DN enhanced negative selection of self-reactive thymic lymphocytes and inhibited T cell activation by increasing apoptosis. Thus signalling through FADD/MORT1 does not lead exclusively to cell death, but under certain circumstances can promote cell survival and proliferation.

Keywords: APO-1/apoptosis/CD95/FADD/MORT1/Fas/T lymphocytes

Introduction

Apoptosis is an evolutionarily conserved cell death mechanism that can be triggered by a broad range of physiological signals or experimentally applied stress conditions (reviewed by Jacobson *et al.*, 1997). Independent signalling cascades converge upon activation of latent intracellular cysteine proteases (caspases), leading to proteolysis of vital cellular constituents and ultimately collapse of the cell. Cytoplasmic adaptors, such as CED-4 or FADD/MORT1 (see below), appear to be critical for recruitment and activation of caspase zymogens and are likely to be the targets of anti-apoptosis proteins, like those of the Bcl-2/CED-9 family or FLIP (reviewed by Jacobson *et al.*, 1997).

Apoptosis plays a prominent role in the development and functioning of the immune system (reviewed by Strasser, 1995). Developing B and T cells that do not express functional antigen receptors fail to receive a positive signal and die by apoptosis, while lymphocytes bearing autoreactive antigen receptors undergo apoptosis in response to a death signal from neighbouring cells (Murphy *et al.*, 1990; Surh and Sprent, 1994). Cell death is also prominent during immune responses in peripheral lymphoid organs. As in immature cells, apoptosis of activated mature lymphocytes can result either from a failure to obtain a positive signal (e.g. via cytokine receptors) or as a consequence of repeated antigen receptor cross-linking provoking a death signal (reviewed by Strasser, 1995). The former is often referred to as 'death by neglect' and the latter as 'activation-induced cell death'. These two physiologically triggered cell death pathways are subject to distinct control. Bcl-2 and its homologues block 'death by neglect' in many circumstances but are ineffective antidotes to antigen receptor-induced apoptosis of immature lymphocytes or cycling mature B and T cells. In contrast, members of the tumour necrosis factor receptor (TNF-R) family play a critical role in 'activation-induced cell death' but are dispensable for 'death by neglect' (reviewed by Strasser, 1995; Jacobson *et al.*, 1997).

Cross-linking of the T cell receptor (TCR) triggers apoptosis in T hybridoma cells and activated normal T cells by inducing CD95 ligand (also called Fas ligand or APO-1 ligand) expression, followed by autocrine or paracrine stimulation of the death-inducing surface receptor CD95 (also called Fas or APO-1) (Alderson *et al.*, 1995; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995). Mice carrying spontaneous or experimentally introduced mutations in CD95 (*lpr* or *CD95* gene knock-out) or CD95 ligand (*gld*) develop progressive lymphadenopathy and autoimmunity, apparently due to their failure to delete chronically activated lymphocytes (reviewed by Nagata, 1997). Deletion of antigen-stimulated peripheral T cells is slowed in mutant mice lacking p55 TNF-R1 or p75 TNF-R2, indicating that these receptors also play a role in activation-induced cell death (Speiser *et al.*, 1996; Sytwu *et al.*, 1996).

Negative selection of autoreactive thymocytes resembles activation-induced cell death of mature T lymphoblasts: it is also a consequence of TCR ligation, occurs through apoptosis (Murphy *et al.*, 1990; Surh and Sprent, 1994) and involves caspase activation (Clayton *et al.*, 1997). Members of the TNF-R family have been implicated in this process as well, but the precise events in this death pathway have yet to be determined. Immature $CD4^+8^+$ thymocytes express CD95 and other members of the TNF-R family and are susceptible to apoptosis induced by agonistic anti-CD95 antibodies (Ogasawara *et al.*, 1995), recombinant soluble CD95L (Suda *et al.*, 1996) or cell surface-bound CD95L (Müller *et al.*, 1995). However, deletion of autoreactive thymocytes occurs normally in mutant *lpr* (Sidman *et al.*, 1992), $CD95^{-/-}$ (Adachi *et al.*, 1995), $TNF\text{-}R1^{-/-}$ (Pfeffer *et al.*, 1993; Rothe *et al.*, 1993) and $TNF\text{-}R2^{-/-}$ mice (Erickson *et al.*, 1994) and is only partially impaired in $CD30^{-/-}$ mice (Amakawa *et al.*, 1996), indicating that negative selection cannot be attributed to ligation of any one of these receptors alone.

The TNF-R family consists of type I transmembrane proteins characterized by cysteine-rich repeats in their extracellular ligand binding domains (reviewed by Wallach

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et al., 1996). A subset of the family, comprising CD95, TNF-R1, p75 NGF-R, DR3 (also called WSL-1, TRAMP, APO-3 or LARD), CAR1 and the two TRAIL receptors (DR4/TRAIL-R1 and TRAIL-R2) also share a conserved motif in their cytoplasmic regions, known as the death domain, because it is essential for transducing the apoptotic signal (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993; Chinnaiyan *et al.*, 1996a; Pan *et al.*, 1997; Walczak *et al.*, 1997; reviewed by Wallach *et al.*, 1996). Upon receptor ligation the death domain acts as the docking site for homotypic interaction with death domain-containing cytoplasmic proteins such as FADD (also called MORT1) (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995) and TRADD (Hsu *et al.*, 1995). FADD/MORT1 binds directly to CD95 (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995) and is recruited to TNF-R1, DR3 and possibly other related receptors via TRADD (Chinnaiyan *et al.*, 1996a; Hsu *et al.*, 1996b). The death effector domain at the N-terminal end of FADD/MORT1 then interacts with a related motif in the prodomain of caspase-8 (also called FLICE, MACH or Mch5) (Boldin *et al.*, 1996; Muzio *et al.*, 1996) or caspase-10b (also called FLICE2) (Vincenz and Dixit, 1997). Activation of these upstream cysteine proteases is thought to trigger a proteolytic cascade which apparently constitutes the 'point of no return' in apoptosis. Under certain circumstances, however, CD95 and other members of the TNF-R family that contain death domains can stimulate alternative signalling pathways which exert positive effects on cell survival and proliferation, rather than triggering apoptosis (see Discussion; reviewed by Wallach *et al.*, 1996).

Thymocyte negative selection may not be perturbed in mutant mice lacking a single member of the TNF-R family because of functional redundancy among these receptors. This hypothesis can be tested by inactivating all of these receptors or a common signal transducer. A mutant of FADD/MORT1 which lacks the death effector domain and therefore cannot transduce a signal to caspase-8 acts as a specific inhibitor of CD95-, TNF-R1-, DR3- and TRAIL-R2-transduced apoptosis in various cultured cell lines (Chinnaiyan *et al.*, 1996a,b; Hsu *et al.*, 1996b; Walczak *et al.*, 1997) and there is, to our knowledge, no indication that it interferes with any other process. We generated transgenic mice expressing a dominant interfering mutant of FADD/MORT1 (hereafter called FADD-DN) under control of the mouse *lck* proximal promoter to investigate the impact of FADD/MORT1-transduced signals *in toto* on T lymphocyte development and function. Unexpectedly, we found that FADD-DN expression enhanced thymocyte negative selection and inhibited peripheral T cell activation by increasing apoptosis. These results provide evidence that signalling pathways operating through FADD/MORT1 do not lead exclusively to apoptosis, but under certain circumstances can promote cell survival and proliferation.

Results

Generation of transgenic mice expressing a dominant interfering mutant of FADD/MORT1

We generated two independent lines of C57BL/6J transgenic mice (strains 60 and 64) expressing a dominant interfering mutant of FADD/MORT1, FADD-DN, which lacks the death effector domain that is needed for activation of caspase-8 (Figure 1A). The N-terminal FLAG epitope tag, utilized to detect protein expression, had no effect on FADD-DN function in CD95L-sensitive CH1 murine B lymphoma cells (data not shown). Consistent with the T cell specificity of the *lck* proximal promoter, the 16 kDa FADD-DN protein was detected by Western blotting (Figure 1B) and flow-cytometric analysis (Figure 1C) in thymus, spleen and lymph nodes, but not in bone marrow. A higher level of the protein was observed in strain 64 than in strain 60 (Figure 1B). Two-colour immunofluorescence staining of surface markers and cytoplasmic FADD-DN in lymph node cells (Figure 1D) and spleen cells (data not shown) confirmed that transgene expression was restricted to T lymphocytes.

FADD-DN blocks CD95-transduced apoptosis in T lymphoid cells

To test whether expression of the FADD-DN transgene could block apoptosis induced by death domain-containing members of the TNF-R family in non-transformed cells, thymocytes from strains 60 and 64 were exposed *in vitro* to recombinant soluble human CD95 ligand (rhCD95L). This ligand carries a FLAG epitope tag and a FLAG-specific antibody was used to enhance cross-linking of receptor–ligand complexes (Apotech Inc). The FADD-DN transgenic cells were completely resistant to ligand-induced apoptosis, while thymocytes from control littermates were killed in a dose-dependent manner (Figure 2E and F). FADD-DN also blocked thymocyte apoptosis induced by treatment with rhCD95L alone or anti-CD95 antibodies plus cycloheximide (data not shown). The level of protection afforded by FADD-DN expression was equivalent to that conferred by transgenic expression of the cowpox virus serpin CrmA, an inhibitor of caspase-8 (Zhou *et al.*, 1997) or by the CD95 loss-of-function mutation *lpr* (Figure 2E and F). In contrast, FADD-DN expression did not inhibit spontaneous death of thymocytes in culture nor did it affect their sensitivity to γ-radiation, phorbol ester, dexamethasone or the calcium ionophore ionomycin (Figure 2A–C and data not shown).

Like thymocytes, mature resting T cells from both FADD-DN transgenic strains were resistant to rhCD95L-induced apoptosis (Figure 2G). Cell surface levels of CD95 were normal in FADD-DN transgenic thymocytes and mature T cells (Figure 2H–K), excluding decreased receptor expression as a basis for the observed resistance. These results show that FADD-DN can prevent CD95-transduced apoptosis in non-transformed cells. The finding that FADD-DN expression does not inhibit apoptosis triggered by DNA damage, glucocorticoid treatment or other cytotoxic stresses provides evidence that these stimuli activate signalling pathways that are independent of FADD/MORT1.

Deletion of autoreactive T cells is enhanced by FADD-DN

To examine specifically the impact of FADD-DN on negative selection of autoreactive thymocytes we investigated the fate of cells responsive to a self-superantigen. Mice expressing Mls-2^a as well as class II MHC I-E molecules delete their TCRVβ3-bearing T cells (Pullen *et al.*, 1988). The *I-E*^{d} and *mls*-2^a genes were introduced

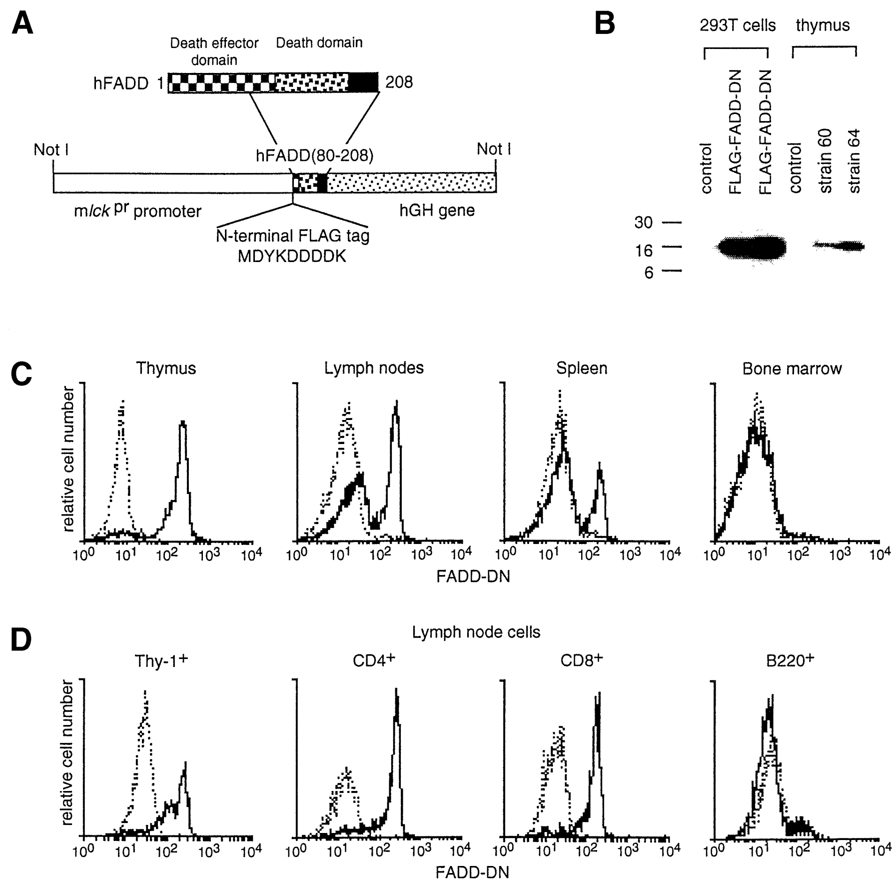

Fig. 1. Generation of transgenic mice expressing FADD-DN in T lineage cells. (**A**) A 5.5 kb DNA fragment encoding FLAG-tagged human FADD/MORT1(αα80–208) between the mouse *lck* proximal promoter, *lck*^{pr}, and the human growth hormone (hGH) gene was introduced into C57BL/6J mouse zygotes by pronuclear microinjection. (**B**) Western blot analysis of FADD-DN transgene expression. Lysates (5×10^6 cell equivalents) from thymocytes of 7-week-old FADD-DN transgenic mice or control littermates were analysed using a FLAG-specific monoclonal antibody. Lysates (1×10^5 cell equivalents) of human 293T cells transfected with pEF-FLAG-FADD-DN or a control vector were used as positive and negative controls respectively. Molecular weight markers are given in kDa. (**C** and **D**) Flow-cytometric analysis of FADD-DN transgene expression. (C) Thymus, spleen, lymph node and bone marrow cells of 10–16-week-old FADD-DN transgenic mice (solid lines) or control littermates (broken lines) were analysed for FADD-DN expression by cytoplasmic immunofluorescence and flow cytometry using anti-FLAG antibody. (D) Lymph node cells surface stained with antibodies to the lymphocyte lineage markers Thy-1, CD4, CD8 or B220 were analysed for FADD-DN expression as in (C). Similar results were obtained with lymphocytes from strain 60. Data are representative of >4 mice of each genotype.

into FADD-DN mice by crossing strain 64 with BALB/c-*bcl*-2 transgenic mice. Offspring inheriting both the FADD-DN and the *bcl*-2 transgenes enabled us to investigate whether these two cell death antagonists synergize in blocking thymocyte negative selection. Figure 3A shows the percentage of TCRVβ3$^+$ lymph node T cells in control C57BL/6 and BALB/c mice and in (C57BL/6×BALB/c)-F$_1$ mice with the genotypes FADD-DN, *bcl*-2, FADD-DN/*bcl*-2 or control wild-type. Consistent with previous observations (Pullen *et al.*, 1988; Strasser *et al.*, 1991), TCRVβ3$^+$ T cells are deleted in mice expressing I-E^d plus Mls-2^a and this is not blocked by Bcl-2. Transgenic expression of FADD-DN or FADD-DN plus Bcl-2 was also unable to prevent deletion of TCRVβ3$^+$ T cells (Figure 3A).

The impact of FADD-DN on negative selection of T cells recognizing conventional antigens was investigated by crossing strain 64 mice with anti-HY TCR transgenic mice. This transgenic TCR recognizes a peptide derived from the male antigen HY presented by class I MHC H-2D^b molecules. Consequently, thymocytes produced in male H-2D^b anti-HY TCR transgenic mice are autoreactive and undergo deletion at the CD4$^+$8$^+$ stage of development (reviewed by von Boehmer, 1990). Surprisingly, FADD-DN expression enhanced negative selection. Male H-2D^b FADD-DN/anti-HY TCR doubly transgenic mice had on average 3-fold fewer CD4$^+$8$^+$ thymocytes than did male littermates expressing just the TCR transgene (Figure 3B and D). HY-TCR$^+$ T cells expressing abnormally low levels of the co-receptor CD8 are unresponsive to male antigen presented by H-2D^b, thereby escape intra-thymic deletion and are found as CD4$^-$8^{low} cells in peripheral lymphoid organs of male H-2D^b anti-HY TCR transgenic mice (reviewed by von Boehmer, 1990). Consistent with the notion that FADD-DN enhances thymocyte negative selection, the number of CD4$^-$8^{low} T cells was reduced 3- to 4-fold in the lymph nodes and spleen of male H-2D^b FADD-DN/anti-HY TCR doubly transgenic mice compared with male littermates expressing just the TCR transgene (Figure 3C and E).

Injection of animals with antibodies to the TCR/CD3 complex triggers deletion of immature CD4$^+$8$^+$ thymocytes (Smith *et al.*, 1989) and this is regarded as a model for negative selection. Consistent with the aforementioned results, anti-CD3 antibody injection led to a 3-fold greater

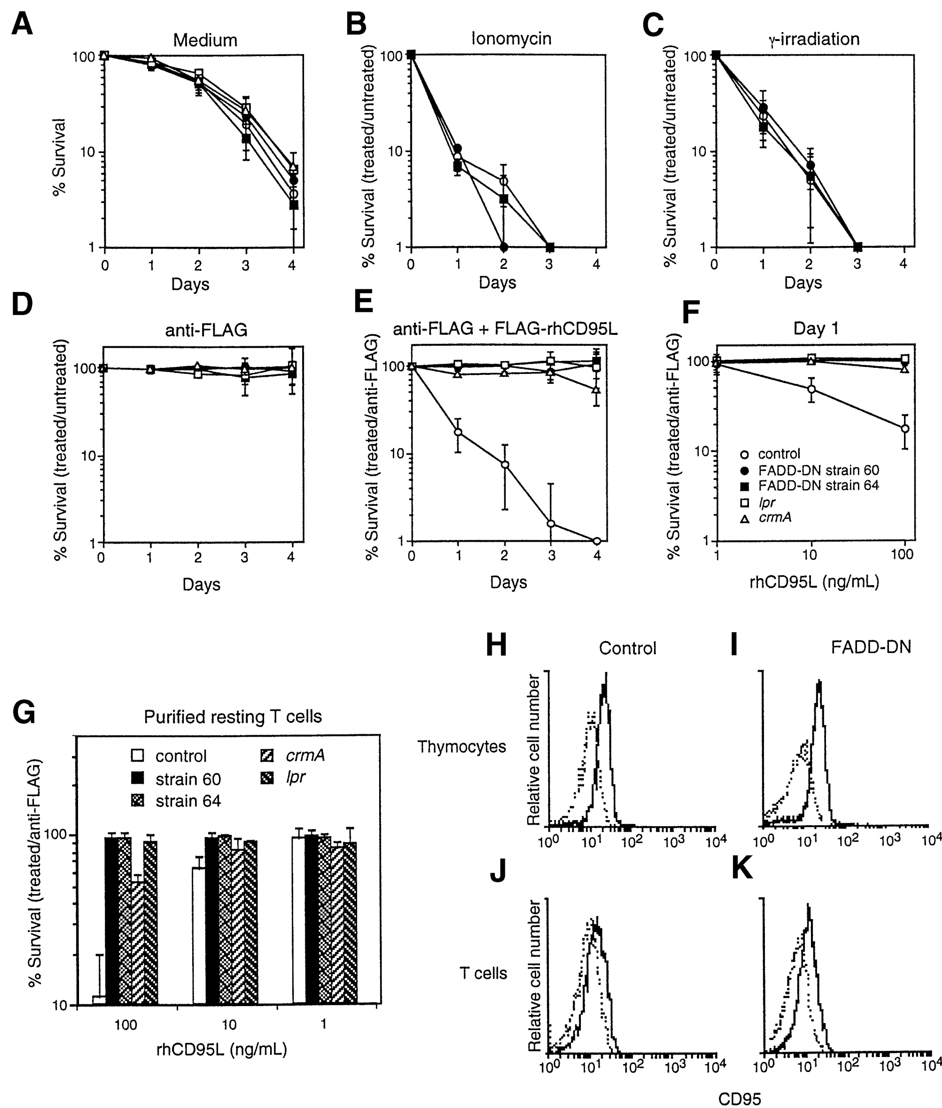

Fig. 2. FADD-DN blocks CD95L-induced killing of thymocytes and mature T cells. (**A–F**) Thymocytes from 6–10-week-old FADD-DN transgenic mice (strain 60, filled circles; strain 64, filled squares), *crmA* transgenic mice (open triangles), mutant *lpr* mice (open squares) and wild-type control mice (open circles), all with a C57BL/6J genetic background, were plated at 1×10^6 cells/ml in tissue culture medium with no additional treatment (A) or were treated with 1 μg/ml ionomycin (B), 10 Gy γ-irradiation (C), 1 μg/ml anti-FLAG antibody (D), 100 ng/ml FLAG–rhCD95L plus 1 μg/ml anti-FLAG antibody (E) or graded concentrations of FLAG–rhCD95L plus 1 μg/ml anti-FLAG antibody (F). (**G**) Purified lymph node T cells were plated at 1×10^5 cells/ml in 1, 10 or 100 ng/ml FLAG–rhCD95L plus 1 μg/ml anti-FLAG antibody. Cell survival was determined after 1 day (F and G) or after 1, 2, 3 and 4 days (A–E) by flow-cytometric analysis of PI stained cells. In (B–D) survival is shown as a percentage of that in culture medium without treatment. In (E–G) survival is a percentage of that in the presence of anti-FLAG antibody. Data points represent the mean ± SD for 3–8 mice. (**H–K**) Surface expression of CD95 is not altered by FADD-DN transgene expression. Thymocytes (H and I) and purified lymph node T cells (J and K) from control littermates (H and J) or FADD-DN transgenic mice (I and K) were stained with Jo2 anti-mouse CD95 monoclonal antibody (solid line) or an isotype-matched control antibody (broken line) and analysed by flow cytometry.

reduction in $CD4^+8^+$ thymocytes in FADD-DN transgenic mice compared with control littermates (data not shown).

Collectively these data demonstrate that FADD/MORT1-transduced signals from death domain-bearing TNF-R family members do not promote thymocyte negative selection. Instead, unexpectedly, they antagonize it.

FADD-DN inhibits mitogen-induced proliferation of mature T cells

Reports that p55 TNF-RI and CD95 can transduce stimulatory signals for cell growth (Alderson *et al.*, 1993; Mackay *et al.*, 1994) led us to examine the effect of FADD-DN expression on T cell activation. To this end spleen cells (Figure 4A–C) or purified T cells (Figure 4D–K) from control and FADD-DN transgenic mice were activated *in vitro* with mitogenic antibodies to CD3 and CD28, concanavalin A or phorbolmyristyl acetate (PMA) and ionomycin. The proliferative response of normal T cells, as measured by $[^3H]$thymidine incorporation, was maximal after 3–4 days stimulation (Figure 4). T cells from FADD-DN transgenic mice responded abnormally to all these stimuli. Thymidine uptake in response to concanavalin A was low throughout an experiment, reaching values that were on average 10-fold lower than the peak response of normal T cells (Figure 4B and C). Maximal thymidine uptake and maximal production of live T cells after CD3 plus CD28 cross-linking or treatment with PMA and ionomycin were the same as in control cultures but

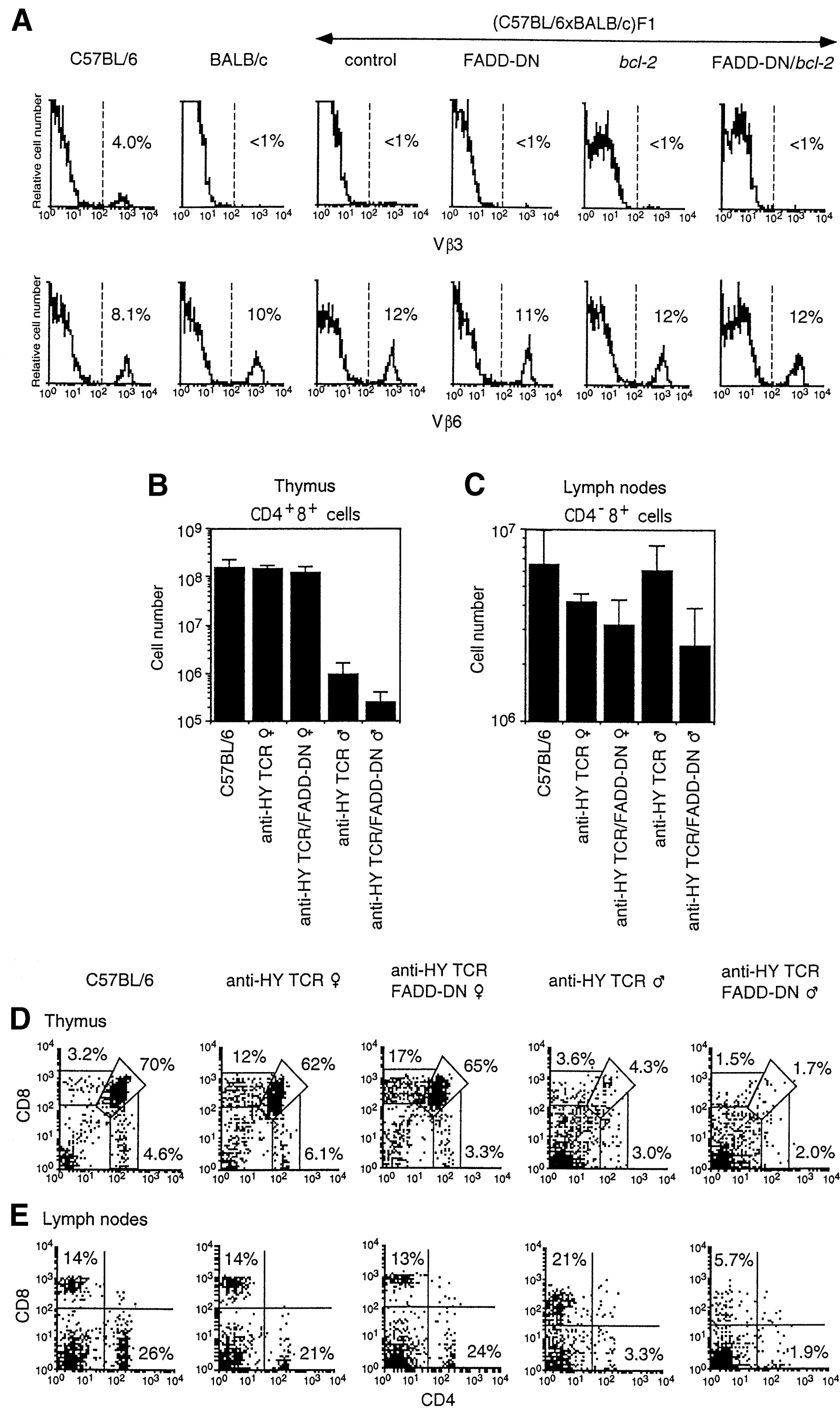

Fig. 3. FADD-DN enhances deletion of autoreactive thymocytes. (**A**) FADD-DN does not prevent deletion of autoreactive Mls superantigen-specific T cells. Lymph node cells from 5–6-week-old C57BL/6J mice, BALB/c mice, FADD-DN transgenic mice (strain 64), *bcl*-2 transgenic mice and FADD-DN (strain 64)/*bcl*-2 doubly transgenic mice, the latter four on a (C57BL/6J×BALB/c)F_1 background, were analysed for the presence of TCRVβ3- and, as a control, for TCRVβ6-bearing T cells by two-colour immunofluorescence and flow cytometry. Histograms show TCRVβ3 or TCRVβ6 expression on T cells (gated for expression of Thy-1) and are representative of analyses of 3–4 mice of each genotype. (**B–E**) FADD-DN enhances deletion of self-reactive thymocytes in anti-HY TCR transgenic mice. Thymocytes and lymph node cells from 6–9-week-old control, anti-HY TCR transgenic or anti-HY/FADD-DN (strain 64) doubly transgenic mice (all with a C57BL/6J genetic background) were counted and the $CD4^+8^+$ thymocyte (B) and $CD4^-8^+$ lymph node T cell (C) populations were determined from two-colour flow-cytometric analysis of cells stained for CD4 and CD8 (**D** and **E**). Expression of the transgenic TCRα and TCRβ chains was confirmed by surface staining with specific monoclonal antibodies (data not shown). Expression of transgenic FADD-DN protein in autoreactive thymocytes was demonstrated by staining fixed and permeabilized cells with the anti-FLAG antibody (data not shown). Results represent the mean ± SD for 3–6 mice.

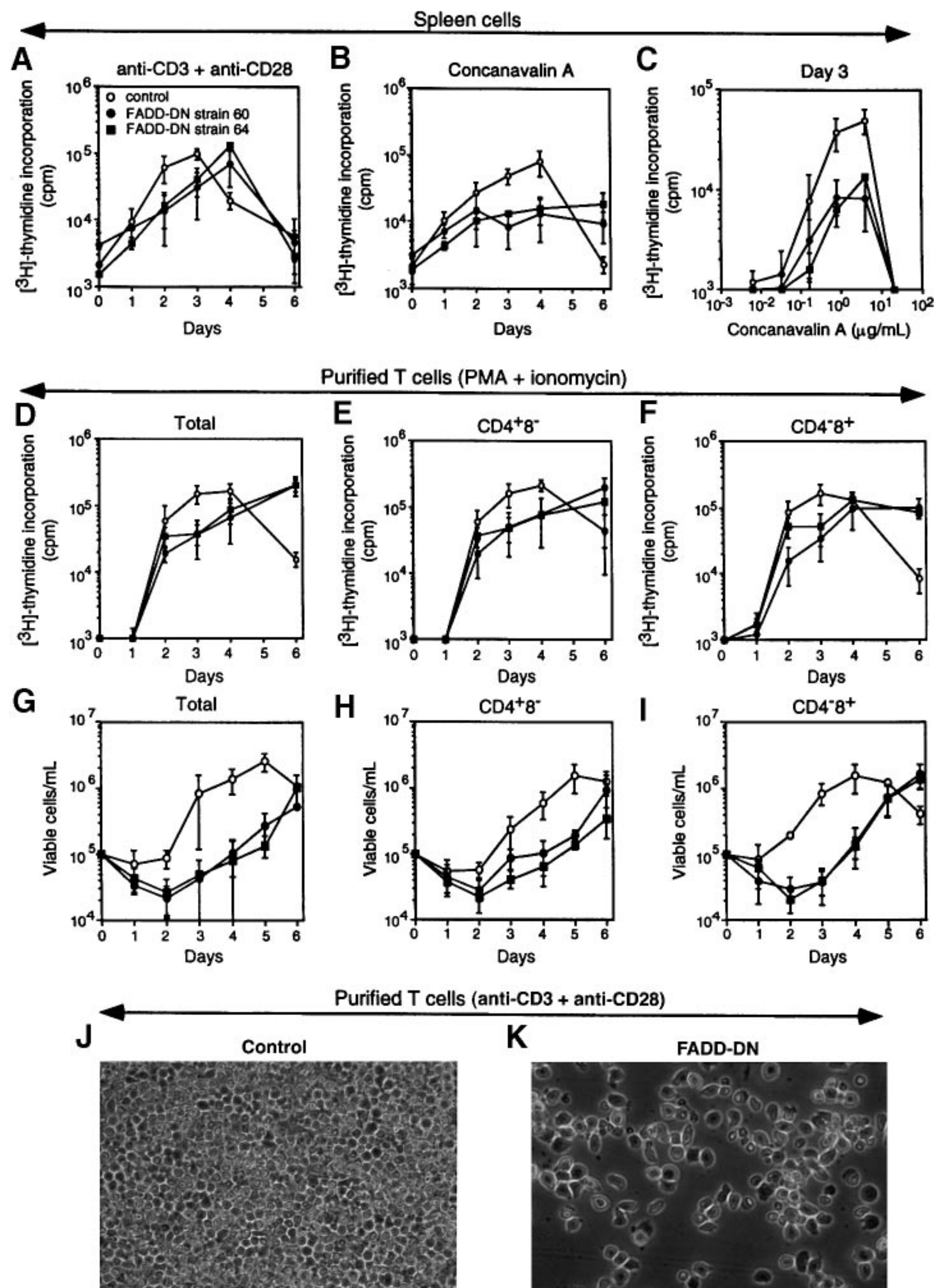

Fig. 4. FADD-DN expression inhibits mitogen-induced proliferation of mature T cells. (**A–C**) Spleen cells from 5–16-week-old FADD-DN transgenic mice (strain 60, filled circles; strain 64, filled squares) or control littermates (open circles) were stimulated with 100 U/ml rmIL-2 plus immobilized antibodies to CD3 and CD28 (A), 4 μg/ml concanavalin A (B) or graded concentrations of concanavalin A (C). Cell proliferation was measured by [^{3}H]thymidine incorporation for 6 h on days 0, 1, 2, 3, 4 and 6 (A and B) or on day 3 (C). Similar differences between FADD-DN transgenic and control T cells were observed when no IL-2 was added to the cultures. (**D–I**) Purified quiescent lymph node T cells (D and G) or CD4$^+$8$^-$ (E and H) and CD4$^-$8$^+$ (F and I) T cell subpopulations were stimulated with 2 ng/ml PMA, 0.1 μg/ml ionomycin and 100 U/ml rmIL-2. Cell proliferation was measured on the days indicated by [^{3}H]thymidine incorporation (D–F) or by cell counting (G–I). Similar results were obtained when purified T cells were stimulated with rmIL-2 plus antibodies to CD3 and CD28. Data points represent the mean ± SD for 3–6 mice. Decreased [^{3}H]thymidine uptake and number of viable cells/ml on day 6 in some cultures was due to overgrowth. The low [^{3}H]thymidine incorporation in T cell cultures stimulated with 20 μg/ml concanavalin A (C) is due to this concentration being toxic to cells. (**J** and **K**) Purified lymph node T cells from control (J) or FADD-DN (strain 64) transgenic mice (K) that have been stimulated with rmIL-2 plus cross-linking antibodies to CD3 and CD28 for 4 days.

occurred 2–3 days later than with normal T cells (Figure 4A and D–I). For example, after 3–5 days PMA plus ionomycin treatment (Figure 4G–I) or CD3- plus CD28-cross-linking (Figure 4J and K) control cultures had reached 1–2$\times 10^6$ viable cells/ml, while FADD-DN cultures had 10- to 20-fold fewer viable cells. Experiments with purified T cells and CD4$^+$8$^-$ and CD4$^-$8$^+$ subsets (Figure 4G–I) demonstrated that the defect caused by FADD-DN expression was intrinsic to the T cells and that both subpopulations were similarly affected. The abnormal growth of FADD-DN T cells was apparently not due to impaired autocrine stimulation by IL-2 because the defect remained when saturating amounts of IL-2 were added to the cultures (Figure 4 and data not shown). Titration of each of the mitogenic reagents demonstrated that normal and FADD-DN T cells had the same requirements for a

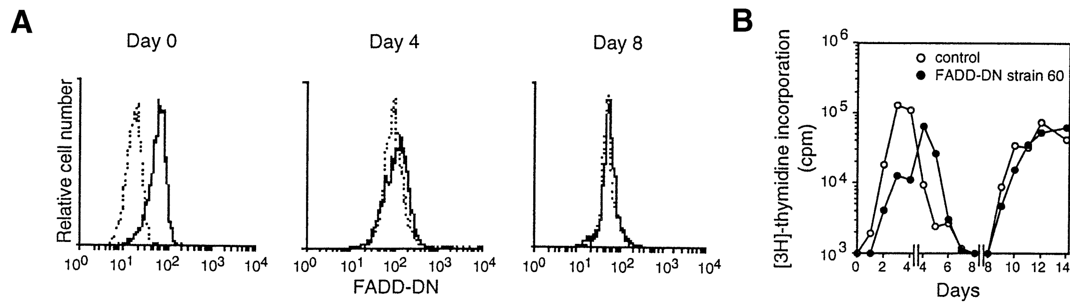

Fig. 5. Mitogenic stimulation selects T cells that do not express the FADD-DN transgene. Purified T cells pooled from the lymph nodes of two FADD-DN transgenic mice (strain 60, filled circles) or two control littermates (open circles) were stimulated with 100 U/ml rmIL-2 plus cross-linking antibodies to CD3 and CD28 (starting concentration 1×10^5 cells/ml). On day 4 the cells were washed and rested at 1×10^5 cells/ml in 10 U/ml rmIL-7. On day 8 the cells (1×10^5 viable cells/ml) were re-stimulated with cross-linking antibodies to CD3 and CD28 plus 100 U/ml rmIL-2. (**A**) Expression of the FADD-DN transgene was examined by cytoplasmic immunofluorescence and flow cytometry using anti-FLAG antibody on days 0, 4 and 8. Solid line histograms represent FADD-DN (strain 60) transgenic T cells and broken lines show staining of control T cells, both gated on expression of Thy-1. Similar results were obtained with cells from strain 64. Selection for T cells that do not express the transgenic FADD-DN protein was also seen when T lymphocytes were activated with concanavalin A or PMA plus ionomycin (data not shown). (**B**) Cell proliferation was measured by [^{3}H]thymidine incorporation for 6 h on the days indicated. Data are typical of two independent experiments.

maximal response (Figure 4C and data not shown), indicating that the proliferative defect of FADD-DN T cells was not due to suboptimal stimulation. In sum, these results show that interference with the normal action of FADD/MORT1 causes a defect in mitogen-induced proliferation of T lymphocytes.

Mitogenic stimulation selects T cells that do not express the FADD-DN transgene

After 4 days of mitogenic stimulation the transgenic T lymphoblasts expressed no detectable FADD-DN protein (Figure 5A), suggesting that stimulation had selected T cells that did not express the transgene. To further investigate the relationship between FADD-DN expression and activation we subjected T cells to alternating phases of mitogenic stimulation and quiescence (Figure 5B). As in the experiments shown in Figure 4A, during 4 days of stimulation with antibodies to CD3 and CD28, proliferation was reduced in cultures of FADD-DN T cells compared with normal T cells (Figure 5B). Following this initial period of stimulation the mitogens were removed and replaced by low levels of IL-7 to promote cell survival. Over the following 4 days the T lymphoblasts ceased proliferating and entered the resting state (Figure 5B). Day 8 quiescent transgenic T cells did not express detectable amounts of FADD-DN (Figure 5A). If the transgenic *lck*^{pr} promoter was active in resting T cells but turned off during mitogenic activation then these cells should have expressed FADD-DN protein. This experiment therefore provided evidence that only those transgenic T cells expressing the lowest levels of FADD-DN could proliferate upon mitogenic stimulation. In accordance with this hypothesis, transgenic T cells responded to secondary stimulation in the same manner as normal T cells (Figure 5B).

FADD-DN expression increases apoptosis and reduces clonogenic growth of mitogen-activated T cells

Delayed and diminished growth of mitogen-activated FADD-DN T cell populations could be due to a reduced frequency of responding cells or a reduced rate of proliferation. The characteristics of the T cell growth curves were consistent with the first hypothesis (Figure 4G–I). In cultures of purified normal T cells the number of viable cells decreased slightly (~10–20%) over the first 1–2 days of stimulation and increased rapidly thereafter. In cultures of FADD-DN T cells a larger fraction of the cells (~70–80%) failed to survive the initial period of stimulation and the number of viable cells did not increase until day 3 (Figure 4G–I). Consistent with these observations, the incidence of apoptosis was considerably higher after 3 days in FADD-DN T cell cultures compared with those from normal animals (Figure 6A). This effect was specific to mitogenic stimulation, since control and transgenic T cells cultured without mitogens underwent apoptosis at the same rate (data not shown).

The maximal growth rates of normal and transgenic T lymphocytes were similar (Figure 4G–I), indicating that those FADD-DN transgenic T cells that survived the initial activation process could proliferate at the normal rate. In accordance with this notion the percentage of viable FADD-DN T cells in S phase of the cell cycle was abnormally low (~2- to 3-fold below control T cells) after 1 and 2 days stimulation (data not shown) but was normal at later time points (Figure 6B). All surviving T cells increased their volume (Figure 6C), demonstrating that they responded to TCR/CD3 ligation. Limiting dilution cultures revealed that FADD-DN diminished the clonal growth of mitogen-activated T cells (Figure 6D). Interestingly, there was no significant difference in clone size (50–200 cells/colony) between control and FADD-DN transgenic T cells. Collectively these results show that FADD-DN reduces mitogenic responses of T lymphocytes by increasing apoptosis early during stimulation.

Neither CrmA expression nor the lpr mutation inhibits T cell proliferation

We next used *crmA* transgenic mice and *lpr* mutant mice to investigate potential mechanisms by which FADD-DN might increase apoptosis and inhibit proliferation of stimulated T cells. A direct comparison of the mitogenic response of splenic T cells from *crmA* transgenic mice, *lpr* mutant mice, FADD-DN transgenic mice and control mice demonstrated that neither CrmA expression nor CD95 deficiency prevents T cells from proliferating normally.

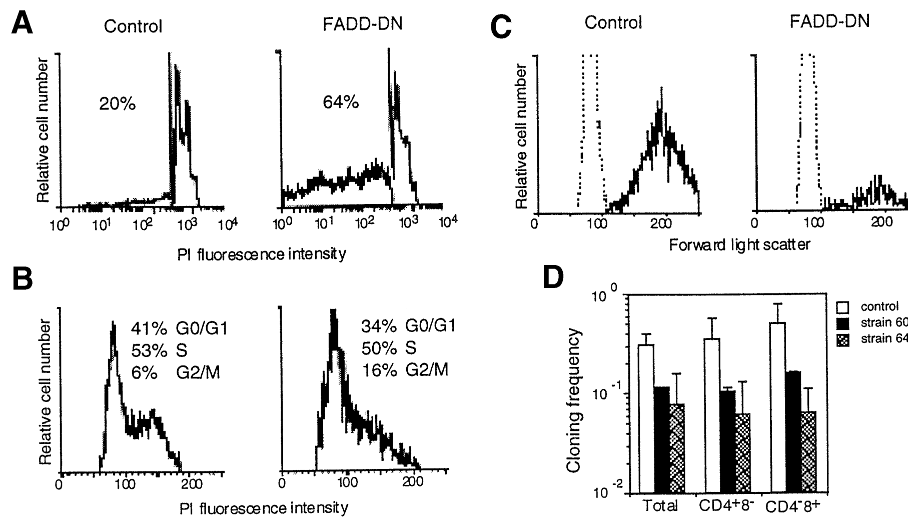

Fig. 6. FADD-DN expression increases apoptosis and reduces clonogenic growth of mitogen-activated T cells. (**A** and **B**) Purified quiescent lymph node T cells from FADD-DN transgenic mice (strain 64) or control littermates were cultured at 1×10^5 cells/ml in medium containing 100 U/ml rmIL-2 in the presence of immobilized antibodies to CD3 and CD28. After 3 days the cells were fixed, stained with PI and analysed by flow cytometry for incidence of apoptosis (A) and cell cycle distribution (gated on viable cells) (B). Similar results were obtained with cells from strain 60 FADD-DN transgenic mice. Histograms are representative of analyses of 3–6 mice of each genotype. (**C**) Forward light scatter of purified T cells from control littermates or FADD-DN mice (strain 64) that had been stimulated for 0 (dotted lines) or 2 days (solid lines) as described above. (**D**) Purified quiescent lymph node T cells or the $CD4^+8^-$ and $CD4^-8^+$ T cell subpopulations from FADD-DN transgenic mice or control littermates were plated at limiting dilution in 96-well trays and stimulated with cross-linking antibodies to CD3 and CD28 plus rmIL-2. The wells were scored as positive or negative for cell growth on day 5 or 6 and the precursor frequency of clonogenic T lymphocytes was determined as described in Materials and methods. Results represent the mean ± SD for 3 mice of each genotype. Similar results were obtained when T cells were activated with PMA and ionomycin plus rmIL-2.

[^{3}H]Thymidine incorporation, growth kinetics and rate of apoptosis of T cells from *crmA* transgenic mice, mutant *lpr* mice and control littermates were equivalent at all times (Figure 7A and B and data not shown). Thus inhibition of caspase-8 (FLICE/MACH) activity or absence of signals from CD95 alone are unlikely to account for increased apoptosis and defective proliferation of mitogen-activated FADD-DN T cells.

Another phenotypic difference between FADD-DN transgenic mice and mutant *lpr* mice was the absence of lymphadenopathy in the former. Six-month-old *lpr* mice had abnormally increased numbers of lymph node cells, many of them bearing the unusual $B220^+TCR/CD3^+$ T cell phenotype (Figure 7C). FADD-DN transgenic mice and *crmA* transgenic animals of the same age did not have these cells and displayed normal lymphoid cellularity. This indicates that FADD/MORT1 and caspase-8 do not transmit all CD95-transduced signals that are responsible for normal T lymphocyte homeostasis and prevention of lymphadenopathy.

Discussion

The FADD/MORT1 protein is an essential signalling intermediary for CD95-, TNF-R1- and DR3-transduced apoptosis in some tumour-derived cell lines (Chinnaiyan *et al.*, 1996a,b; Hsu *et al.*, 1996b). Two receptors for TRAIL have been identified; one induces apoptosis via FADD/MORT1 (Walczak *et al.*, 1997) and the other by an alternative route (Pan *et al.*, 1997). We show here that thymocytes and mature T cells from FADD-DN transgenic mice are resistant to CD95L-induced apoptosis (Figure 2), demonstrating for the first time that FADD/MORT1 is essential for this process in non-transformed cells. The impact of FADD-DN on TNF- and TRAIL-induced apoptosis could not be assessed, since thymocytes and mature T cells are normally resistant to these ligands (Wiley *et al.*, 1995; our unpublished observations). As more members of the TNF-R family and their corresponding ligands are discovered these animals will serve as useful tools for assessing the role of FADD/MORT1 in signal transduction in non-transformed cells.

In accordance with previous findings (Suda *et al.*, 1996; Smith *et al.*, 1997), we found substantial differences in biological activity between CD95L and agonistic anti-CD95 antibodies. A 100-fold higher concentration of Jo2 monoclonal antibody than of CD95L was required to induce apoptosis in thymocytes (Smith *et al.*, 1997; our unpublished observations). Mature T cells from normal mice were killed by CD95L (Figure 2G) but resisted treatment with Jo2 antibody, even in the presence of cycloheximide, which facilitates killing in thymocytes (Smith *et al.*, 1997; our unpublished observations). A possible explanation is offered by the recent finding that CD95L and agonistic antibodies can stimulate different signalling pathways (Thilenius *et al.*, 1997). Differences in binding affinity between soluble and membrane-anchored TNF for its two receptors, TNF-R1 and TNF-R2, are responsible for differences in biological responses (Grell *et al.*, 1995). It is possible that in the case of CD95L, as for other members of the TNF ligand family, usage of alternative receptors and differences in the extent of receptor oligomerization determine which signalling pathways are activated.

Possible cell death mechanisms involved in thymocyte negative selection

Six death-domain-bearing TNF-R family members have been identified to date and it has been speculated that there is some functional redundancy between them in

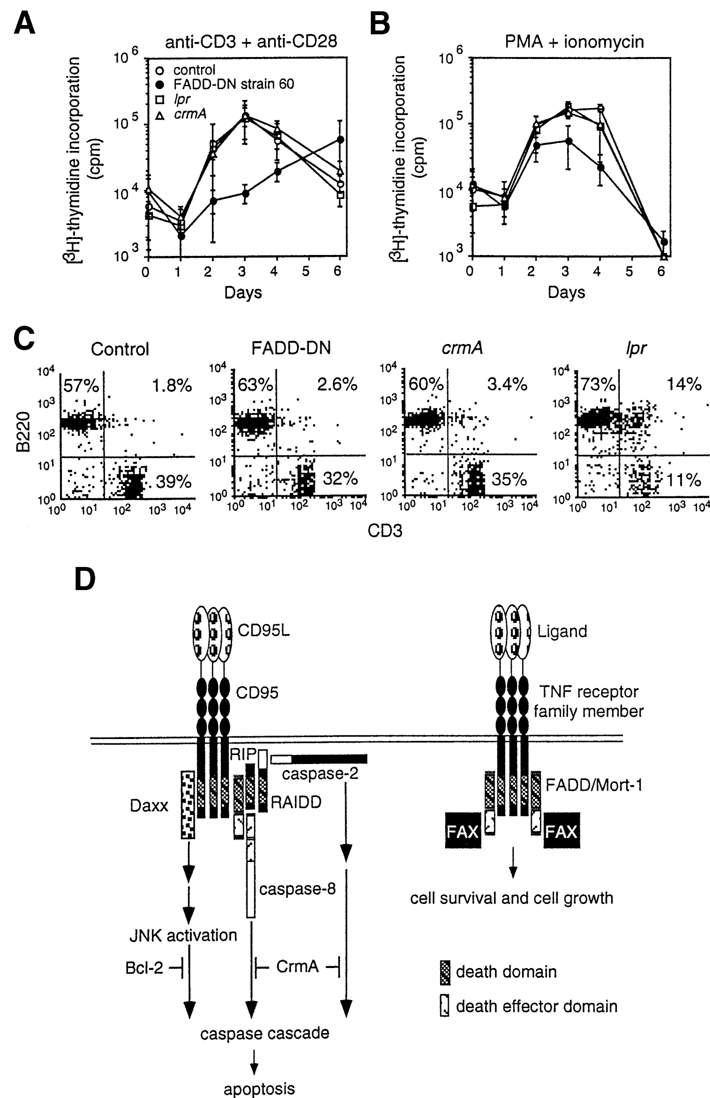

Fig. 7. Mechanism of FADD-DN-mediated inhibition of T cell proliferation. (**A–C**) Phenotypic differences between T cells from FADD-DN transgenic mice, *crmA* transgenic mice and mutant *lpr* mice. (A and B) Expression of a *crmA* transgene or loss-of-function mutation of CD95 (*lpr*) does not inhibit proliferation of mitogen-activated T cells. Spleen cells from 6–10-week-old FADD-DN transgenic mice (strain 60, filled circles), control non-transgenic mice (open circles), *crmA* transgenic mice (open triangles) and *lpr* mutant mice (open squares), all with a C57BL/6J genetic background, were stimulated with 100 U/ml rmIL-2 plus either cross-linking antibodies to CD3 and CD28 (A) or 2 ng/ml PMA plus 0.16 μg/ml ionomycin. Cell proliferation was measured by [^{3}H]thymidine incorporation for 6 h on the days indicated. Data points represent the mean ± SD for 3 mice. (C) FADD-DN expression does not cause *lpr*-like lymphadenopathy. Immunofluorescence and flow-cytometric analysis of lymph node cells from a control non-transgenic mouse, a FADD-DN transgenic mouse (strain 64), a *crmA* transgenic mouse and a mutant *lpr* mouse, all with a C57BL/6J genetic background. Mice were aged 20–28 weeks. Dot plots are representative of analyses of 3–4 mice of each genotype. (**D**) Model for signalling from death domain-bearing TNF-R family members. At least three pathways lead from CD95 to apoptosis. The FADD/MORT1→caspase-8 pathway can be blocked by CrmA but is insensitive to Bcl-2 (Strasser *et al*., 1995; Chinnaiyan *et al*., 1996b; Hsu *et al*., 1996b). The RIP→RAIDD→caspase-2 pathway is sensitive to CrmA (Duan and Dixit, 1997). The Daxx→Jun kinase pathway is the only one that can be inhibited by Bcl-2 (Yang *et al*., 1997). All these pathways may have to be inhibited to reproduce the *lpr* CD95 loss-of-function phenotype. Alternatively, CD95L may have a second receptor, CD95L-R2, which contributes to the *lpr* phenotype by transducing abnormally increased growth-stimulatory signals in the absence of competition by CD95 for CD95L binding. Early during T cell activation FADD/MORT1 can transmit a survival and/or growth-stimulatory signal via a mechanism that is insensitive to CrmA, thus excluding caspase-8. We propose the existence of an adaptor, called FAX, that can bind to FADD/MORT1 and transmit a survival- and/or growth-stimulating signal. It is likely that there is similar complexity in signals emanating from other members of the TNF-R family.

thymocyte negative selection. Our results (Figure 3) show that signals from the TNF-R family which engage the cell death machinery via FADD/MORT1 are dispensable for this process. Unexpectedly, FADD-DN enhanced the deletion of autoreactive thymocytes (Figure 3), indicating that FADD/MORT1 can be part of a signal that promotes survival of T lymphocytes activated through their TCR. The 1.5- to 2-fold reduction in mature $CD4^{-}8^{+}$ T cells that we saw in FADD-DN mice may therefore be due to a defect in positive selection or a consequence of impaired proliferative expansion in peripheral lymphoid organs.

The failure of FADD-DN to inhibit thymocyte negative selection does not exclude other FADD/MORT1-independent pathways that lead from TNF-R family members to caspase activation and apoptosis. Two FADD/MORT1-independent pathways from oligomerized CD95 receptors have been uncovered in tumour-derived cell lines: (i) RIP can interact with the CD95

death domain (Stanger *et al.*, 1995) and bind the adaptor RAIDD, which in turn recruits and activates caspase-2 (Nedd2/Ich-1) (Duan and Dixit, 1997); (ii) Daxx interacts with the CD95 death domain and triggers apoptosis by causing Jun kinase activation (Yang *et al.*, 1997). It is possible that other death domain-bearing members of the TNF-R family can also trigger multiple independent signalling routes to apoptosis.

Two members of the TNF receptor subfamily that lack a death domain have been implicated as regulators of thymocyte negative selection. Impaired deletion of autoreactive T cells was observed in CD30-deficient mice (Amakawa *et al.*, 1996) and in animals injected with neutralizing antibodies to CD40 ligand (Foy *et al.*, 1995). However, in both instances negative selection was not completely abrogated and it has not been established whether CD30 and CD40 act as 'death receptors' within the doomed thymocytes or function within thymic antigen-presenting cells. Members of the TNF-R subfamily that lack a death domain can recruit proteins of the TRAF and IAP families and thereby activate Rel/NF-κB, which is thought to promote cell growth and survival rather than apoptosis (Rothe *et al.*, 1994). One such receptor, CD27, binds to a death domain-containing cytoplasmic protein termed Siva and triggers apoptosis (Prasad *et al.*, 1997). It is likely that related signalling intermediaries exist for other members of this TNF-R subfamily.

Deletion of autoreactive thymocytes is delayed in *bcl*-2 transgenic mice (Sentman *et al.*, 1991; Strasser *et al.*, 1991, 1994). This may be due to the fact that Bcl-2 protects highly sensitive $CD4^+8^+$ thymocytes against the toxic effects of calcium flux caused by TCR/CD3 ligation (Strasser *et al.*, 1991, 1994). Alternatively, Bcl-2 may exert its effect on thymocyte negative selection by blocking another signalling route, such as the one transduced via Daxx and Jun kinase (Yang *et al.*, 1997).

We speculate that multiple signalling pathways contribute to elimination of autoreactive thymocytes. Generation of transgenic mice expressing dominant interfering mutants of Siva, RAIDD or Daxx and crosses between those animals, FADD-DN mice, *bcl*-2 transgenic mice and animals lacking TNF-R family members may clarify the mechanisms of thymocyte negative selection.

The effect of FADD-DN on T cell activation

There is ample evidence that death domain-bearing members of the TNF-R family can promote cell growth and survival. TNF-R1 transduces a proliferative signal in primary mouse fibroblasts (Mackay *et al.*, 1994) and is essential for liver regeneration after partial hepatectomy (Yamada *et al.*, 1997). The p75 NGF-R delivers a survival signal in some types of neurons (Kaplan and Miller, 1997). Antibody-mediated cross-linking of CD95 enhances cell growth in some cases of chronic lymphocytic leukemia (Mapara *et al.*, 1993), solid tumour cell lines (Owen-Schaub *et al.*, 1994), normal human diploid fibroblasts (Aggarwal *et al.*, 1995) and T cells that have been triggered via the TCR/CD3 complex (Alderson *et al.*, 1993). Interestingly, anti-CD3 antibody-induced T cell proliferation is enhanced by membrane-anchored CD95L (M.Alderson, personal communication) but reduced by soluble ligand (Suda *et al.*, 1996), indicating that the degree of CD95 receptor oligomerization may determine whether a growth or a death signal is transmitted.

The growth stimulatory effects of CD95, TNF-R1 and DR3 are thought to be mediated via activation of members of the Rel/NF-κB transcription factor family (Kruppa *et al.*, 1992; Mackay *et al.*, 1994; Chinnaiyan *et al.*, 1996a; reviewed by Wallach *et al.*, 1996). Experiments on cultured cell lines indicated that the pathway activating Rel/NF-κB and the signal leading to cell death are distinct. CrmA and FADD-DN are specific inhibitors of the pathway to apoptosis, while dominant interfering mutants of TRAF2 and RIP selectively block Rel/NF-κB activation (Chinnaiyan *et al.*, 1996a,b; Hsu *et al.*, 1996a,b). Consistent with these observations, we found that FADD-DN and CrmA inhibit CD95-transduced apoptosis (Figure 2) and so far have no indication that these molecules inhibit Rel/NF-κB activation in mitogen-stimulated T cells.

In light of this knowledge it was surprising that FADD-DN inhibited mitogen-induced proliferation of mature T lymphocytes and increased apoptosis (Figures 4–6). These findings indicate that normal T cells must have a growth-promoting pathway which is transduced via FADD/MORT1 and/or a close homologue that can be blocked by FADD-DN. Unlike the pathway leading to apoptosis, this growth-promoting signal does not require activation of caspase-8, since T lymphocytes from *crmA* transgenic mice respond normally to mitogens (Figure 7). We speculate that in activated T cells FADD/MORT1 can bind one or several growth-stimulating proteins (denoted FAX for FADD/MORT1-associated protein X in Figure 7D). An attractive candidate is FLIP (also called I-FLICE, FLAME-1, CASH and Casper), which can bind to the death effector domain of FADD/MORT1 and inhibit its pro-apoptotic activity (Irmler *et al.*, 1997).

It is interesting to contemplate why no growth inhibitory effect of FADD-DN has been observed in stably transfected cell lines (Chinnaiyan *et al.*, 1996a,b; Hsu *et al.*, 1996b; our unpublished observations). Transformed cells are continuously cycling, while mitogen-stimulated normal T cells must first complete the G_0–G_1 transition to enter the cell cycle. Bearing this in mind, plus the differences in the rate of apoptosis and kinetics of proliferation between normal and FADD-DN transgenic T cells (Figure 4), it appears most likely that FADD/MORT1 transduces a survival- and/or growth-stimulatory signal during the G_0–G_1 transition. Human peripheral blood T cells are resistant to anti-CD95 antibody-induced apoptosis during the first 2 days of mitogenic stimulation and only later become susceptible (Owen-Schaub *et al.*, 1992; Klas *et al.*, 1993). These differences in CD95 signalling are consistent with FADD/MORT1 changing its partner from the growth-promoting FAX to the death-inducing caspase-8 during the course of the G_0–G_1 transition.

The source of the ligand which stimulates the FADD-DN-inhibitable growth stimulatory signal must be T lymphocytes themselves, since transgene expression inhibits proliferation in cultures of purified cells (Figure 4). The nature of the ligand is less clear. We observed no proliferative defect in T cells from *lpr* mice (Figure 7) and T cell activation was reported to be normal in TNF-$R1^{-/-}$ mice (Pfeffer *et al.*, 1993; Rothe *et al.*, 1993), thus excluding these two receptors as the sole trigger of the

stimulatory signal. The physiological roles of DR3 and the two TRAIL receptors are unknown, but their expression pattern is indicative of a function in lymphocyte activation and/or death (Chinnaiyan *et al.*, 1996a; Pan *et al.*, 1997; Walczak *et al.*, 1997). We believe that several members of the death domain-bearing TNF-R family may transduce a growth-stimulatory signal via FADD/MORT1 early during T cell activation.

Implications for CD95L/CD95-induced cell death signalling

Why do FADD-DN and CrmA completely block CD95-transduced apoptosis in thymocytes and mature T cells (Figure 2) but fail to elicit *lpr*-like lymphadenopathy (Figure 7C; Smith *et al.*, 1996)? Insufficient levels of transgene expression appears to be an unlikely explanation, since the phenotype of the incomplete loss of function mutation in *lpr* mice is only quantitatively different from that in $CD95^{-/-}$ animals (Adachi *et al.*, 1995). Absence of transgene expression in B lymphocytes also cannot account for this observation, since T cell hyperplasia does develop in B cell-deficient *lpr* mice (Shlomchik *et al.*, 1994). At least two explanations could account for the finding that $TCR^{+}B220^{+}$ T cells do not accumulate in FADD-DN and *crmA* transgenic mice. While all *in vitro* killing by CD95L or anti-CD95 antibodies appears to be critically dependent on FADD/MORT1 and a CrmA-sensitive cysteine protease, presumably caspase-8, alternative cell death pathways from CD95 could operate in activated T lymphocytes *in vivo*. As mentioned above, recruitment and activation of caspase-2 via RIP and RAIDD (Duan and Dixit, 1997) or binding of Daxx leading to Jun kinase activation and ultimately apoptosis (Yang *et al.*, 1997) stand out as likely candidates. Mice that are doubly transgenic for *crmA* and *bcl*-2 or FADD-DN and *bcl*-2 do not develop lymphadenopathy (Smith *et al.*, 1996; our unpublished observations), indicating that other functions of CD95L/CD95 besides the CrmA- and FADD-DN-inhibitable FADD/MORT1→caspase-8 signal and the Bcl-2-inhibitable Daxx→Jun kinase pathway are important for T cell homeostasis (Figure 7D).

Another, more speculative, explanation is that CD95L, like TNF and TRAIL, has two receptors, CD95 and another, which can trigger cell growth. According to this model (Figure 7D) T cells in *lpr* and $CD95^{-/-}$ mice would not only fail to receive a death stimulus via CD95 but also obtain abnormally increased growth stimulation via the second putative CD95L receptor, since it would not compete with CD95 for binding of the common ligand. Two logical predictions of this idea are that the *gld* point mutation in CD95L abolishes binding to CD95 but maintains interaction with its second putative receptor and that $CD95L^{-/-}$ animals should have a different phenotype than *gld* mutant mice.

In conclusion, our results reveal a novel complexity of signalling from members of the TNF-R family by showing that FADD/MORT1 does not always transduce an apoptotic signal but can, under certain circumstances, transmit survival and growth stimulatory signals.

Materials and methods

Transgene construct

A *Hinc*II–*Xba*I fragment encoding truncated human FADD/MORT1(αα80–208) was isolated from pcDNA3 AUI-FADD (Chinnaiyan *et al.*, 1995) and inserted into the *Bam*HI (blunt ended)–*Xba*I site of pEF FLAG B (Huang *et al.*, 1997). The *Bgl*II–*Bam*HI fragment encoding FLAG–FADD/MORT1(αα80–208) was then subcloned into the *Bam*HI site of the expression vector p1017 (Chaffin *et al.*, 1990). Inbred C57BL/6J mouse zygotes were injected with the assembled sequences as a *Not*I fragment, which contained the proximal promoter of the mouse *lck* gene (lck^{pr}), FLAG epitope-tagged FADD/MORT1(αα80–208) and the human growth hormone gene. Transgene-positive mice were identified by direct PCR amplification of DNA from whole blood (McCusker *et al.*, 1992) using oligonucleotide primers specific to the human growth hormone gene (5′-TAG GAA GAA GCC TAT ATC CCA AAG G and 5′-ACA GTC TCT CAA AGT CAG TGG GG).

Mice

FADD-DN transgenic mice (strains 60 and 64) were propagated by serially mating heterozygous transgenic animals with C57BL/6J mice. Other transgenic strains, Eμ-*bcl*-2-36, expressing human Bcl-2 constitutively in B and T lymphoid cells (Strasser *et al.*, 1991), anti-HY TCR (von Boehmer, 1990), CD2-*crmA* (strain 65), expressing the cowpox virus serpin CrmA (Ray *et al.*, 1992) in T lineage cells (Smith *et al.*, 1996), and mutant CD95-deficient *lpr* mice (Cohen and Eisenberg, 1993), have been described previously. All transgenes were used on a C57BL/6J genetic background, with the exception of Eμ-*bcl*-2-36, which was on a BALB/c background. FADD-DN/*bcl*-2 doubly transgenic mice were generated by crossing heterozygous FADD-DN transgenic mice (strain 64) with heterozygous Eμ-*bcl*-2-36 transgenic mice. Inheritance of the *bcl*-2 transgene was determined by PCR using oligonucleotide primers (5′-GGA ACT GAT GAA TGG GAG CAG TGG and 5′-GCA GAC ACT CTA TGC CTG TGT GG) to amplify the SV40 element of the transgene construct. FADD-DN/anti-HY TCR doubly transgenic mice were generated by crossing heterozygous FADD-DN mice with homozygous anti-HY TCR transgenic mice.

Western blot analysis

Western blots were prepared as described previously (Strasser *et al.*, 1995). Proteins were solubilized from cells in lysis buffer (0.25M Tris–HCl, pH 6.8, 10% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue and 0.5 μg/ml Pefabloc) and resolved by electrophoresis through 4–20% gradient Tris–glycine polyacrylamide gels (Novex) in the presence of SDS. Proteins were then transferred to nitrocellulose membranes by electroblotting. Non-specific binding sites were blocked (>1 h) in phosphate-buffered saline containing 5% skimmed milk, 1% casein and 0.1% Tween-20, and the membranes were then incubated with 3 μg/ml anti-FLAG M2 monoclonal antibody (IBI). Bound antibody was detected with affinity purified rabbit anti-mouse IgG (Fcγ-specific) antibodies (Jackson ImmunoResearch) and ^{125}I-labelled protein A ($1–2\times10^{6}$ c.p.m./ml).

Immunofluorescence staining, flow cytometric analysis and cell sorting

Dispersed cells from bone marrow, lymph nodes, spleen and thymus were surface stained with monoclonal antibodies that had been conjugated in our laboratory according to the manufacturers' instructions with fluorescein isothiocyanate (FITC), R-phycoerytherin (PE) or biotin (Molecular Probes). The monoclonal antibodies used and their specificities were: RA3-6B2, anti-B220; GK1.5, anti-CD4; H129.19.6.8, anti-CD4; 53.6.72, anti-CD8; YTS 169, anti-CD8; 8C5, anti-Gr-1; T3.70, anti-HY TCRα chain; HB58, anti-Igκ; JC5, anti-Igλ; MI/70, anti-Mac-1; Ter119, anti-erythroid cell surface marker; T24.31.2, anti-Thy-1; KJ25-606.4, anti-TCRVβ3; RR4-7, anti-TCRVβ6; F23.2, anti-TCRVβ8.2; and T3/70, anti-HY TCRα chain (for references see Strasser *et al.*, 1991). Bound biotinylated antibodies were detected with PE–streptavidin or Tricolor–streptavidin (Caltag). Hamster monoclonal antibody Jo2 anti-mouse CD95 (Fas/APO-1) (PharMingen) was detected with a FITC-conjugated mouse anti-hamster IgG (Fcγ-specific) monoclonal antibody cocktail (PharMingen). Between 5000 and 10 000 viable cells (not stained by propidium iodide) were analysed in a FACScan flow cytometer (Becton Dickinson). Lymph node T cells were purified in a FACS II or $FACStar^{+}$ sorter by negative cell sorting. FITC-negative, propidium iodide (PI)-negative cells were selected following surface staining with a cocktail of FITC-conjugated monoclonal antibodies specific for B220, Igκ, Igλ, Mac-1, Gr-1 and Ter119. FITC-conjugated monoclonal antibodies to CD4 or CD8 were included to isolate $CD4^{-}8^{+}$ and $CD4^{+}8^{-}$ T lymphocytes respectively. For long-term cultures T lymphocytes were purified from suspensions of lymph node cells, using magnetic beads coated with goat anti-rat IgG (Paesel and Lorei) to remove unwanted cells stained with rat monoclonal antibodies to B220, Mac-1, Gr-1 and

Ter119. Staining of the enriched T cell population with a biotinylated antibody to Thy-1 plus PE–streptavidin revealed that the purity was 90–95%. Expression of FLAG–FADD-DN or Bcl-2 protein was determined by cytoplasmic immunofluorescence staining and flow-cytometric analysis. Cells were fixed for 10 min at room temperature in 1% paraformaldehyde and stained for 30 min on ice with 3 μg/ml mouse anti-FLAG M2 monoclonal antibody (IBI) or mouse anti-human Bcl-2 monoclonal antibody (Bcl-2-100) in the presence of 0.3% saponin (Sigma). FITC-conjugated goat anti-mouse IgG (Fcγ-specific) antibodies (Southern Biotechnology) were used at 10 μg/ml as secondary reagent. Cells were analysed in a FACScan, live and dead cells being discriminated on the basis of their forward and side light scattering properties.

Cell survival and proliferation assays

Thymocytes, spleen cells and purified T cells were cultured in the high glucose version of Dulbecco's modified Eagle's (DME) medium supplemented with 13 μM folic acid, 250 μM L-asparagine, 50 μM 2-mercaptoethanol and 10% fetal calf serum (Biosciences). Cell viability was determined by flow cytometric analysis of PI stained cells in a FACScan. Recombinant soluble human CD95L (rhCD95L) carried a FLAG epitope tag and a FLAG-specific monoclonal antibody was used to enhance cross-linking of receptor–ligand complexes (Apotech Inc). T cell proliferation following mitogenic stimulation was measured as [^{3}H]thymidine incorporation after 100 μl cultures (starting concentration: spleen cells, 1×10^6 cells/ml; purified lymph node T cells, 1×10^5 cells/ml) were pulsed for 6 h with 0.5 μCi [^{3}H]thymidine (Amersham). Viable cell numbers were determined by trypan blue exclusion or by staining T cell cultures with PI, adding a known number of FACS calibration beads (Flow Cytometry Standards) and analysing the samples on a FACScan. Beads and viable (PI$^-$) lymphocytes were distinguished by their different forward and side light scattering properties and the ratio of the two was used to calculate the concentration of live T cells in the cultures.

Cell cycle and apoptosis analysis

T cells were fixed overnight at 4°C in 70% ethanol and stained for 20 min at 37°C with 69 μM PI in 38 mM sodium citrate, pH 7.4, containing 5 μg/ml RNase A. Between 5000 and 10 000 cells were analysed in a FACScan and their cell cycle distribution was determined using DNA Cell-Cycle Analysis Software Version C (Becton Dickinson). Apoptotic cells were identified within the PI stained population by virtue of exhibiting an apparent subdiploid DNA content.

Limiting dilution analysis of T cell growth

Using the single cell deposition unit of the FACSstar$^+$ sorter, purified lymph node T cells were distributed at limiting dilution (1, 3, 9, 27 or 81 cells/well) in flat-bottomed 96-well plates (Falcon) containing optimal concentrations (20 μg/ml in the coating solution) of immobilized monoclonal antibodies (KT3 anti-CD3 and 37N51 anti-CD28) plus 100 U/ml rmIL-2. Wells were scored by microscopy as positive or negative for growth after 5–6 days incubation at 37°C. The precursor frequency of clonogenic cells was estimated from the slope of a graph plotting ln(fraction of wells negative for growth) versus the starting number of cells/well.

Acknowledgements

We thank Drs V.Dixit, S.Cory, D.Vaux, J.Tschopp, H.von Boehmer, R.Perlmutter, K.Tomonari, M.Krummel and J.Allison for gifts of transgenic mice, expression vectors, cytokines and monoclonal antibody-producing hybridoma cells. We are grateful to L.Gibson for generation of the transgene construct, to A.Mifsud and J.De Winter for animal husbandry, to Dr F.Battye, R.Muir and D.Kaminaris for operating the FACS, to M.Stanley and M.Pakusch for expert technical assistance and to J.Tyers for editorial assistance. We gratefully acknowledge Drs S.Cory, M.Grell, D.Vaux and M.Krummel for insightful discussions and Drs S.Cory, J.Adams, K.Shortman, J.Miller, D.Huang and D.Vaux for critical review of the manuscript. K.N. is supported by a Melbourne University PhD scholarship and A.S. is a Scholar of the Leukemia Society of America and a recipient of a Clinical Investigator Award from the Cancer Research Institute. This work was supported by the National Health and Medical Research Council (Canberra), the Anti-Cancer Council of Victoria and the US National Cancer Institute (CA43540).

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Received October 10, 1997; revised November 10, 1997; accepted November 17, 1997