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TNF Receptor 2-Deficient CD8 T Cells Are Resistant to Fas/Fas Ligand-Induced Cell Death[1]

Hung-Sia Teh,[2] Andrew Seebaran, and Soo-Jeet Teh

Apoptotic cell death plays a fundamental role in the maintenance of tissue homeostasis in complex biological systems. It is also a major mechanism for keeping immune reactions in check. Members of the TNF family of receptors and cytokines are implicated in the regulation of apoptotic signals that shape the immune system. In this study, we have examined the role of three members of the TNFR family, Fas (CD95), TNFR1 (p55), and TNFR2 (p75), in inducing cell death in Con A-activated CD4 and CD8 T cells. It was found that Con A-activated p55$^{-/-}$ CD4 or CD8 T cells were highly resistant to TNF-induced cell death. By contrast, although activated p75$^{-/-}$ CD4 or CD8 T cells were killed by TNF, they were more resistant to TNF-induced killing when compared with p75$^{+/+}$ cells, particularly at higher concentrations of TNF. We also determined whether activated p55$^{-/-}$ and p75$^{-/-}$ T cells differ in their sensitivity to cell death induced by TCR cross-linking. We found that activated p55$^{-/-}$ CD4 or CD8 T cells were equally susceptible to TCR-induced cell death. More interestingly, the loss of the p75 receptor conferred resistance to TCR-induced death in activated CD8, but not CD4 T cells. This resistance to TCR-induced death in activated p75$^{-/-}$ CD8 T cells correlated with the resistance of these cells to Fas/Fas ligand-induced cell death. ***The Journal of Immunology,*** **2000, 165: 4814–4821.**

Fas (CD95) and its ligand (FasL)[3] are members of the TNFR and TNF families, respectively (1, 2). This receptor-ligand pair plays an important role in the homeostasis of the peripheral immune system (3). FasL is expressed predominantly on activated lymphocytes and is able to induce programmed cell death on virtually all Fas-expressing cells, if they are receptive to its signal (3, 4). The importance of this interaction for the maintenance of lymphocyte homeostasis is demonstrated in the generalized lymphoproliferative disorder associated with natural loss-of-function mutations of Fas (*lpr*) and its ligand (*gld*) (2, 5). Mice homozygous for *lpr* or *gld* develop remarkably similar progressive nonmalignant lymphoproliferative diseases characterized by splenomegaly, severe lymphadenopathy, hypergammaglobulinemia, circulating autoantibodies, and premature death.

TNF, the prototype member of this family, is also potentially important in the induction of programmed cell death (1). This cytokine is a product of many cell types, but particularly of leukocytes, and is an important mediator in the early stages of the inflammatory response (1). Experiments with gene knockout mice have also shown that the two TNFRs, TNFR1 (p55) (6) and TNFR2 (p75) (7), participate in the induction of apoptosis and subsequent deletion of Ag-specific mature T cells. However, p55$^{-/-}$ and p75$^{-/-}$ mice are normal with respect to size and the composition of their lymphocyte compartments (8, 9). Both Fas and TNFR1 contain conserved death domains in their cytoplasmic tails, which mediate defined protein-protein interactions (10, 11), allowing the recruitment of other death domain-containing proteins such as Fas-associated death domain protein (FADD) (MORT1), TNFR-associated death domain protein, or receptor interacting protein (RIP) (12–17). The association of FADD to Fas or TNFR1 results in the recruitment of Fas-associated death domain-like IL-1-converting enzyme/MORT1-associated CED-3 homologue (caspase 8), the activation of which in turn leads to cell death (18, 19). In contrast to Fas or TNFR1, the cytoplasmic tail of TNFR2 does not contain a death domain and does not interact with death domain-containing proteins such as FADD, RIP, or TNFR-associated death domain protein. However, several studies have shown that TNFR2 can also transduce TNF-dependent apoptotic signals (7, 20). More recently, it was shown that cell death mediated by TNFR2 is dependent on the presence of RIP, a protein Ser/Thr kinase previously shown to be required for NF-κB activation by TNFR1 (21). These studies suggest that other signaling molecules mediate the induction of cell death by this receptor. The cytoplasmic tail of TNFR2 has been shown to interact with signal transduction molecules known as TNFR-associated factor (TRAF) proteins, particularly TRAF1 and TRAF2 (22). In some cases, the TRAF proteins have been shown to mediate various biological effects exerted by their cognate receptors. For example, TRAF2 was shown to mediate NF-κB activation (23–25).

In this study, we have used Con A-activated CD4 and CD8 T cells from *lpr/lpr*, TNFR1$^{-/-}$, or TNFR2$^{-/-}$ mice to determine the relative contribution of the TNF and Fas signaling pathways in inducing cell death in these cells. Our findings are consistent with the hypothesis that TNFR2 plays distinct regulatory roles in cell death induced by TNF or FasL in activated CD4 and CD8 T cells.

Materials and Methods

Mice

Breeders for C57BL/6 (B6), B6-p55$^{-/-}$, B6-p75$^{-/-}$, and B6-*lpr/lpr* mice were obtained from The Jackson Laboratory (Bar Harbor, ME). These mice

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[3] Abbreviations used in this paper: FasL, Fas ligand; 7-AAD, 7-amino actinomycin D; FLIP, Fas-associated death domain-like IL-1-converting enzyme-inhibitory protein; c-FLIP, cellular FLIP; c-IAP, cellular inhibitor of apoptosis protein; FADD, Fas-associated death domain protein; RIP, receptor interacting protein; TRAF, TNFR-associated factor.

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were bred at the Animal Unit in our department. Mice of 6–8 wk of age were used for the experiments described.

Abs and flow cytometry

Abs and their specificities were as follows: Jo2, Fas (CD95) (PharMingen, San Diego, CA); K10, Fas ligand (PharMingen); 53.67, CD8α (American Type Culture Collection (ATCC), Manassas, VA); 53.58, CD8β (ATCC); GK1.5, CD4 (ATCC); 145-2C11, CD3ϵ (ATCC); 55R-593, p55 (Genzyme, Cambridge, MA); TR75-32, p75 (Genzyme). A FACScan equipped with the LYSYS II software (Becton Dickinson) was used to acquire and analyze the data. For three-color analysis, a total of 25,000 events was acquired.

Cell cultures

$CD4^+CD8^-$ (CD4) and $CD4^-CD8^+$ (CD8) T cells were isolated from the lymph nodes of the indicated mouse lines by incubating the cells with biotinylated anti-CD4 or anti-CD8β mAb, respectively, followed by positive selection using a MACS MS^+ Separation Column and MiniMACS magnet following the procedure provided by the manufacturer (Miltenyi Biotech, Auburn, CA). The purified CD4 or CD8 lymph node cells were of >95% purity, and 5×10^5/ml of purified cells were cultured with 2.5 μg/ml of Con A and 20 U/ml of IL-2 for 48 h. All cells were cultured in Iscove's DMEM (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% (v/v) FBS and antibiotics (I-medium). The cultures were then split 1 in 2 in I-medium containing 20 U/ml of IL-2 and cultured for an additional 24 h. After this culture period, the cells were assessed for the expression of cell surface markers or in TCR-induced cell death assays, as indicated. For assessment of TCR-induced cell death, day 3 activated cells (1×10^5) were plated onto flat-bottom microtiter wells that had been precoated with 1 μg/ml of 2C11 mAb in 0.20 ml of I-medium containing 20 U/ml of IL-2. After an incubation period of 16 h, the cells were collected from individual wells and assessed for cell death. This was done by incubating the cells with 7-amino actinomycin D (7-AAD; Calbiochem, La Jolla, CA), as previously described (26, 27). The cells were then fixed with paraformaldehyde and analyzed by FACS. Cells staining 7-AAD^- were considered nonapoptotic and viable. The percentage of specific kill was determined as $100 \times (1 -$ (% of viable cells cultured with 2C11)/(% of viable cells that were cultured without 2C11)).

Lysis of Fas-expressing T cells by $FasL^+$ fibroblasts

The 3T3 fibroblasts that have been mock transfected (3T3-PSRγ) or Fas ligand transfected (FasL-3T3) were kindly provided by Dr. Nick Crispe (Yale University, New Haven, CT). Expression of FasL by the 3T3-PSRγ and the FasL-3T3 cell lines was determined by FACS analysis. For assessment of Fas/FasL-mediated killing, day 3 CD4 or CD8 Con A blasts from various mouse lines were ^{51}Cr labeled. A total of 1×10^4 ^{51}Cr-labeled cells was plated onto wells containing either 3×10^4, 1×10^4, or 3×10^3 3T3-PSRγ or FasL-3T3 cells and incubated for 6 h at 37°C. Spontaneous release was determined by incubating ^{51}Cr-labeled Con A blasts for the same time period in the absence of fibroblasts. Percentage of specific kill was determined as $100 \times$ ((counts released with fibroblasts − spontaneous release)/(maximum release − spontaneous release)). Maximum release was determined by freezing and thawing the ^{51}Cr-labeled Con A blasts three times and determining the radioactivity released into the culture supernatant.

Results

Activated $p75^{-/-}$ CD8 T cells are resistant to TCR-induced cell death

Cross-linking of the TCR on activated T cells leads to programmed cell death. This form of programmed cell death is referred to as propiocidal apoptosis and is dependent on culturing the activated T cells in IL-2 (28, 29). Propiocidal apoptosis is dependent at least in part on the expression of Fas on the activated T cells (30). To determine whether p55 and p75 play any role in propiocidal apoptosis, we activated purified CD4 and CD8 lymph node T cells from B6, B6-*lpr/lpr*, B6-$p55^{-/-}$, and B6-$p75^{-/-}$ mice with Con A and IL-2. Propiocidal apoptosis was induced by TCR cross-linking. The 7-AAD assay (see *Materials and Methods*) was used for distinguishing live from dead cells. The results in Fig. 1*A* indicate that Con A-activated CD4 T cells from B6 and B6-$p75^{-/-}$ mice were highly susceptible to propiocidal apoptosis. As expected, activated CD4 T cells from B6-*lpr/lpr* mice were relatively resistant

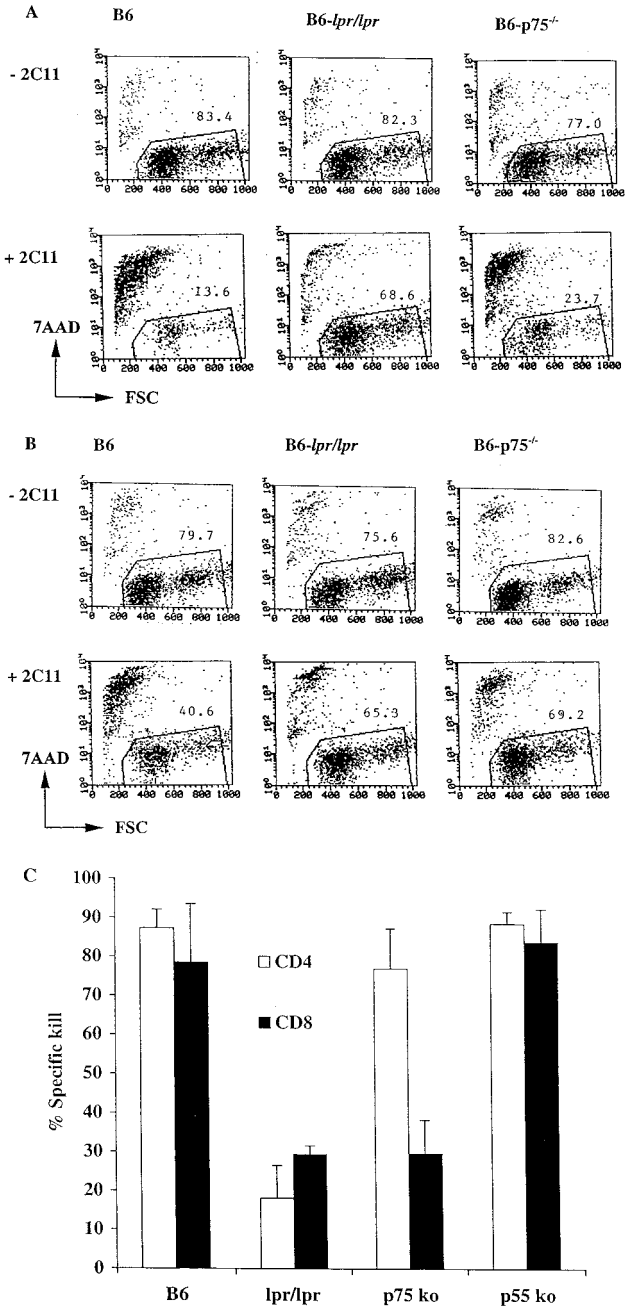

FIGURE 1. CD8 Con A blasts from $p75^{-/-}$ mice are resistant to TCR-induced cell death. Day 3 Con A blasts from the indicated mouse line were incubated with and without immobilized 2C11 mAb and IL-2 for 16 h at 37°C. IL-2 was included in these cultures to prevent cell death as a result of IL-2 deprivation. After this incubation period, the cells from individual cultures were collected and stained with 7-AAD and analyzed by FACS. Dot plots of 7-AAD fluorescence vs forward scatter (FSC) for CD4 (*A*) and CD8 (*B*) Con A blasts after the 16-h incubation period are indicated. The numbers in *A* and *B* indicate the percentage of viable cells after the 16-h incubation period. Percentage of specific kill (*C*) is calculated as described in *Materials and Methods*. The error bars are SDs of triplicate cultures.

to propiocidal apoptosis (Fig. 1*A*). Activated CD8 T cells from B6 and B6-*lpr/lpr* mice were similar to CD4 T cells in their susceptibility and resistance to propiocidal apoptosis, respectively (Fig. 1*B*). Unexpectedly, activated CD8 T cells from B6-$p75^{-/-}$ mice were highly resistant to propiocidal apoptosis (Fig. 1*B*). These results are also expressed as percentage of specific kill (Fig. 1*C*) because this form of data presentation offers a more quantitative

way for comparing results between the various groups. We also found that similar to cells from B6 mice, activated CD4 and CD8 T cells from B6-p55$^{-/-}$ mice were also highly susceptible to propiocidal apoptosis (Fig. 1*C*). Therefore, only the p75$^{-/-}$, and not the p55$^{-/-}$ mutation confers resistance of activated CD8 T cells to propiocidal apoptosis.

Because TCR-induced cell death is mediated in large part by Fas/FasL interactions, we determined whether the resistance of activated p75$^{-/-}$ CD8 T cells to TCR-induced cell death could be due to alteration in Fas expression by these cells. The results in Fig. 2 indicate that Fas expression in Con A-activated CD4 and CD8 T cells is not affected by the p75$^{-/-}$ mutation. Similarly, the expression of p75 in Con A-activated CD4 and CD8 T cells is not affected by the *lpr* mutation. The fluorescence data also indicate that CD4 and CD8 Con A blasts from B6-*lpr/lpr* and B6-p75$^{-/-}$ mice did not express detectable level of Fas and p75, respectively.

Activated p75$^{-/-}$ CD8 T cells are resistant to FasL-induced cell death

Propiocidal apoptosis is also dependent on the induction of FasL in activated T cells as a result of TCR cross-linking (31–35). Therefore, one potential explanation for the resistance of activated p75$^{-/-}$ CD8 T cells to propiocidal apoptosis is that these cells do not up-regulate FasL upon TCR cross-linking. To determine whether activated p75$^{-/-}$ CD8 T cells can up-regulate FasL as a result of TCR cross-linking, Con A-activated CD8 T cells from B6, B6-*lpr/lpr*, and B6-p75$^{-/-}$ mice were stimulated with an anti-CD3ϵ mAb for 6 h, and the induction of FasL expression on these cells was quantitated by flow cytometry. The 6-h incubation time is insufficient for most cells to undergo propiocidal apoptosis and offers a suitable time point for assessment of FasL expression. The results in Fig. 3 indicate that p75$^{-/-}$ CD8 T cells were able to up-regulate FasL at least as efficiently as B6 CD8 T cells after TCR stimulation. Thus, the resistance of p75$^{-/-}$ CD8 T cells to propiocidal apoptosis is not due to the inability of these cells to up-regulate FasL after TCR stimulation. We also noted that B6-*lpr/lpr* CD8 T cells were the most efficient in expressing FasL after TCR stimulation, an observation previously reported by other investigators (36).

The observation that FasL induction is not defective in p75$^{-/-}$ CD8 T cells raises the possibility that these cells are resistant to cell death mediated by Fas/FasL interactions. To test this possibility, we determined the extent of lysis that was caused by incubating activated T cells with a FasL-expressing fibroblast cell line in a ^{51}Cr release assay. The level of FasL expression in the parental fibroblast cell line and the FasL transfectant is shown in Fig. 4*A*. It is clear from this figure that FasL expression is only detectable in the transfectant cell line. The data in Fig. 4*B* showed that CD4 Con A blasts from B6 and B6-p75$^{-/-}$ mice were equally susceptible to killing by the FasL^{+} cell line. As expected, CD4 blasts from B6-*lpr/lpr* mice were resistant to killing by the FasL^{+} cell line. By contrast, CD8 blasts from B6-p75$^{-/-}$ mice were much more resistant than CD8 blasts from B6 mice to killing by the FasL^{+} cell line (Fig. 4*C*). This result suggests that the resistance of CD8 blasts from B6-p75$^{-/-}$ mice to propiocidal apoptosis is most likely due to the resistance of this cell population to Fas/FasL-mediated killing.

Activated p75$^{-/-}$ CD4 and CD8 T cells are more resistant to killing by TNF

Previous studies have suggested that TCR-induced apoptosis in activated T cells is mediated in part by TNF and killing by TNF is mediated by the p75 receptor (7). The availability of B6-p55$^{-/-}$ and B6-p75$^{-/-}$ mice allows us to determine more directly whether TNF-induced killing of CD4 and CD8 Con A blasts is mediated by either the p55 and/or p75 receptor. The sensitivity of CD4 and CD8 Con A blasts to killing by TNF was determined by culturing these blasts with various concentrations of TNF in the presence or absence of a low concentration (1 μg/ml) of the protein synthesis inhibitor, cycloheximide. Previous studies have shown that the addition of cycloheximide is necessary for the apoptotic effects of TNF (37, 38). We also found that between 0.1 and 10 ng/ml, TNF by itself does not induce apoptosis in either CD4 or CD8 blasts from these three lines of mice (data not shown). In the presence of cycloheximide, CD4 or CD8 Con A blasts from B6 mice were highly sensitive to killing by TNF (Fig. 5). At the concentrations tested, CD4 or CD8 blasts from p55$^{-/-}$ mice were completely resistant to killing by TNF plus cycloheximide (Fig. 5). Although CD4 or CD8 blasts from p75$^{-/-}$ mice were susceptible to killing by TNF plus cycloheximide, the dose-response curve to TNF differs from that observed for the corresponding blasts from B6 mice. At the lowest concentration of TNF (0.1 ng/ml), there was no

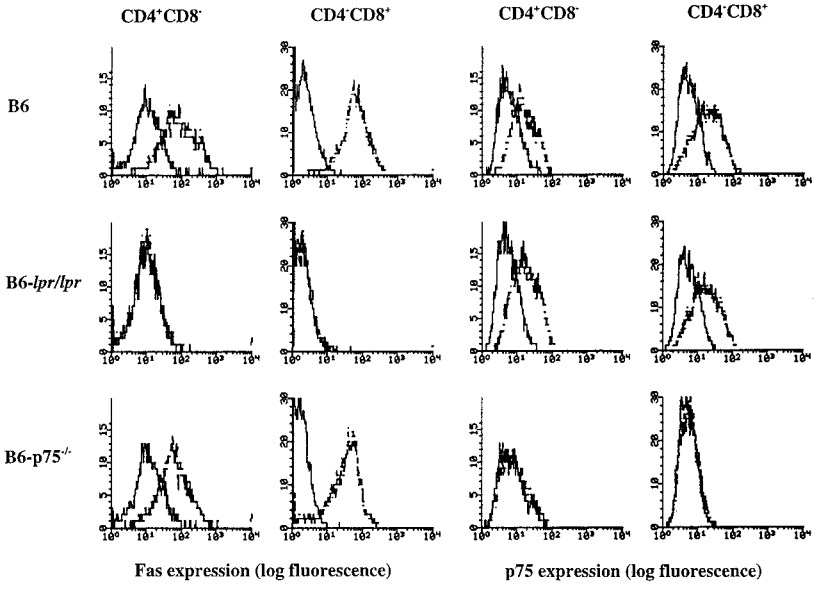

FIGURE 2. Fas (CD95) and TNFR2 (p75) expression on Con A blasts. Purified CD4 or CD8 T cells from the indicated mouse line were activated with Con A and IL-2 for 3 days. The level of Fas expression on these cells was determined by staining with biotinylated anti-Fas (Jo2) mAb, followed by streptavidin-Tricolor. The level of p75 expression was determined by staining the cells with unlabeled anti-p75 mAb, followed by anti-mouse Ig FITC. The solid lines indicate the base level of Fas or p75 expression obtained by staining of the indicated cells with either streptavidin-Tricolor (for Fas expression) or by anti-mouse Ig FITC (for p75 expression).

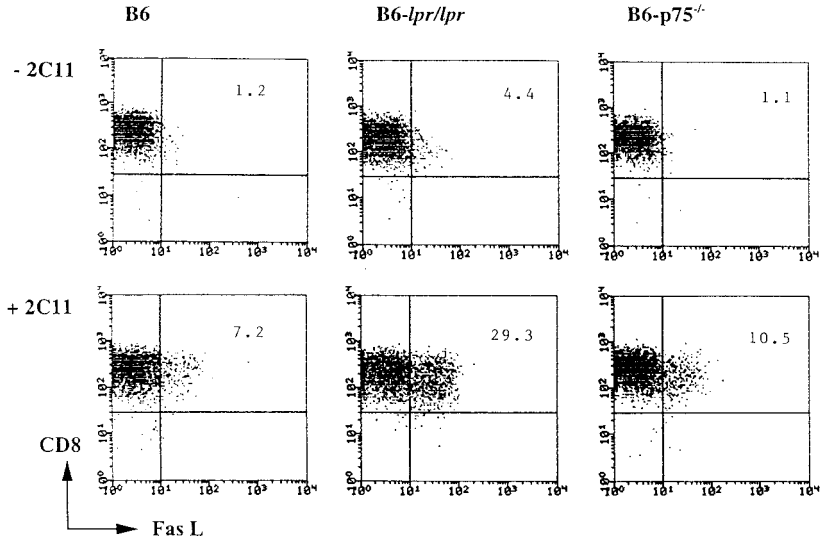

FIGURE 3. Normal induction of FasL in TCR-stimulated p75$^{-/-}$ CD8 T cells. Day 3 CD8 Con A blasts from the indicated mouse line were stimulated with immobilized 2C11 mAb and IL-2 for 6 h at 37°C. The cells were then recovered and stained with biotinylated anti-FasL mAb, followed by streptavidin-Tricolor. The numbers indicate percentages of cells in the quadrant.

difference in susceptibility to killing between CD4 and CD8 blasts from B6 or B6-p75$^{-/-}$ mice. However, at higher concentrations of TNF (1 and 10 ng/ml), CD4 and CD8 blasts from p75$^{-/-}$ mice were more resistant than those from B6 mice to killing by TNF plus cycloheximide. These results indicate that expression of the p75 receptor is insufficient for TNF-mediated apoptosis in both CD4 and CD8 blasts. Furthermore, the absence of the p75 receptor in these blasts renders them less susceptible to killing at high concentrations of TNF.

The p75 receptor is required for the optimal activation of CD4 and CD8 T cells

The results in Fig. 5 suggest that the p75 receptor is incapable of transducing TNF-mediated death signals in CD4 and CD8 Con A blasts. Other studies have suggested that the p75 receptor is required for optimal proliferation of thymocytes and cytotoxic CD8 T cells (39). To further define the function of the p75 receptor in CD4 and CD8 T cells, we determined whether the p75 receptor is required for the optimal proliferative response of CD4 and CD8 T cells in response to TCR stimulation. This was done by purifying CD4 and CD8 T cells from the lymph nodes of B6 and B6-p75$^{-/-}$ mice and activating them with an anti-TCR mAb plus IL-2 and measuring their proliferative response 2 days later. The data in Fig. 6 indicate that the proliferative responses of purified CD4 and CD8 T cells from B6-p75$^{-/-}$ mice in response to anti-TCR stimulation were about 2-fold less than that observed for corresponding cells from B6 mice. By contrast, the proliferative responses of CD4 and CD8 T cells from B6-p55$^{-/-}$ mice in response to stimulation by anti-TCR mAb + IL-2 were similar to that of CD4 or CD8 T cells from B6 mice (data not shown). Thus, the p75, but not the p55, receptor is required for optimal proliferative responses of anti-TCR-stimulated CD4 and CD8 T cells.

Cell recovery from cell cultures at various times after TCR stimulation is affected by both the rate of cell proliferation and cell death. Because we have observed that activated CD8 T cells from B6-p75$^{-/-}$ mice were more resistant to TCR-induced cell death, the expectation is that even though anti-TCR-stimulated p75$^{-/-}$ CD8 T cells proliferated less optimally than B6 CD8 T cells, the higher resistance of activated p75$^{-/-}$ CD8 T cells to TCR-induced cell death will confer a survival benefit, which may be reflected by higher cell recoveries at later time points of the culture. To test this possibility, we stimulated unpurified spleen cells from B6 and B6-p75$^{-/-}$ mice, which contained a mixture of CD4 and CD8 T cells, with anti-TCR mAb plus IL-2 and determined the number of CD4 and CD8 T cells recovered at various times after culture initiation. The data in Fig. 7 show that fairly similar number of CD8 T cells were recovered from anti-TCR-stimulated during the first 3 days of culture. However, on day 4, significantly more number of CD8 T cells were recovered from cultures of p75$^{-/-}$ cells relative to B6 cultures. By contrast, fewer CD4 T cells were recovered from p75$^{-/-}$ cultures relative to B6 cultures, and this lower recovery was evident by day 3 after TCR stimulation. The increase in yield of p75$^{-/-}$ CD8 cells after 4 days of culture was also reflected in the CD8/CD4 ratios of the cultured cells. Before culture, the CD8/CD4 ratios of spleen cells from B6 and p75$^{-/-}$ mice were 0.58 and 0.50, respectively. After 4 days of culture, the CD8/CD4 ratio was 2.3 for B6 and 4.1 for p75$^{-/-}$ spleen cells. These observations are consistent with our conclusions that the absence of the p75 receptor confers a survival advantage for CD8, but not CD4, T cells with regard to TCR-induced cell death.

Discussion

The role of TNFR1 (p55) and TNFR2 (p75) in TNF-induced killing of activated CD4 and CD8 T cells

In the presence of low concentrations of cycloheximide, activated CD4 and CD8 T cells are killed by TNF (Fig. 5). Under our assay conditions, TNF-mediated killing of these cells is dependent on two factors: 1) the presence of the p55 receptor, and 2) the inclusion of cycloheximide in the killing assay. Zheng et al. (7) have previously reported that the killing of activated CD8 T cells by TNF is mediated by the p75 receptor. The authors' conclusion was based in part on the failure to detect expression of the p55 receptor on activated CD4 and CD8 T cells. We have also been unable to detect expression of the p55 receptor on activated CD4 and CD8 T cells by FACS analysis (data not shown). However, the abrogation of killing by TNF plus cycloheximide in activated p55$^{-/-}$ CD4 or CD8 T cells (Fig. 5) indicates that p55 is essential for this process. Therefore, p55 must be expressed on these cells at a level that is below detection by FACS analysis. Our data also indicate that TNF is unable to induce killing of either activated CD4 or CD8 T cells via the p75 receptor because activated p55$^{-/-}$ CD4 or CD8 T cells express the same level of p75 as activated cells from B6 mice (data not shown) and these cells are resistant to killing by TNF plus cycloheximide (Fig. 5). Speiser et al. (6) have also shown that the p55 receptor is required for the deletion of activated CD8 cytotoxic

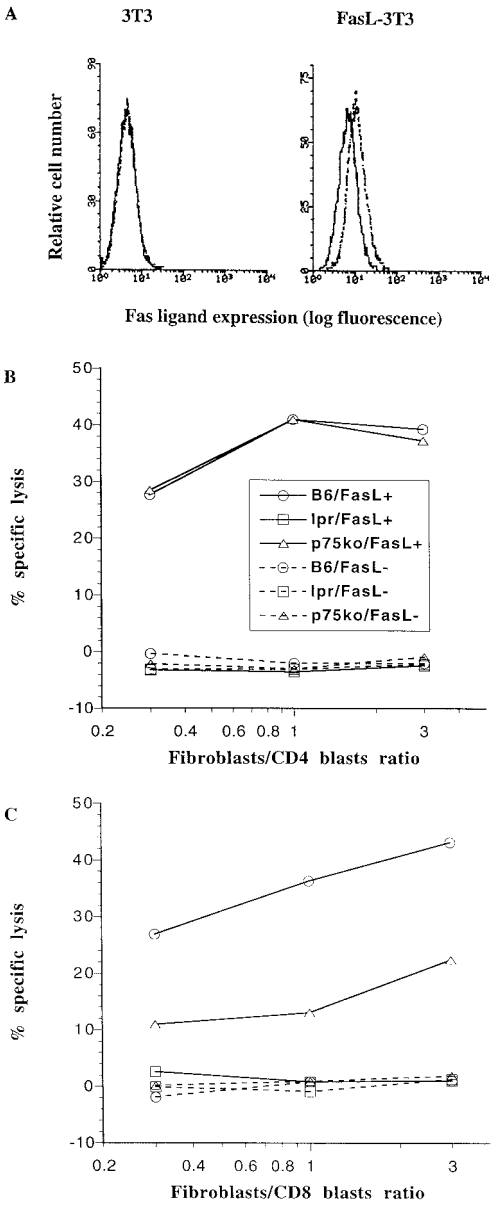

FIGURE 4. CD8, but not CD4, Con A blasts from $p75^{-/-}$ mice are resistant to Fas/FasL-mediated killing. Day 3 CD4 or CD8 Con A blasts from the indicated mouse line were ^{51}Cr labeled and incubated either with $FasL^{-}$ (3T3-PSRγ) or $FasL^{+}$ (FasL-3T3) fibroblasts. *A*, The level of FasL expression on these cell lines was determined by staining the cells with a biotinylated anti-FasL mAb, followed by streptavidin-Tricolor. Only FasL-3T3 fibroblasts express detectable level of FasL. *B*, The ratios of fibroblasts to CD4 Con A blasts are as indicated. IL-2 was included in the assay medium to prevent cell death resulting from IL-2 deprivation. After a 6-h incubation period, the amount of ^{51}Cr release into the culture supernatant was determined. The percentage of specific lysis values are the mean of duplicate cultures. *C*, Similar to *B*, except CD8 Con A blasts from the indicated mouse lines were substituted for CD4 Con A blasts.

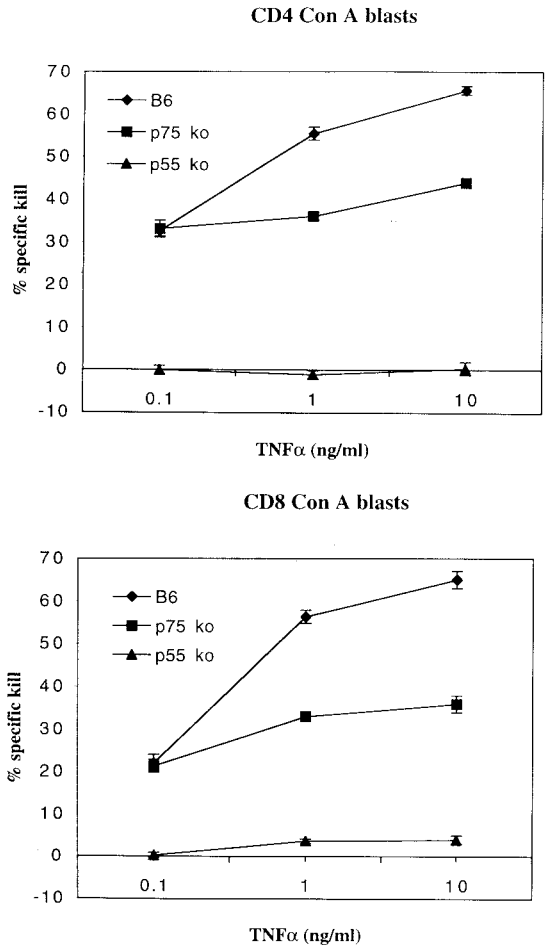

FIGURE 5. TNF-induced cell death in activated CD4 or CD8 cells is dependent on p55 expression and is regulated by p75. Day 3 CD4 or CD8 Con A blasts from the indicated mouse line were cultured with the indicated concentration of TNF, 20 U/ml of IL-2, and 1 μg/ml of cycloheximide for 16 h at 37°C. Percentage of specific kill is calculated as described in *Materials and Methods*. The error bars denote SDs of triplicate cultures. In the absence of cycloheximide, little or no TNF-induced killing can be detected (data not shown).

T cells in vivo. We found that the role of p75 is to augment TNF-induced, p55-mediated killing at high concentrations of TNF (Fig. 5). The augmentation of TNF-induced, TNFR1-mediated cell death by TNFR2 has also been observed by Weiss et al. (37). Previous studies have shown that TNFR1 can mediate both apoptotic and antiapoptotic signals (15, 40). Weiss et al. (37) proposed that the augmentation of TNF/TNFR1-mediated cell death by TNFR2 may be due to the sequestration of antiapoptotic proteins by the p75 receptor complex. p75 may also augment TNF/TNFR1-mediated killing of CD4 and CD8 T cells by a similar mechanism.

The role of p75 in TCR- and Fas-mediated killing of activated CD4 and CD8 T cells

We found that TCR-mediated killing of activated CD8, but not CD4, T cells is drastically reduced in the absence of the p75 receptor (Fig. 1). Zheng et al. (7) proposed that there are two effector mechanisms associated with cell death induced by cross-linking the TCR on activated T cells. The first is the induction of FasL in activated T cells as a result of TCR cross-linking, and this can lead to activation of the Fas death pathway as a result of Fas-FasL interaction. The second mechanism is that TCR stimulation of activated T cells leads to the production of TNF, which initiates the TNF-dependent death pathway. Zheng et al. (7) proposed that the Fas/FasL-mediated pathway is sufficient for TCR-induced killing of activated CD4 T cells. They also proposed that Fas/FasL-mediated killing of activated CD8 T cells is a minor pathway, and the major pathway for TCR-induced cell death of activated CD8 T cells is mediated by a p75-dependent TNF pathway. Our data are inconsistent with these conclusions for the following reasons: 1)

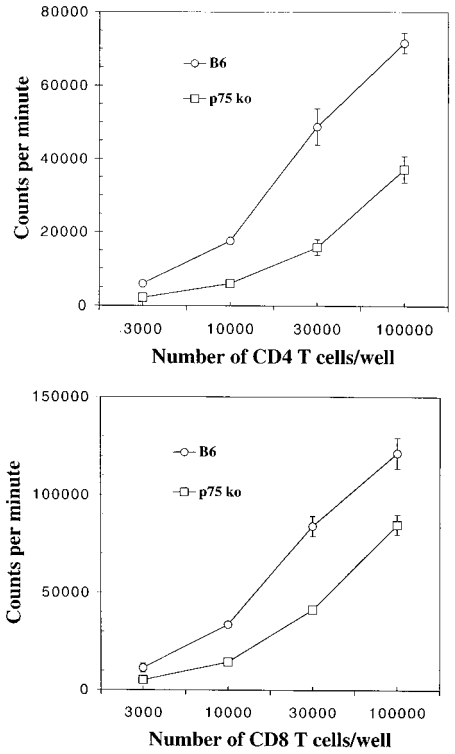

FIGURE 6. p75 is required for the optimal proliferation of anti-TCR-stimulated CD4 and CD8 T cells. The indicated number of purified CD4 and CD8 T cells from B6 and B6-p75$^{-/-}$ mice were cultured in wells coated with 10 μg/ml of 2C11 in I-medium containing 20 U/ml of IL-2. One μCi of [^{3}H]thymidine was added to each culture during the last 6 h of a 48-h culture period. The error bars represent the SDs of triplicate cultures.

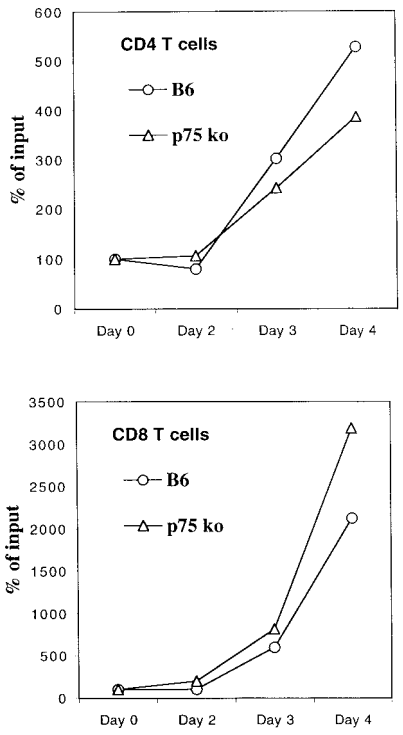

FIGURE 7. Higher recovery of anti-TCR-stimulated p75$^{-/-}$ CD8 T cells after 4 days of culture. Spleen cells (1×10^5) from B6 and B6-p75$^{-/-}$ mice were cultured in wells coated with 10 μg/ml of 2C11 in I-medium containing 20 U/ml of IL-2. The percentage of CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells recovered after 2, 3, or 4 days of culture was determined by FACS analysis. The total number of CD4 or CD8 cells recovered from each culture at these times was determined and expressed as a percentage of the starting population.

Activated CD8 T cells lacking the p55 receptor are not killed by TNF plus cycloheximide (Fig. 5); this observation suggests that TNF-induced cell death in activated CD8 T cells operates through the p55 receptor. 2) Activated B6 CD8 T cells, which express Fas, can be killed very efficiently by FasL$^+$ fibroblasts (Fig. 4). This observation indicates that the Fas/FasL-mediated death pathway is not defective in activated CD8 T cells. 3) Activated p55$^{-/-}$ CD8 T cells, which express the same level of the p75 receptor as activated B6 CD8 T cells, are not killed by TNF plus cycloheximide (Fig. 5), suggesting that TNF-dependent death signals are not transmitted through the p75 receptor.

Instead of mediating TNF-induced cell death, we found that the p75 receptor is required for efficient Fas/FasL-mediated killing of activated CD8 T cells (Fig. 4). Together with the finding that the p75 receptor augments TNF/p55-mediated killing, our data are more consistent with the following alternative explanations for the resistance of activated p75$^{-/-}$ CD8 T cells to TCR-induced cell death: 1) activated p75$^{-/-}$ CD8 T cells are highly resistant to Fas/FasL-mediated killing, and 2) the reduced sensitivity of these cells to TNF/p55-mediated killing.

What are the potential explanations for the relative resistance of activated p75$^{-/-}$ CD8 T cells to Fas/FasL-mediated death pathway? Previous studies have shown that Fas-mediated killing as a result of TCR stimulation is dependent on the induction of FasL expression after TCR stimulation (31–33). However, we found that FasL induction is not defective in TCR-stimulated p75$^{-/-}$ CD8 T cells (Fig. 3). Mature T cells have been shown to express high levels of Fas-associated death domain-like IL-1-converting enzyme-inhibitory proteins (FLIPs) that block death receptor-induced cell death (41). Significantly, T cells down-regulate FLIP when they are activated in vitro, and this down-regulation correlated with the sensitivity of the activated T cells to Fas/FasL-mediated cell death (42–44). Retrovirus-mediated expression of cellular FLIP (c-FLIP) blocks Fas-induced apoptosis of activated T lymphocytes, but does not affect cell death resulting from cytokine withdrawal (44). It remains to be determined whether the p75 receptor complex may recruit and sequester c-FLIP away from the Fas receptor complex and in this way interferes with Fas/FasL-mediated killing. The p75 receptor complex can also recruit cellular inhibitors of apoptosis proteins (c-IAPs) (45), which contribute to antiapoptotic signals (46, 47). Therefore, another potential mechanism for the regulation of Ag-induced cell death by the p75 receptor is through the sequestration of proteins such as c-IAPs away from the Fas receptor complex.

It is interesting to note that Fas/FasL-mediated killing of activated CD4 T cells is not regulated by the p75 receptor. A potential explanation for the independence on the p75 receptor of activated CD4 T cells to Fas-mediated killing is that inhibitors of Fas-mediated killing such as c-FLIP and/or c-IAPs may be present at relatively low levels in activated p75$^{-/-}$ CD4 T cells, and the sequestration of these factors by the p75 receptor complex may not be required for Fas-mediated killing of activated CD4 T cells. This possibility is being investigated.

The lpr *and* gld *phenotypes are affected by p55$^{-/-}$ and TNF$^{-/-}$ mutations*

The *lpr* phenotype is accelerated in p55$^{-/-}$ *lpr/lpr* mice (48). More recently, it was shown that TNF$^{-/-}$ *gld/gld* mice have a much less

severe *gld* phenotype (49). The lymphoproliferative disorder in *lpr* and the *gld* mice is due to the accumulation of the $CD4^-CD8^-\alpha\beta$ TCR^+ $B220^+$ (double-negative) T cell population in the peripheral lymphoid organs of these mice (5). It is conceivable that in $p55^{-/-}$ *lpr/lpr* mice, there is a lack of signaling through p55, but signaling through the p75 receptor can still occur. We speculate that signaling through the p75 receptor may promote the proliferation of double-negative cells and exacerbates the *lpr* phenotype. The requirement for the p75 receptor for optimal proliferative responses is most likely due to the activation of NF-κB-mediated signaling pathways (23, 24), which has been shown to antagonize TNF-mediated apoptosis (50–52). By contrast, in $TNF^{-/-}$ *gld/gld* mice, signaling through the p75 receptor cannot occur and proliferative signals offered by the p75 receptor are lacking for these cells, and this may account for the less severe *gld* phenotype.

The studies in the *lpr* and *gld* mice and our present work illustrate that Fas and TNF signaling pathways are intimately related. We have demonstrated that signaling through TNFR1 and the Fas receptor can be regulated by TNFR2 signals. Delineation of the nature of the cross-talk between distinct receptors of the TNFR family will undoubtedly lead to novel insights regarding the multiple functions of these receptors and the molecular basis for the multifaceted manifestations of the lymphoproliferative and autoimmune disorders associated with the *gld* and *lpr* mutations.

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References

1. Smith, C. A., T. Farrah, and R. G. Goodwin. 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death. *Cell 76:959.*
2. Nagata, S., and T. Suda. 1995. Fas and Fas ligand: *lpr* and *gld* mutations. *Immunol. Today 16:39.*
3. Krammer, P. H. 1999. CD95 (APO-1/Fas)-mediated apoptosis: live and let die. *Adv. Immunol. 71:163.*
4. Lynch, D. H. 1996. The role of FasL and TNF in the homeostatic regulation of immune responses. *Adv. Exp. Med. Biol. 406:135.*
5. Cohen, P. L., and R. A. Eisenberg. 1991. *Lpr* and *gld*: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol. 9:243.*
6. Speiser, D. E., E. Sebzda, T. Ohteki, M. F. Bachmann, K. Pfeffer, T. W. Mak, and P. S. Ohashi. 1996. Tumor necrosis factor receptor p55 mediates deletion of peripheral cytotoxic T lymphocytes in vivo. *Eur. J. Immunol. 26:3055.*
7. Zheng, L., G. Fisher, R. E. Miller, J. Peschon, D. H. Lynch, and M. J. Lenardo. 1995. Induction of apoptosis in mature T cells by tumor necrosis factor. *Nature 377:348.*
8. Pfeffer, K., T. Matsuyama, T. M. Kundig, A. Wakeham, K. Kishihara, A. Ahahinian, K. Wiegmann, P. S. Ohashi, M. Kronke, and T. W. Mak. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell 73:457.*
9. Erickson, S. L., F. J. de Sauvage, K. Kikly, K. Carver-Moore, S. Pitts-Meek, N. Gillett, K. C. F. Sheehan, R. D. Schreiber, D. V. Goeddel, and M. W. Moore. 1994. Decreased sensitivity to tumor-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature 372:560.*
10. Itoh, N., and S. Nagata. 1993. A novel protein domain required for apoptosis: mutational analysis of human Fas antigen. *J. Biol. Chem. 268:10932.*
11. Tartaglia, L. A., T. M. Ayres, G. H. W. Wong, and D. V. Goeddel. 1993. A novel domain within the 55 kd TNF receptor signals cell death. *Cell 74:845.*
12. Chinnaiyan, A. M., K. O'Rourke, M. Tewari, and V. M. Dixit. 1995. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell 81:505.*
13. Boldin, M. P., E. E. Varfolomeev, Z. Pancer, I. L. Mett, J. H. Camonis, and D. Wallach. 1995. A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J. Biol. Chem. 270:7795.*
14. Hsu, H., J. Huang, H.-B. Shu, V. Baichwal, and D. V. Goeddel. 1996. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity 4:387.*
15. Hsu, H., J. Xiong, and D. V. Goeddel. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. *Cell 81:495.*
16. Hsu, H., H.-B. Shu, M.-G. Pan, and D. V. Goeddel. 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transcription pathways. *Cell 84:299.*
17. Stanger, B. Z., P. Leder, T. H. Lee, E. Kim, and B. Seed. 1995. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell 81:513.*
18. Boldin, M. P., T. M. Goncharov, Y. V. Goltsev, and D. Wallach. 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell 85:803.*
19. Muzio, M., A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scarridi, J. D. Bretz, M. Zhang, and R. Gentz. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell 85:817.*
20. Alexander-Miller, M. A., M. A. Derby, A. Sarin, P. A. Henkart, and J. A. Berzofsky. 1998. Supraoptimal peptide-major histocompatibility complex causes a decrease in Bcl-2 levels and allows TNFR2-mediated apoptosis of cytotoxic T lymphocytes. *J. Exp. Med. 188:1391.*
21. Pimentel-Muinos, F. X., and B. Seed. 1999. Regulated commitment of TNF receptor signaling: a molecular switch for death or activation. *Immunity 11:783.*
22. Rothe, M., S. C. Wong, W. J. Henzel, and D. V. Goeddel. 1994. A novel family of putative signal transducers associated with the cytoplasmic domain of the p75 kDa tumor necrosis factor receptor. *Cell 78:681.*
23. Rothe, M., V. Sarma, V. M. Dixit, and D. V. Goeddel. 1995. TRAF2-mediated activation of NF-κB by TNR receptor 2 and CD40. *Science 269:1424.*
24. Cheng, G., and D. Baltimore. 1996. TANK, a co-inducer with TRAF2 of TNF- and CD40L-mediated NF-κB activation. *Genes Dev. 10:963.*
25. Lee, S. Y., S. Y. Lee, G. Kandala, M.-L. Liou, H.-C. Liou, and Y. Choi. 1996. CD30-TRAF interaction: NF-κB activation and binding specificity. *Proc. Natl. Acad. Sci. USA 93:9699.*
26. Philpott, N. J., A. J. C. Turner, J. Scopes, M. Westby, J. C. W. Marsh, E. C. Gordonsmith, A. G. Dagleish, and F. M. Gibson. 1996. The use of 7-amino-actinomycin D in identifying apoptosis: simplicity of use and broad spectrum of application compared with other techniques. *Blood 87:2244.*
27. Motyka, B., and H.-S. Teh. 1998. Naturally occurring low affinity peptide/MHC class I ligands can mediate negative selection and T cell activation. *J. Immunol. 160:77.*
28. Lenardo, M. J. 1991. Interleukin-2 programs mouse $\alpha\beta$ T lymphocytes for apoptosis. *Nature 353:858.*
29. Boehme, S. A., and M. J. Lenardo. 1993. Propiocidal apoptosis of mature T cells occurs at S phase of the cell cycle. *Eur. J. Immunol. 23:1552.*
30. Russell, J. H., B. Rush, C. Weaver, and R. Wang. 1993. Mature T cells of autoimmune *lpr/lpr* mice have a defect in antigen-stimulated suicide. *Proc. Natl. Acad. Sci. USA 90:4409.*
31. Dhein, J., H. Walczak, C. Baumler, K.-M. Debatin, and P. H. Krammer. 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature 373:438.*
32. Ju, S.-T., D. J. Panka, H. Cui, R. Ettinger, M. El-Khatib, D. H. Sherr, B. Z. Stanger, and A. Marshak-Rothstein. 1995. Fas (CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature 373:444.*
33. Brunner, T., R. J. Mogil, D. LaFace, N. J. Yoo, A. Mahboubi, F. Echeverri, S. J. Martin, W. R. Force, D. H. Lynch, C. F. Ware, and D. R. Green. 1995. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature 373:441.*
34. Reap, E. A., D. Leslie, M. Abrahams, R. A. Eisenburg, and P. L. Cohen. 1995. Apoptosis abnormalities of splenic lymphocytes in autoimmune *lpr* and *gld* mice. *J. Immunol. 154:936.*
35. Alderson, M. R., T. W. Tough, T. Davis-Smith, S. Braddy, B. Falk, K. A. Schooley, R. G. Goodwin, C. A. Smith, F. Ramsdell, and D. H. Lynch. 1995. Fas ligand mediates activation-induced cell death in human T lymphocytes. *J. Exp. Med. 181:71.*
36. Chu, J. L., P. Ramos, A. Rosendorff, J. Nikolic-Zugic, E. Lacy, A. Matsuzawa, and K. B. Elkon. 1995. Massive up-regulation of the Fas ligand in *lpr* and *gld* mice: implications for Fas regulation and the graft-versus-host disease-like wasting syndrome. *J. Exp. Med. 181:393.*
37. Weiss, T., M. Grell, K. Siemienski, F. Muhlenbeck, H. Durkop, K. Pfizenmaier, P. Scheruich, and H. Wajant. 1998. TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. *J. Immunol. 161:3136.*
38. Tang, D., J. M. Lahti, J. Grenet, and V. J. Kidd. 1999. Cycloheximide-induced T-cell death is mediated by a Fas-associated death domain-dependent mechanism. *J. Biol. Chem. 274:7245.*
39. Tartaglia, L. A., R. F. Weber, I. S. Figari, C. Reynolds, M. A. Palladino, Jr., and D. V. Goeddel. 1991. The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc. Natl. Acad. Sci. USA 88:9292.*
40. Liu, Z.-G., H. Hsu, D. V. Goeddel, and M. Karin. 1996. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. *Cell 87:565.*
41. Thome, M., P. Schneider, K. Hofmann, H. Fickenscher, E. Meini, F. Neipel, C. Mattmann, K. Burns, J.-L. Bodmer, M. Schroter, et al. 1997. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature 386:517.*
42. Irmler, M., M. Thome, M. Hahne, P. Schneider, K. Hofmann, V. Steiner, J.-L. Bodmer, M. Schroter, K. Burns, C. Mattmann, et al. 1997. Inhibition of death receptor signals by cellular FLIP. *Nature 388:190.*
43. Refaeli, Y., L. Can Parijs, C. A. London, J. Tschopp, and A. K. Abbas. 1998. Biochemical mechanisms of IL-2-regulated Fas-mediated T cell apoptosis. *Immunity 8:615.*

44. Van Parijs, L., Y. Refaeli, A. K. Abbas, and D. Baltimore. 1999. Autoimmunity as a consequence of retrovirus-mediated expression of c-FLIP in lymphocytes. *Immunity 11:763.*
45. Rothe, M., M.-G. Pan, W. J. Henzel, T. M. Ayres, and D. V. Goeddel. 1995. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell 83:1243.*
46. Liston, P., N. Roy, K. Tamai, C. Lefebvre, S. Baird, G. Cherton-Horvat, M. M. R Farahani, J. E. Ikeda, A. MacKenzie, and R. G. Korneluk. 1996. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature 379:349.*
47. Duckett, C. S., V. E. Nava, R. W. Gedrich, R. J. Clem, J. L. Van Dongen, M. C. Gilfillan, H. Shiels, J. M. Hardwick, and C. B. Thompson. 1996. A conserved family of cellular genes related to the baculovirus IAP gene and encoding apoptosis inhibitors. *EMBO J. 15:2685.*
48. Zhou, T., C. K. Edwards III, P. Yang, Z. Wang, H. Bluethmann, and J. D. Mountz. 1996. Greatly accelerated lymphadenopathy and autoimmune disease in *lpr* mice lacking tumor necrosis factor receptor I. *J. Immunol. 156:2661.*
49. Korner, H., E. Cretney, P. Wilhelm, J. M. Kelly, M. Rollinghoff, J. D. Sedgwick, and M. J. Smyth. 2000. Tumor necrosis factor sustains the generalized lymphoproliferative disorder (*gld*) phenotype. *J. Exp. Med. 191:89.*
50. Beg, A. A., and D. Baltimore. 1996. An essential role for NF-κB in preventing TNFα-induced death. *Science 274:782.*
51. Van Antwerp, D. J., S. J. Martin, T. Kafri, D. R. Green, and I. M. Verma. 1996. Suppression of TNF-α-induced apoptosis by NF-κB. *Science 274:787.*
52. Wang, C.-Y., M. W. Mayo, and A. S. Baldwin, Jr. 1996. TNF-α and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. *Science 274:784.*