Immunity, Vol. 8, 615–623, May, 1998, Copyright ©1998 by Cell Press

Biochemical Mechanisms of IL-2–Regulated Fas-Mediated T Cell Apoptosis

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Summary

Activation-induced cell death (AICD) of lymphocytes is an important mechamism of self-tolerance. In $CD4^+$ T cells, AICD is mediated by the Fas pathway and is enhanced by IL-2. To define the mechanisms of this pro-apoptotic action of IL-2, we analyzed $CD4^+$ T cells from wild-type and IL-$2^{-/-}$ mice expressing a transgenic T cell receptor. T cells become sensitive to AICD after activation by antigen and IL-2. IL-2 increases transcription and surface expression of Fas ligand (FasL) and suppresses transcription and expression of FLIP, the inhibitor of apoptosis. The ability of IL-2 to enhance expression of a pro-apoptotic molecule, FasL, and to suppress an inhibitor of Fas signaling, FLIP, likely accounts for the role of this cytokine in potentiating T cell apoptosis.

Introduction

Apoptotic death of lymphocytes is an important homeostatic mechanism in the immune system and is involved in the maintenance of peripheral tolerance to self antigens. One form of apoptosis is induced by repeated stimulation of lymphocytes and is called activation-induced cell death (AICD). In $CD4^+$ T cells, AICD results from coexpression of the death receptor, Fas (CD95), and its ligand, FasL (CD95L) (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). Mutations in Fas or FasL result in systemic lupus-like autoimmune disease in mice (Nagata and Suda, 1995) and humans (Fisher et al., 1995; Rieux-Laucat et al., 1995), illustrating the critical role of this death pathway in the maintenance of self-tolerance.

A striking feature of Fas-mediated apoptosis is that it is tightly regulated, since it is dependent on prior activation. Furthermore, Fas-mediated AICD is potentiated by IL-2 (Lenardo, 1991), which is a surprising finding, given that IL-2 is traditionally thought to be a survival and growth-promoting cytokine. In fact, IL-2 may actually be required for triggering this death pathway, because T cells from knockout mice lacking IL-2 (Kneitz et al., 1995) or the α chain of the IL-2 receptor, CD25 (Van Parijs et al., 1997), are resistant to AICD. Knockout mice lacking IL-2 or the α or β chain of the IL-2 receptor develop severe lymphoproliferation and manifestations of autoimmunity (Sadlack et al., 1993; Suzuki et al., 1995; Willerford et al., 1995), illustrating the importance of this cytokine in lymphocyte homeostasis.

The biochemical pathway of Fas signaling had been analyzed extensively in a variety of cell lines and transfectants, providing a framework for investigation of the regulation of this pathway by IL-2. In the simplest scheme, Fas signaling is initiated by oligomerization of the receptor by FasL (Nagata, 1997). The cytoplasmic domain of cross-linked Fas then binds the adaptor molecule FADD/MORT-1 via a protein–protein interaction domain (Boldin et al., 1995; Chinnaiyan et al., 1995). This is followed by the binding of another protein, FLICE/MACH-1 (caspase-8), which also has a protein interaction domain, and a separate caspase domain (Boldin et al., 1996; Muzio et al., 1996). The caspase is activated, leading to a cascade of catalytic activation of caspases that culminates in apoptosis.

The process can be inhibited by a FLICE-like inhibitor protein, FLIP, which was discovered as a viral product (Golstev et al., 1997; Hu et al., 1997; Shu et al., 1997; Srinivasula et al., 1997; Thome et al., 1997) and later shown to exist in mammalian cells (Irmler et al., 1997). The resistance of naive T cells to AICD has been attributed to high levels of intracellular FLIP, which competitively inhibits the binding of FLICE and shuts off Fas signaling (Irmler et al., 1997). However, in some cell lines, FLIP has been shown to induce apoptosis (Shu et al., 1997), suggesting that its actions may be complex. Although some studies suggest that anti-apoptotic members of the Bcl family can also block Fas-mediated apoptosis (Boise and Thompson, 1997), recent biochemical and functional analyses indicate that Bcl-regulated apoptosis is a pathway different from Fas-mediated death, and the anti-apoptotic actions of Bcl-2 and Bcl-X_L are probably proximal to the activation of caspases (Chinnaiyan et al., 1997; Susin et al., 1997; Vander Heiden et al., 1997).

In view of this likely sequence of events in Fas-mediated AICD, one can hypothesize that IL-2 potentiates AICD either by up-regulating levels of the pro-apoptotic proteins of the Fas signaling complex or by suppressing expression of the inhibitors. To address this question, it was necessary to establish the biochemical features of Fas signaling in normal, IL-2–responsive T cells, since much of the earlier analysis was done with tumor lines that are not responsive to physiologic stimuli such as IL-2. The model system we chose uses mice expressing a transgenic T cell receptor (TCR), called 3A9, specific for the hen egg lysozyme (HEL) peptide 46-61 plus I-A^k,

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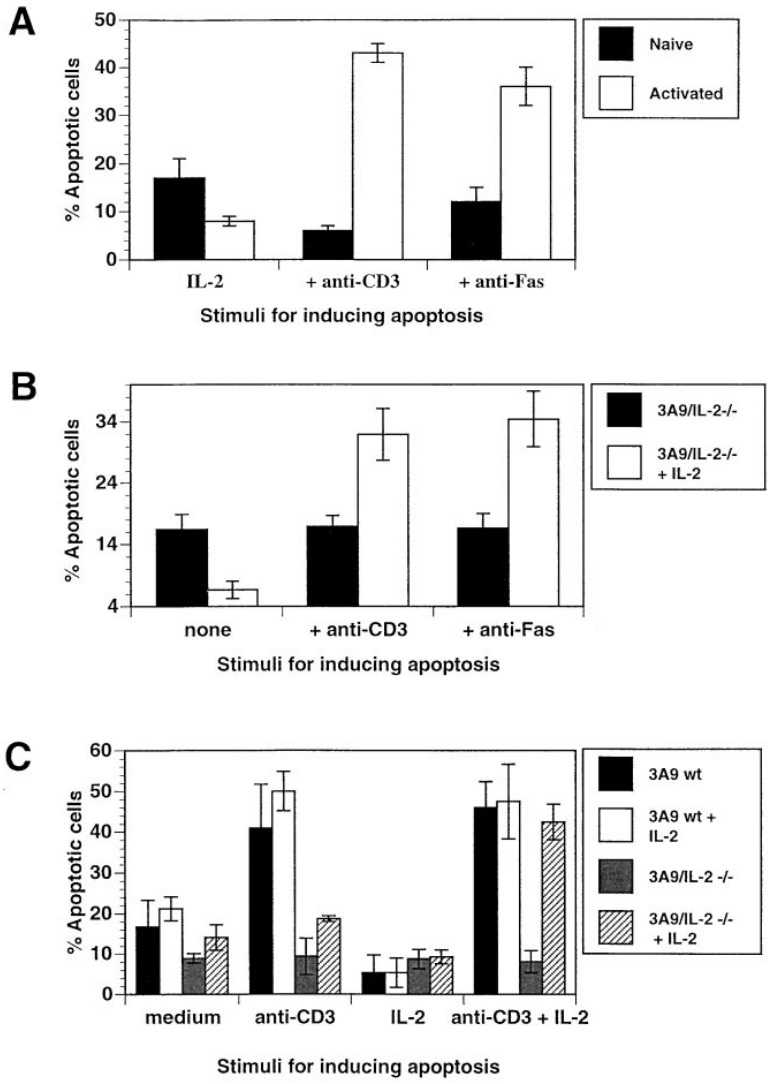

Figure 1. T Cell Activation and IL-2 Are Required for AICD in $CD4^+$ T Cells

The cell populations compared were (A) freshly harvested (naive) 3A9 $CD4^+$ T cells and cells activated for 3 days with HEL(46-61) peptide plus APCs, with IL-2 (50 U/ml); (B) 3A9/IL-$2^{-/-}$ T cells activated for 3 days with antigen plus APCs, with or without IL-2; and (C) wild-type and IL-$2^{-/-}$ 3A9 T cells activated and restimulated, with or without IL-2. To induce AICD, cells were cultured in wells coated with anti-CD3, or incubated with anti-Fas and cultured in wells coated with an anti-hamster IgG antibody (A and B only), in the presence or absence of 50 U/ml IL-2. After approximately 20 hr, cell death was determined by propidium iodide staining. Data shown are from one representative experiment of three (A and B) or pooled from three experiments (C).

bred into an IL-$2^{-/-}$ background. The advantage of this system is that normal function can be restored simply by adding IL-2 to the T cells. In this article we show that IL-2 is a transcriptional activator of FasL and a transcriptional inhibitor of FLIP. This finding demonstrates that a cytokine can exert simultaneous and opposite effects on the expression of pro-apoptotic and anti-apoptotic proteins and is the likely molecular explanation for the critical role of IL-2 in potentiating Fas-mediated AICD.

Results

Activated IL-$2^{-/-}$ $CD4^+$ T Cells Are Resistant to Fas-Mediated AICD

The sensitivity of T cells to Fas-mediated AICD is tightly regulated, since it is dependent on prior activation (Irmler et al., 1997; Peter et al., 1997) and on IL-2–induced signals (Lenardo, 1991; Kneitz et al., 1995; Van Parijs et al., 1997). To establish the conditions that make T cells sensitive to AICD, we isolated naive 3A9 TCR transgenic T cells and activated wild-type 3A9 T cells and 3A9/IL-$2^{-/-}$ T cells with the cognate peptide HEL(46-61) and syngeneic splenocytes as antigen-presenting cells (APCs), with or without added IL-2. Naive and activated T cells were then exposed to anti-CD3 or anti-Fas antibody with or without IL-2, and apoptosis was assayed approximately 20 hr later.

Recently activated, but not naive, T cells undergo apoptosis upon exposure to anti-CD3 or upon direct cross-linking of Fas (Figure 1A). Similarly, IL-$2^{-/-}$ T cells show markedly increased sensitivity to apoptosis if they are first activated in the presence of IL-2 (Figure 1B). More detailed analysis of the role of IL-2 showed that activated wild-type 3A9 T cells die upon restimulation with anti-CD3, with or without additional IL-2, presumably because the cells themselves produce IL-2. In striking contrast, the 3A9/IL-$2^{-/-}$ T cells undergo AICD only if IL-2 is added both during the activation (priming) stage and the effector stage, at which AICD is induced by repeated engagement of the TCR (Figure 1C). Kinetic analysis showed that approximately 3 days of activation

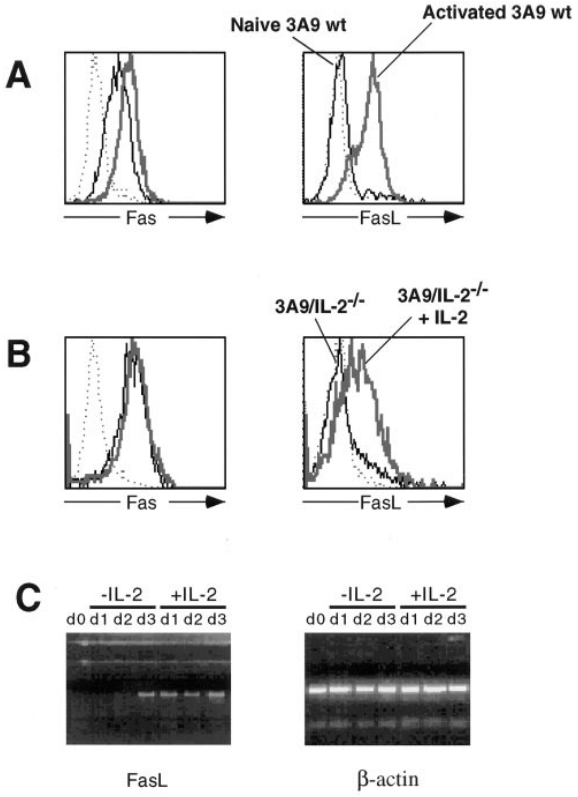

Figure 2. Effect of Cell Activation and IL-2 on Expression of Fas and FasL

(A and B) Naive and in vitro activated 3A9 $CD4^+$ T cells (A) and 3A9/IL-$2^{-/-}$ T cells activated with or without IL-2 (B) were restimulated in wells coated with anti-CD3 for 8 hr, stained with antibodies against Fas and FasL or a control antibody (dotted line in all histograms) and analyzed by flow cytometry. The expression of these proteins on gated $CD4^+$ cells is shown.

(C) $CD4^+$ cells from 3A9/IL-$2^{-/-}$ mice were activated with antibodies to CD3 and CD28, with or without 50 U/ml IL-2. Cells were harvested at indicated times, and RNA was prepared from all samples, reverse transcribed, amplified using primers specific for FasL and β-actin, and electrophoresed in 2% agarose gels.

in the presence of endogenously produced or exogenously added IL-2 is required to make cells sensitive to AICD (data not shown). Moreover, sensitivity to Fas-mediated AICD is also acquired by activating T cells with anti-CD3 plus anti-CD28 antibodies in the presence of IL-2 (Van Parijs et al., 1996, and data not shown). This activation protocol is particularly useful when T cells need to be analyzed without the potentially complicating influence of APCs. Phenotypic analysis showed that both wild-type and IL-$2^{-/-}$ 3A9 T cells down-regulate L-selectin and up-regulate CD69 upon activation, indicating that they respond comparably to antigen (data not shown).

These results show that in the absence of IL-2, $CD4^+$ T cells are activated by antigen but do not acquire sensitivity to Fas-mediated AICD, and normal sensitivity can be restored by culturing the cells in the presence of IL-2. Therefore, this experimental model provides a system for analyzing the mechanisms of IL-2 regulation of the Fas-mediated death pathway.

Effect of Cell Activation and IL-2 on the Expression of Fas and FasL

T cell activation and IL-2 may induce sensitivity to AICD by enhancing expression of the death receptor, Fas, or its ligand, FasL. To test this possibility, naive T cells and cells activated with or without IL-2 were stained with antibodies to Fas and FasL and analyzed by flow cytometry. Naive wild-type T cells express modest levels of Fas and undetectable FasL in response to 20 hr of stimulation with anti-CD3. Prior activation with antigen and APCs has a small effect on Fas, but markedly increases FasL expression (Figure 2A). In IL-$2^{-/-}$ T cells, activation with IL-2 has little effect on Fas but increases FasL expression (Figure 2B). Therefore, one effect of T cell activation and IL-2 is to increase FasL expression upon restimulation.

To determine whether this IL-2–stimulated increase of FasL expression is due to enhanced transcription, 3A9/IL-$2^{-/-}$ T cells were activated with a combination of anti-CD3 and anti-CD28 antibodies with or without IL-2 for 3 days. Aliquots of cells were harvested daily, and RNA was extracted and assayed for FasL transcripts by reverse transcriptase polymerase chain reaction (RT-PCR). As shown in Figure 2C, in the absence of IL-2, FasL RNA is detected within 3 days of activation, whereas in the presence of IL-2, FasL transcripts are present within 1 day of stimulation. Naive cells (day 0) do not contain detectable FasL mRNA. Thus, IL-2 is not absolutely required for FasL expression, but it markedly enhances the rate and levels of expression.

Although a deficiency of FasL may account in part for the AICD-resistant phenoype of IL-$2^{-/-}$ T cells, this cannot be the sole explanation, because IL-$2^{-/-}$ T cells do not die upon direct cross-linking of Fas, which bypasses FasL (Figure 2B). We therefore postulated that the action of IL-2 must be distal to Fas engagement and involves either formation of the Fas-associated signaling complex or the expression of inhibitors of the Fas pathway.

IL-2 Is Not Required for Association of FADD with Cross-Linked Fas

To establish that we could specifically detect FADD in association with cross-linked Fas by immunoprecipitation and Western blotting, we activated wild-type and gld T cells with anti-CD3, immunoprecipitated Fas, and probed blots with a rabbit anti-FADD antibody. Fas-associated FADD was detected in wild-type but not in gld T cells (data not shown), presumably because the mutant FasL cannot transduce apoptotic signals through Fas. We then examined this association in 3A9/IL-$2^{-/-}$ T cells activated in the presence and absence of IL-2. As shown in Figure 3, FADD associates with Fas upon cross-linking, either by anti-CD3 indirectly (via induced FasL), or directly by cross-linking with anti-Fas, in T cells that are activated with or without IL-2. The amount of Fas-associated FADD is apparently increased by IL-2. We do not know whether this is because IL-2 enhances the synthesis of FADD or promotes the formation of the death-inducing signaling complex. However, IL-2 is not required for association of the proximal components of the complex. We have not yet examined association

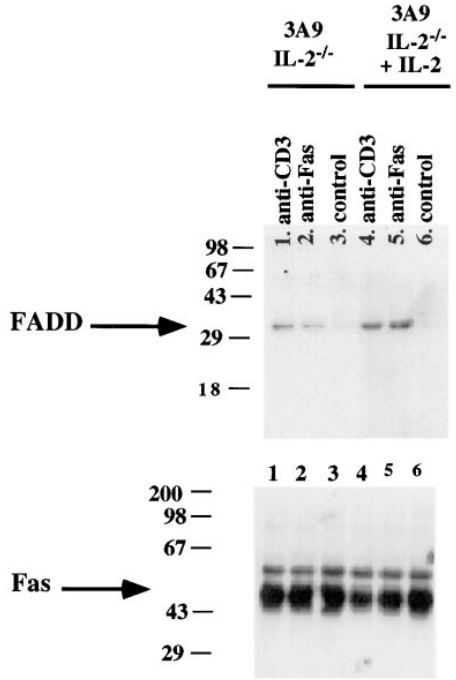

Figure 3. The Association of FADD with Fas Does Not Require IL-2

3A9/IL-2$^{-/-}$ CD4^{+} T cells were activated with HEL(46-61) peptide plus APCs, alone or with 50 U/ml of IL-2, for 3 days. Activated cells were incubated on anti-CD3–coated plates for 6 hr at 37°C or with anti-Fas antibody (Jo2) or control hamster IgG, followed by a mouse anti-hamster IgG antibody for 30 min; washed; and incubated at 37°C for 10 min. Cells were lysed in 0.1% Brij56 and lysates immunoprecipitated with an anti-Fas antibody. A 12% SDS-PAGE gel was run and blotted for Fas, and a 15% SDS-PAGE gel was run and blotted for FADD.

of the third component of the death complex, FLICE, because a reliable antibody specific for murine FLICE is not available.

We also asked whether IL-2 affects transcription of various components of the Fas signaling complex. RNase protection assays showed that the levels of FADD and FLICE are moderately increased by day 3 of activation and that this increase is enhanced by IL-2 (Figure 4). In addition, the levels of FasL transcript observed in these assays reflect those detected by RT-PCR (Figure 2C).

T Cell Activation in the Presence of IL-2 Suppresses Transcription and Synthesis of FLIP

Since the proximal steps in the formation of the death complex are not dependent on IL-2, we postulated that IL-2 may function by removing inhibitors of the Fas pathway. The first such inhibitor we examined is FLIP, because its critical role in regulating sensitivity to AICD has been demonstrated in numerous recent studies (Hu et al., 1997; Irmler et al., 1997; Thome et al., 1997). 3A9/IL-2$^{-/-}$ T cells were activated in the presence or absence of IL-2 for 3 days, and aliquots were cultured with IL-2 for an additional 48 hr. RNA was extracted at different times during the cultures and assayed for FLIP mRNA by RT-PCR. As shown in Figure 5A, in the absence of IL-2, high levels of FLIP mRNA are present throughout the 3 day activation. Addition of IL-2 during the initial activation, or in a second culture after activation with

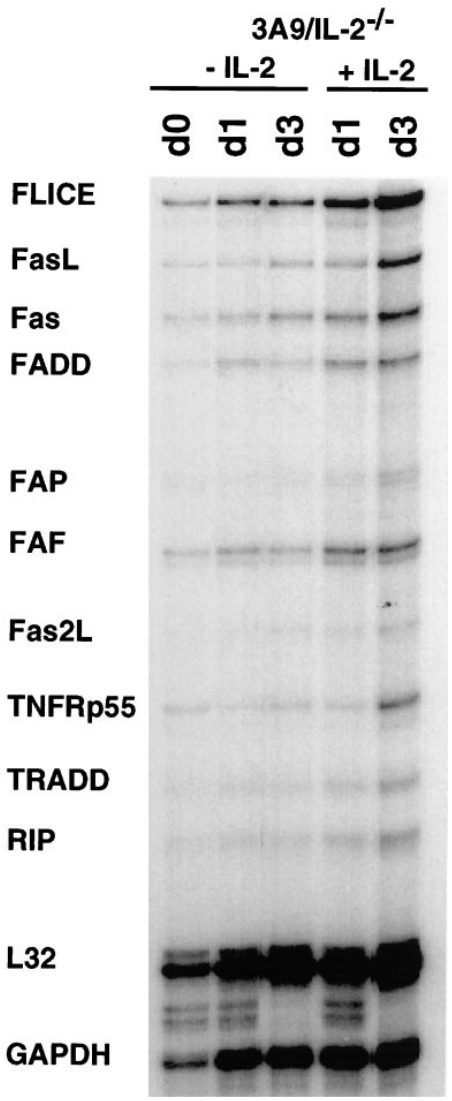

Figure 4. T Cell Activation and IL-2 Increase Transcription of FADD and FLICE

CD4^{+} cells from 3A9/IL-2$^{-/-}$ mice were activated with antibodies to CD3 and CD28, with or without 50 U/ml IL-2. Cells were collected on days 1 and 3. RNA was extracted and used for RNAse protection using the mAPO-3 probe kit (PharMingen) and developed by autoradiography for 6 hr. By densitometry and scanning comparison with control probes (GAPDH and L32), the levels of FLICE RNA increased about 2-fold over that in the starting population in cells activated for 3 days with IL-2; levels of FasL RNA increased by about 4-fold; and levels of Fas and FADD RNAs increased less than 1.5-fold. d, day.

antigen and APCs, leads to the complete disappearance of FLIP mRNA. This suggests that IL-2 is a transcriptional inhibitor of FLIP. Western blot analysis of whole-cell lysates, using an anti-FLIP antibody, showed that intracellular FLIP protein also persists in T cells activated without IL-2 but is lost by 3 days of activation with IL-2 (Figure 5B).

Constitutive Expression of FLIP Inhibits AICD in T Cells

These experiments strongly suggest that the resistance of IL-2$^{-/-}$ T cells to AICD is due to the presence of FLIP

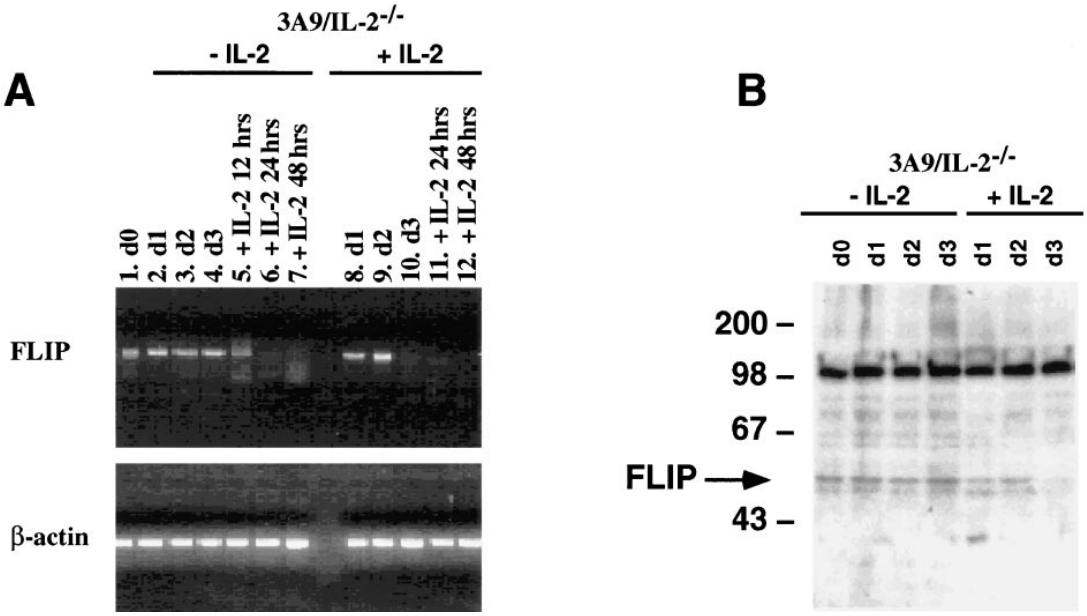

Figure 5. FLIP Transcription and Synthesis Are Suppressed by IL-2

$CD4^+$ cells from 3A9/IL-$2^{-/-}$ mice were activated with antibodies to CD3 and CD28, with or without 50 U/ml IL-2.

(A) RNA was assayed by RT-PCR using primers specific for for FLIP and β-actin. Lanes 5–7 and lanes 11 and 12 contain RT-PCR products obtained from cells that were activated for 3 days without or with IL-2 and then cultured with 50 U/ml IL-2 for the indicated times. d, day.

(B) Whole-cell lysates from 3A9/IL-$2^{-/-}$ T cells activated with anti-CD3 plus anti-CD28 with or without IL-2 were electrophoresed and Western blots probed with anti-FLIP followed by anti-rabbit IgG-HRP. The top band is a nonspecific band that serves as an internal control for protein loading.

in these cells. However, the issue is complicated by findings in some cell types that FLIP may be an inducer of apoptosis (Shu et al., 1997). Therefore, we wanted to establish whether FLIP functions as an inhibitor or inducer of AICD in normal, activated T cells. Murine and human T cell lines stably transfected with FLIP are resistant to Fas-mediated AICD (L. V. P. and J. T., unpublished data), suggesting that FLIP functions predominantly as an inhibitor of this pathway of cell death in T cell lines. To determine the effects of FLIP in an IL-2–regulated pathway of AICD, we used a retroviral gene transfer system to express FLIP cDNA in activated wild-type 3A9 T cells. The retrovirus construct used expresses both FLIP and human placental alkaline phosphatase (PLAP) from a dicistronic transcript (see Experimental Procedures), so that PLAP can be used as a surface marker to identify infected T cells and as an indicator of the levels of retrovirus-derived FLIP present in individual T cells. Following infection with the FLIP-expressing retrovirus, MSCV-FLIP-IRES-PLAP, activated 3A9 T cells that express high levels of PLAP are resistant to AICD (39.3% ± 8.2% apoptotic cells in two representative experiments; Figure 6) compared to cells that express low levels of PLAP (79.8% ± 5.6% apoptotic cells). T cells that have been infected with a control virus, MSCV-IRES-PLAP, that does not contain FLIP remain sensitive to AICD, independent of PLAP expression ($PLAP^{hi}$ cells show 60.2% ± 3.9% apoptosis and $PLAP^{lo}$ cells show 76.5% ± 4.7% apoptosis in two representative experiments; Figure 6). These data support the hypothesis that in primary T cells, FLIP functions as an inhibitor of IL-2–regulated AICD.

Discussion

The principal goal of this study was to analyze the mechanisms by which IL-2 potentiates Fas-mediated apoptosis in T cells. Using T cells from TCR transgenic mice lacking IL-2, we show that this cytokine has two important actions that regulate sensitivity to Fas-mediated AICD. First, IL-2 enhances the transcription and surface expression of FasL (Figure 2). The induction of FasL is known to require TCR engagement (Combadiere et al., 1996), indicating that TCR signals and IL-2 generated signals cooperate to induce maximal levels of FasL. As a result, T cell activation in the presence of IL-2 is required for maximal cross-linking of Fas by endogenously synthesized FasL. However, this cannot be the only action of IL-2, because in the absence of IL-2 T cells do not become sensitive to apoptosis induced by directly cross-linking Fas, without a requirement for endogenous FasL (Figure 1). IL-2 is not essential for the recruitment of the adaptor molecule, FADD, to oligomerized Fas (Figure 3), indicating that it does not play an obligatory role in the formation of the Fas signaling complex. The second important action of IL-2 is to suppress the transcription of the inhibitor of Fas-mediated apoptosis, FLIP (Figure 5). Thus, the prediction is that in wild-type T cells, oligomerized Fas associates with FADD and the caspase FLICE, whereas in the absence of IL-2 the complex consists of Fas, FADD, and the inhibitor, FLIP. Formal establishment of this difference will require antibodies specific for murine FLICE and FLIP, and these are currently being generated and tested.

The mechanisms by which IL-2 enhances FasL transcription and inhibits FLIP transcription are not known.

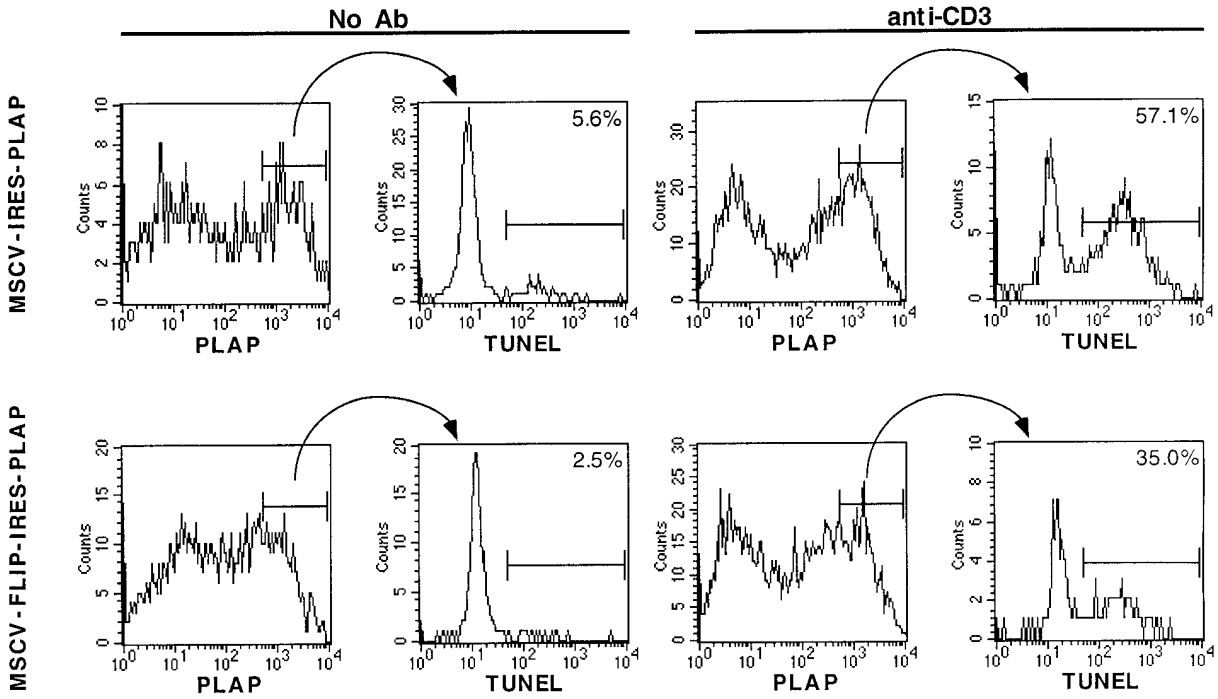

Figure 6. Retrovirus-Mediated Expression of FLIP Protects Activated T Cells from AICD

$CD4^+$ cells from wild-type 3A9 mice were activated for 3 days with antibodies to CD3 and CD28 with 50 U/ml IL-2 and infected with a FLIP-expressing retrovirus (MSCV-FLIP-IRES-PLAP) or a control virus (MSCV-IRES-PLAP). To induce AICD, the activated T cells were cultured in wells coated with anti-CD3 in the presence of 50 U/ml IL-2. After approximately 20 hr, cell death was determined by staining with an antibody against PLAP (to distinguish infected and non-infected cells) and TUNEL. Histograms show the expression of PLAP on all cells, the gates used to identify infected cells, and the TUNEL staining profile of $PLAP^{hi}$ (infected) cells. Numbers in histograms are the percentage of cells that are TUNEL-positive (apoptotic). Results shown are from one representative experiment of two, each done in duplicate. Mock transfections yielded no $PLAP^+$ cells (data not shown).

The FasL promoter (Latinis et al., 1997) contains a consensus binding site for the IL-2–induced signal transducer and activator of transcription, STAT5 (Lin et al., 1995), but we do not know whether this is involved in FasL transcriptional activation. In fact, it has recently been shown that STAT5a knockout mice do not have a defect in superantigen-induced T cell deletion (Nakajima et al., 1997), which is largely due to Fas-mediated AICD. The ability of IL-2 to shut off FLIP transcription is perhaps even more intriguing and is difficult to explain based on current understanding of cytokine-induced transcription factors.

Our results and studies from other laboratories (Irmler et al., 1997; Peter et al., 1997) have clearly shown that T cell activation is required for triggering Fas-mediated AICD. Naive T cells express low levels of FasL and high levels of FLIP (Figures 2 and 5), which is strikingly similar to the situation in $IL\text{-}2^{-/-}$ T cells. This suggests that during T cell activation, the critical factor that induces sensitivity to AICD is IL-2 itself.

The finding that IL-2 promotes T cell death seems paradoxical, considering that its first and most clearly established function is as a growth and survival factor. The unregulated lymphoid accumulation seen in mice lacking IL-2 suggests that the major obligatory function of this cytokine may be in T cell homeostasis. In fact, we and others have suggested that the growth-promoting effects of IL-2 can be replaced by physiological levels of other cytokines but that the pro-apoptotic effects cannot (Fournel et al., 1996; Van Parijs et al., 1997). This would explain the accumulation of activated and cycling cells in mice deficient in IL-2 or IL-2 signaling. Also consistent with this hypothesis is our finding that IL-2 inhibits transcription of FLIP, but IL-4 does not (data not shown). High concentrations of cytokines other than IL-2 may promote AICD under some conditions as long as they are able to drive cells into cycle (Zheng et al., 1998). In any event, it is now clear that IL-2 is capable of inducing lymphocyte death as well as growth. It may be that the growth-promoting activity of this cytokine is dominant early during an immune response, and if T cell stimulation is persistent or IL-2 concentrations increase above a threshold, the potentiation of AICD becomes dominant, leading to feedback regulation of the immune response. Defining the dual functions of this cytokine will require better understanding of the biochemical signals used by IL-2 to promote T cell proliferation versus apoptosis.

Experimental Procedures

Mice

$IL\text{-}2^{-/-}$ mice (Sadlack et al., 1993) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice expressing the transgenic 3A9 TCR, specific for HEL(46-61) in association with $I\text{-}A^k$, were obtained from Mark Davis (Stanford University Medical School, Stanford, CA). These mice were backcrossed with $IL\text{-}2^{-/-}$ mice and interbred to

produce TCR transgenic wild-type (3A9$^+$, IL-2$^{+/+}$ or IL-2$^{+/-}$) and knockout (3A9$^+$, IL-2$^{-/-}$) H-2^k offspring. Progeny were genotyped by PCR using the following primers: 3A9 transgene (5′-GCAGTCACC CAAAGCCCAAG-3′ and 5′-CCCCAGCTCACCTAACACTG-3′) and IL-2 null allele (5′-TCGAATTCGCCAATGACAAGACGCT-3′, 5′-CTAG GCCACAGAATTGAAAGATCT-3′, and 5′-GTAGGTGGAAATTCTAG GATCATCC-3′). All mice used in these experiments were 3–4 weeks old and were maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical School and those of the Institute of Laboratory Animal Resources, National Research Council.

T Cell Purification and In Vitro Activation

Naive CD4$^+$ TCR transgenic T cells were purified as described elsewhere (Van Parijs et al., 1998). In brief, pooled lymph node and spleen cells harvested from 3A9 TCR transgenic animals were incubated with anti-CD4–coated magnetic beads (Dynal, Oslo, Norway) for 45 min at 4°C. The adherent cells were washed twice and incubated for 45 min at 4°C with the Dynal Detach antibody. For in vitro activation, 2×10^5 naive CD4$^+$ T cells were cultured with 2×10^6 mitomycin C (Sigma, St. Louis, MO) treated H-2^k spleen cells in the presence of 1 μg/ml of HEL(46-61) peptide, with or without 50 U/ml IL-2, in a total volume of 1 ml of RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (Sigma); L-glutamine, penicillin, streptomycin, nonessential amino acids, and sodium pyruvate, all at 2 mM; 10 mM HEPES (all from Gibco–BRL Laboratories, Grand Island, NY), and 5×10^{-5} M 2-mercaptoethanol. To activate T cells without APCs, 10^6 CD4$^+$ cells were cultured with 1 μg/ml of soluble anti-CD3 antibody (2C11) and 10 μg/ml of soluble anti-CD28 (37N1), with or without 50 U/ml IL-2.

Assays for Apoptosis

Activated T cells (10^6) were cultured in duplicate wells coated with 1 μg/ml anti-CD3, in the presence or absence of 50 U/ml IL-2. In some experiments, 10^6 T cells were incubated for 30 min at 4°C with 1 μg/ml anti-Fas antibody (Jo2, PharMingen, San Diego, Ca) or hamster immunoglobulin G (IgG); washed; and incubated with 1 μg/ml goat anti-hamster IgG (PharMingen), with or without IL-2. Controls included cells cultured in medium or IL-2 alone. Cells were collected 20–24 hr later, washed in phosphate-buffered saline (PBS) plus 0.1% D-glucose; fixed in 70% ethanol for 12 hr at 4°C; and incubated in 50 μg/ml propidium iodide for 30 min at room temperature. Apoptosis was determined by flow cytometry using a Becton Dickinson FACScalibur, gating on the subdiploid population.

Phenotypic Analysis

The surface expression of Fas was assayed by staining cells with fluorescein isothiocyanate (FITC)–conjugated anti-Fas and phycoerythrin (PE)–conjugated anti-CD4 (PharMingen) on ice for 30 min. Cells were washed and fixed in 1% paraformaldehyde. Staining for FasL was done using a biotinylated rat anti-murine FasL (A11; Hahne et al., 1995), followed by fluorescein isothiocyanate–conjugated streptavidin and PE-conjugated anti-CD4. All samples were analyzed by flow cytometry.

RT-PCR Assays

Cells that were activated as indicated were frozen at −80°C as pellets. All RNA extractions were done simultaneously for all samples in a given experiment. RNA was extracted from about 10^6 cells from each group, using Trizol reagent (Gibco–BRL) according to the manufacturer's instructions. The resulting RNA was reverse transcribed by adding one third of the total RNA in a volume of 7.5 μl to 1.5 μl of dNTPs (10 nM), 0.5 μl of RNAsin (Promega, Madison, WI), 0.5 μl DTT (100 mM), 0.5 μl oligo d(T), 3 μl of 5× reaction buffer, and 1.5 μl of murine Moloney leukemia virus reverse transcriptase (MMLV-RT, Gibco–BRL). Samples were incubated at 42°C for 2 hr, phenol-chloroform extracted twice, and precipitated in ethanol. The cDNA pellet was resuspended in 40 μl of diethyl pyrocarbonate–treated water. The reaction mixture consisted of 4 μl of cDNA template, 4 μl of 50 mM $MgCl_2$, 2 μl of 10 nM dNTPs, 2 μl of each primer (10 ng/ml), 32.5 μl of distilled-deionized water, 0.5 μl of Taq-DNA polymerase (5 U/μl), and 5 μl of 10× PCR buffer (Gibco–BRL). The PCR protocol consisted of 1 min at 94°C, 1 min at 59°C, 1.5 min at 72°C, and 40 cycles of this sequence. All reactions were performed in an MJ-Research thermal cyler. The oligonucleotides used to amplify murine FasL were 5′-CAGCTCTTCCACCTGCAGAAGG-3′, and 5′-AGATTCCTCAAAATTGATCAGAGAGAG-3′; for murine β-actin, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGC TCAGTAACAGTCCG-3′; and for murine FLIP, 5′-GTCACATGACATA ACCCAGATTGT-3′ and 5′-GTACAGACTGCTCTCCCAAGCACT-3′. PCR products were run on 2% agarose–Tris-borate-EDTA gels and stained with ethidium bromide (Sigma).

Immunoprecipitations and Western Blots

The immunoprecipitations for Fas were done by lysing 10^7 activated T cells in 1 ml of Brij56 lysis buffer (100 mM NaCl, 20 mM Tris [pH 7.4], 0.1% Brij56 [Pierce, Rockford IL]), freshly supplemented with a small protease inhibitor cocktail containing 2 μg/ml of aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 100 μg/ml phenylmethylsulfonyl fluoride (all Boehringer Mannheim, Indianapolis, IN), 10 μM sodium orthovanadate, 10 μM sodium fluoride, and 10 μM sodium pyrophosphate (all Sigma). Cells were lysed on ice for 20 min and centrifuged at 12,000 rpm for 10 min at 4°C. Clear supernatants were transfered to a fresh 1.5 ml Eppendorf tube and incubated with a hamster anti-mouse Fas antibody (Jo2, PharMingen), on a rocker at 4°C for 1 hr. Recombinant protein G–agarose beads (Gibco–BRL), which had been recently equilibrated in fresh Brij56 lysis buffer, supplemented with protease inhibitors, were added to the tubes (40 μl of bead suspension per tube) and incubated for 1 hr at 4°C on a rocker. At the end of this incubation period, samples were washed five times for 5–10 min each in Brij56 lysis buffer, supplemented with protease inhibitors, and boiled for 5 min in 50 μl of 2× sodium dodecyl sulfate (SDS) gel loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol).

Twenty microliters of sample was added to each lane on the SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gels. Sister gels were run for the Western blots: 12% SDS-PAGE for Fas and 15% SDS-PAGE for FADD. For FLIP Western blots, whole-cell lysates were electrophoresed in 12% SDS-PAGE gels. The Western blot procedures used here consist of transferring the protein to polyvinylidene difluoride membranes (New England Nuclear, Boston, MA) as previously described (Sambrook, 1989). Membranes were blocked overnight in 5% nonfat dry milk dissolved in 1× Tris-buffered saline plus 0.05% Tween-20 (Sigma) at 4°C. Blots were then rinsed in 1× Tris-buffered saline plus 0.05% Tween-20 three times and incubated with the primary antibodies. For murine Fas, we used 7C10 (a generous gift from B. Stanger [Stanger et al., 1995]), followed by a horseradish peroxidase (HRP)–conjugated goat anti-rat immunoglobulin antibody (ICN Biochemicals, Costa Mesa, CA). For FADD, we used a rabbit polyclonal antibody made to human FADD (a generous gift from V. Dixit), followed by HRP-conjugated goat anti-rabbit immunoglobulin. For FLIP, we used a rabbit polyclonal antibody (L109) made to human FLIP (Irmler et al., 1997), followed by HRP-conjugated goat anti-rabbit immunoglobulin.

Western blots were developed using enhanced chemiluminescence (ECL kit, Amersham, Buckinghamshire, England), following the manufacturer's specifications. Size markers used in SDS-PAGE gels were prestained molecular weight markers (High and Low, Gibco–BRL).

RNAse Protection Assays

RNA was extracted from 10^7 3A9/IL-2$^{-/-}$ T cells activated with anti-CD3 plus anti-CD28 with or without IL-2, using Trizol reagent (Gibco–BRL). The RNAse protection was performed using 5 μg of RNA with the RiboQuant multi-probe RNAse protection assay system (PharMingen), following the manufacturer's specifications. In brief, RNA was hybridized overnight with the in vitro–translated ^{32}P-labeled probe (mAPO-3 kit, PharMingen). Following hybridization, samples were treated with RNase A and proteinase K, phenol/chloroform extracted, and ethanol precipitated. The protected fragments were resolved by electrophoresis on a 5% acrylamide/urea gel, and autoradiograms were exposed for 48 hr.

Retroviral Constructs and T Cell Infection

FLIP cDNA (Irmler et al., 1997) was cloned into MSCV-IRES-PLAP (from J. Jacob, Massachusetts Institute of Technology, Cambridge,

MA) upstream of the internal ribosome entry site (IRES). High-titer retrovirus was generated by transient tranfection of the BOSC 23 packaging cell line (Pear et al., 1993). 3A9/+ T cells were activated with 1 μg/ml of anti-CD3 and anti-CD28. After 24 hr of activation, T cells were spun at 2500 rpm for 1.5 hr at 30°C in the presence of supernatant derived from transfected BOSC 23 cells supplemented with 4 μg/ml polybrene (Sigma). This procedure was repeated 24 hr later. After 72 hr of activation, T cells were collected and cultured in duplicate wells coated with 1 μg/ml anti-CD3 in the presence or absence of 50 U/ml IL-2. The induction of apoptosis in infected and noninfected T cells was assayed 20 hr later by surface staining with an antibody to human PLAP (Zymed) and TdT-mediated dUTP-biotin nick-end labeling (TUNEL) (Boehringer Mannheim), following the manufacturers' specifications. In brief, treated cells, which had been surface stained for PLAP, were washed twice in PBS supplemented with 1% bovine serum albumin (Sigma) and permeabilized in 0.1% Triton X-100 (Boehringer Mannheim) in 0.1% sodium citrate (Sigma). TUNEL staining was performed using 50 μl of TUNEL mix per sample, at 37°C for 1 hr. Cells were then washed in PBS and analyzed by flow cytometry on a FACScalibur apparatus (Becton Dickinson), gating on populations expressing high and low levels of PLAP.

Acknowledgments

The authors thank Joshy Jacob for his generous gift of the MSCV-IRES-PLAP construct. This work was supported by National Institutes of Health grants AI32531 and AI35297 (A. K. A.) and HL07627 and CA51462 (L. V. P.) and a predoctoral fellowship from the Howard Hughes Medical Institute (Y. R.).

Received December 15, 1997; revised March 31, 1998.

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