The caspase-8 inhibitor FLIP promotes activation of NF-κB and Erk signaling pathways

T. Kataoka*, R.C. Budd*†, N. Holler*, M. Thome*, F. Martinon*, M. Irmler*, K. Burns*, M. Hahne*, N. Kennedy†, M. Kovacsovics* and J. Tschopp*

Background: Activation of Fas (CD95) by its ligand (FasL) rapidly induces cell death through recruitment and activation of caspase-8 via the adaptor protein Fas-associated death domain protein (FADD). However, Fas signals do not always result in apoptosis but can also trigger a pathway that leads to proliferation. We investigated the level at which the two conflicting Fas signals diverge and the protein(s) that are implicated in switching the response.

Results: Under conditions in which proliferation of CD3-activated human T lymphocytes is increased by recombinant FasL, there was activation of the transcription factors NF-κB and AP-1 and recruitment of the caspase-8 inhibitor and FADD-interacting protein FLIP (FLICE-like inhibitory protein). Fas-recruited FLIP interacts with TNF-receptor associated factors 1 and 2, as well as with the kinases RIP and Raf-1, resulting in the activation of the NF-κB and extracellular signal regulated kinase (Erk) signaling pathways. In T cells these two signal pathways are critical for interleukin-2 production. Increased expression of FLIP in T cells resulted in increased production of interleukin-2.

Conclusions: We provide evidence that FLIP is not simply an inhibitor of death-receptor-induced apoptosis but that it also mediates the activation of NF-κB and Erk by virtue of its capacity to recruit adaptor proteins involved in these signaling pathways.

Addresses: *Institute of Biochemistry, University of Lausanne, BIL Biomedical Research Center, Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland. †Immunobiology Program, The University of Vermont College of Medicine, Given Medical Building, D-305 Burlington, Vermont 05405-0068, USA.

Correspondence: J. Tschopp
E-mail: jurg.tschopp@ib.unil.ch

Received: **26 October 1999**
Revised: **26 January 2000**
Accepted: **7 April 2000**

Published: **16 May 2000**

Current Biology 2000, 10:640–648

0960-9822/00/$ – see front matter

Background

Members of the family of receptors for tumour necrosis factor (TNF) and their ligands are critical regulators of apoptosis and various other cellular processes. The receptors have characteristic cysteine-rich motifs in the extracellular domain. Their ligands are a family of type II membrane glycoproteins with homology to TNF, but they can also exist as soluble trimers after processing by metalloproteases [1]. Much has been learned about signal transduction by Fas (CD95) and by TNF receptor 1 (TNF-R1) [2]. These receptors, and four other members of the family, TRAIL-R1, TRAIL-R2, TRAMP/DR3 and DR6, contain a cytoplasmic region, called the death domain, that is essential for cell death signaling [3,4]. When the receptor is activated, the death domain undergoes homotypic interaction with a death domain in the adaptor proteins Fas-associated death domain protein (FADD) or TNF-receptor-associated death domain protein (TRADD). FADD binds directly to Fas and indirectly to TNF-R1 via TRADD but is essential for cell death signaling from both receptors [2]. The death effector domain at the amino terminus of FADD recruits pro-caspase-8 via homotypic interaction with its two death effector domains. A high local concentration of caspase-8 zymogens is thought to facilitate self-processing and assembly of the mature enzyme. Activated caspase-8 then initiates apoptosis by cleavage of downstream caspases 3, 6 and 7.

Some γ-herpesviruses and molluscipoxviruses encode a molecule that has two death effector domains, FLIP; this protein controls sensitivity towards FasL-mediated apoptosis [5,6]. Viral FLIP is recruited via FADD to the Fas signaling complex and prevents caspase-8 recruitment, thereby inhibiting apoptosis. Two cellular homologues have been characterized [7,8]. FLIP(L), the full-length 55 kDa form of FLIP, shows overall structural homology to caspase-8, containing two death effector domains that interact with FADD, and an inactive caspase-like domain. FLIP(S), an alternatively spliced short form of FLIP, contains only the two death effector domains and has lower anti-apoptotic capacity.

Because most of the death-domain-containing members of the TNF receptor family use the adaptor protein FADD to couple to caspases [2], the finding that T cells without functional FADD do not proliferate in the presence of mitogens or on stimulation of the T-cell receptor (TCR)–CD3 complex was unexpected and suggested that TCR-induced proliferation of T cells requires activation of death receptors [9–12]. One of several candidates is Fas, as TCR stimulation leads to upregulation of FasL [13], and antibodies to Fas mediate costimulatory signals for T-cell proliferation [14]. FasL-induced signals also have a dual role in B cells [15]. FasL is not only essential for triggering

B-cell deletion *in vivo*, but is also required for the promotion of B-cell clonal proliferation. This switch is modulated by signals from the B-cell receptor [15]. We undertook a series of experiments to investigate the mechanisms that allow the switch from a FasL-induced death pathway to a pathway that ultimately results in proliferation and/or differentiation.

Results

Recombinant FasL costimulates T-cell growth

Cross-linked soluble FasL added to suboptimal doses of anti-CD3 antibody promoted a dose-dependent increase in T-cell growth by as much as fourfold (Figure 1a), confirming previous results [14] and indicating that the reported costimulation of T cells by anti-Fas antibody was due to the direct activation of proliferative signals and not merely to blocking of FasL-induced cell death [16]. The costimulatory effect was similar in size to that of anti-CD28 antibody (Figure 1a). FasL costimulation was observed only when soluble FasL was oligomerized through cross-linking of its FLAG tag by anti-FLAG antibody to mimic active membrane-bound FasL [17]. No proliferation was observed in the absence of the CD3 costimulatory signal. Cross-talk between the two signaling pathways is therefore required.

We further examined signal pathways that might be involved in Fas costimulation using mice transgenic for the DNA binding sites of the transcription factors AP-1 or NF-κB linked to a luciferase gene reporter [18,19]. As in human T cells, Fas activation by agonistic antibodies (Figure 1b) or FasL (data not shown) also promoted the proliferation of CD3-activated T cells from the transgenic mice and from wild-type mice (data not shown). CD3 activation of T cells from these mice induced AP-1–luciferase and NF-κB–luciferase activities that were much increased in the presence of Fas costimulation (Figure 1c). The costimulation was at least as large as that seen with CD28 costimulation. Thus, in primary T cells that are resistant to Fas-induced death, Fas costimulatory signals enhance signaling pathways, such as NF-κB and AP-1, that are important for T-cell growth.

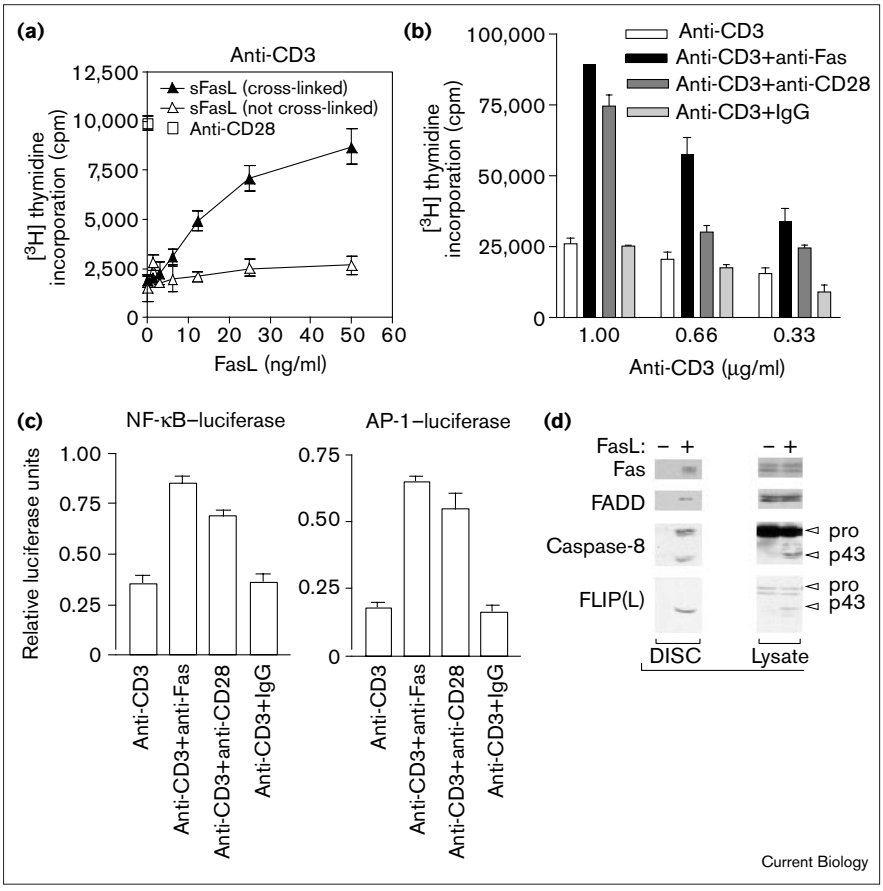
We next examined the point at which FasL-induced NF-κB and AP-1 signals are diverted from the proapoptotic pathway which, after Fas oligomerization, is initiated by FADD recruitment and caspase-8 activation. After 1 h of stimulation of resting human T cells with anti-CD3 antibody and FasL, partial caspase-8 processing into the p43 fragment was observed (Figure 1d). During this period there was no evidence of cell death as detected with propidium iodide (data not shown). The early appearance of caspase-8 cleavage during CD3–FasL-induced T-cell activation in the absence of cell death suggested that a specific endogenous inhibitor of signals downstream of caspase-8 might be pivotal in diverting a potential death signal towards cell growth. The only known endogenous inhibitor of caspase-8 is FLIP. FLIP(L) acts as a substrate trap in that it is cleaved by caspase-8 after Asp 376, generating a 43 kDa fragment [20,21]. FLIP constitutes the first potential substrate of caspase-8, and its recruitment to the Fas–FADD–caspase-8 complex can therefore be followed by monitoring of its proteolytic processing [20,21]. Resting T cells express predominantly FLIP(L) (Figure 1d and data not shown). After T-cell activation via anti-CD3 antibody and FasL, FLIP was processed to the 43 kDa form (Figure 1d); this finding suggests that FLIP is involved in the non-apoptotic Fas signaling pathway. Assembly of the Fas death-inducing signaling complex [22] (DISC) in proliferating T cells was examined using cross-linked FLAG-tagged soluble FasL. FLIP was present in the DISC, in addition to Fas, FADD, and caspase-8 (Figure 1d). Only the cleaved, processed form of FLIP was detectable in the DISC. In a previous study, DISC formation was not observed in short-term activated T cells [21]. In that study, T cells were analyzed with anti-Fas antibody rather than FasL after 1 day of phytohaemagglutinin stimulation (and not directly after TCR/Fas costimulation); this finding suggests a complex cross-talk between the Fas and TCR signaling pathways. Thus, under conditions in which FasL induces not cell death but proliferation, FADD-recruited FLIP probably binds to caspase-8 thereby stopping further progression of the death signal. This idea accords with the previously suggested role of FLIP [7].

FLIP binds components of the NF-κB and Erk signaling pathways

The observation that FasL-treated resting T cells were not only resistant to apoptosis but also activated NF-κB and AP-1 signaling pathways and thus proliferated more vigorously with CD3 costimulation suggested an additional more active role of FLIP in T-cell signaling than simply as an inhibitor of apoptosis. We therefore investigated the possibility that FLIP could augment signals that lead to the activation of these transcription factors. Initially, various forms of FLIP were expressed in 293T human embryonic kidney cells (Figure 2). Cotransfection of reporter plasmids with various FLIP constructs showed that NF-κB could be activated in a dose-dependent manner by overexpressed FLIP(L) as well as weakly by FLIP(S), but not by the carboxy-terminal fragment of FLIP (Figure 2a,b).

NF-κB activation depends on the formation of a multiprotein complex comprising TNF-receptor-associated factors (TRAFs), NIK, IKKα, IKKβ, NEMO, IκB-α, IκB-β and IKAP, resulting in phosphorylation and degradation of IκBα and the release of NF-κB for nuclear translocation [23,24]. Dominant-negative versions of some of these proteins can block NF-κB-activating signals triggered by upstream receptors. Indeed, the dominant-negative forms of IKKβ, TRAF1, and to a lesser extent TRAF2, inhibited FLIP-mediated NF-κB activation (Figure 2c), indicating that the overexpression of FLIP initiated NF-κB activation

Figure 1



CD3-induced T cell proliferation is augmented by FasL and leads to FLIP processing. **(a)** Resting human T cells were stimulated with suboptimal soluble anti-CD3 antibody (3 μg/ml) in the presence of the indicated concentrations of soluble FLAG-tagged FasL (sFasL) with or without cross-linking by anti-FLAG antibody (1 μg/ml). Proliferation was monitored by [^{3}H]thymidine incorporation after 3 days. Error bars show the SD. **(b)** Resting murine T cells were stimulated with suboptimal plastic-immobilized anti-CD3 antibodies at the indicated concentrations in the presence of agonistic antibodies to Fas or CD28 (3 μg/ml). **(c)** CD3/Fas costimulation of primary murine T cells induced AP-1–luciferase and NF-κB–luciferase activities. T cells were purified from mice transgenic for the DNA binding sites of the transcription factors AP-1 or NF-κB linked to a luciferase reporter gene. **(d)** Resting human T cells were stimulated with anti-CD3 antibody (3 μg/ml) plus cross-linked soluble FasL (50 ng/ml). DISC analysis was made after 1 h of culture by adding additional crosslinked FasL before (+) and after (–) lysis. Western blots of the DISC or cell lysates are shown.

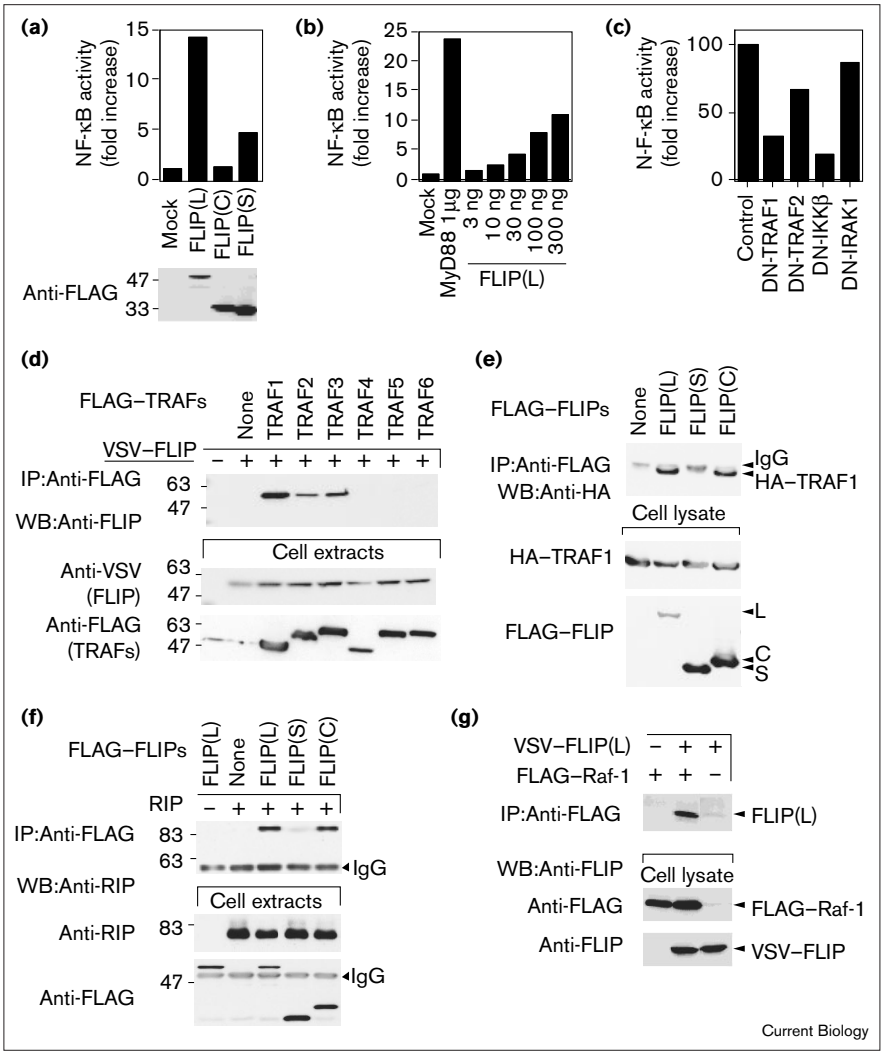
upstream of TRAFs. The dominant-negative form of IRAK1, a kinase implicated in NF-κB signaling induced by interleukin-1 [25], did not inhibit NF-κB activation. We found that FLIP interacted with TRAFs 1, 2 and 3, but not with the other known TRAFs (TRAF4–6) when overexpressed in 293T cells (Figure 2d). TRAF1 bound to the large subunit of the caspase-like region of FLIP (Figure 2e and data not shown). In addition to TRAFs, the presence of the serine/threonine kinase RIP is required for the activation of NF-κB [26]. FLIP was also able to recruit RIP, an interaction predominantly mediated by the caspase-like domain (Figure 2f). Thus, FLIP, by virtue of its capacity to sequester both RIP and TRAFs [27] can recruit proteins that are involved in IκB degradation.

In addition to NF-κB, we observed that in 293T cells, FLIP also spontaneously engaged the signaling pathway leading to the activation of the mitogen-activated protein (MAP) kinase Erk (which is ultimately required for AP-1 activation; data not shown). Erk activation is most frequently a result of the Ras-initiated membrane recruitment of the MAP kinase kinase kinase, Raf-1, which leads to the activation of the MAP kinase kinase, Mek, and then of Erk. In agreement with this notion, we found that in 293T cells, FLIP bound to Raf-1 (Figure 2g), whereas it associated only slightly or not at all with activated Ras, Erk and Mek (data not shown).

FLIP recruits TRAF1, TRAF2, RIP and Raf-1 to the Fas signaling complex

As overexpression of proteins in 293T cells can lead to aberrant protein complex formation that is not found under physiological conditions, we next wanted to validate the observed interactions by studying the assembly of the endogenous proteins. We therefore studied DISC assembly. We analyzed the complex 15 minutes after FasL addition in mock-transfected and FLIP(L)-transfected Jurkat T cells [20] (cells destined to undergo apoptosis and cells that are protected from cell death, respectively). FADD and caspase-8 were readily coprecipitated from FasL-activated cells but not from untreated cells (Figure 3a). In agreement with previous reports [21,22], both pro-caspase-8 and cleaved caspase-8 were found in the DISC in mock-transfected, Fas-sensitive cells (Figure 3a). By contrast, in the DISC isolated from FLIP-expressing cells, we detected only caspase-8 that had been cleaved between the large and small subunits of the caspase domain but not between the death effector domains and the caspase-like

Figure 2

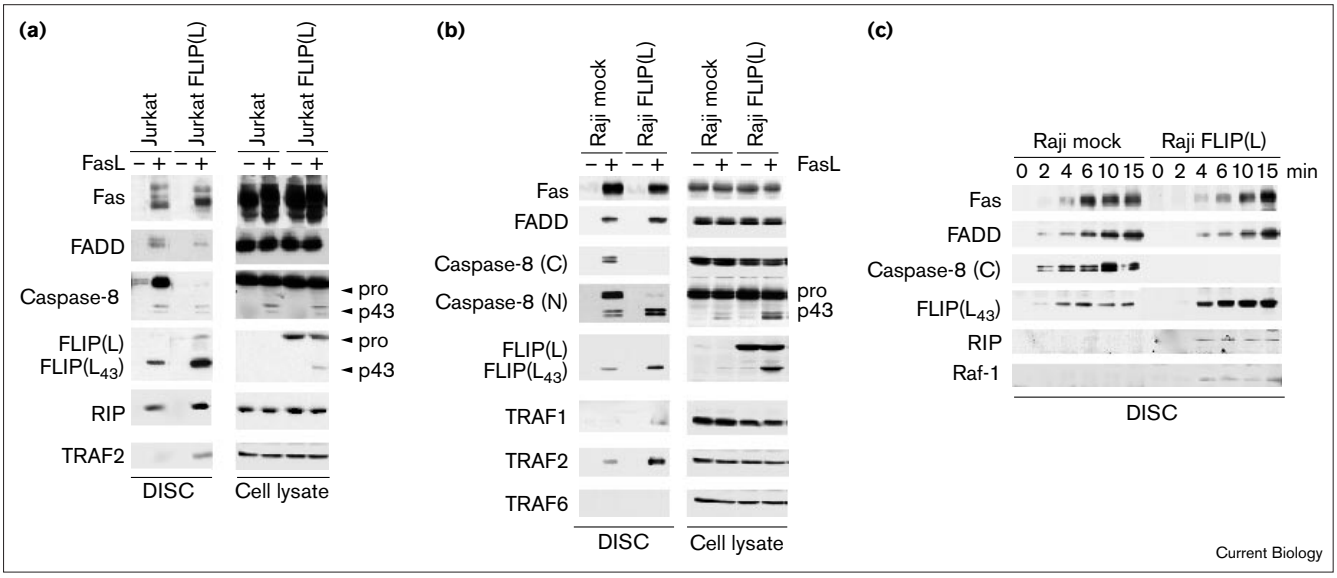


FLIP recruits TRAFs, RIP and Raf-1, and activates the NF-κB pathway. **(a)** Human embryonic kidney 293T cells were cotransfected with NF-κB luciferase reporter plasmid, β-galactosidase expression vector and the FLIP constructs consisting of either 100 ng FLIP(L), FLIP(S), or the carboxy-terminal portion of FLIP(L), FLIP(C). Luciferase activities were determined 24 h after transfection and normalized on the basis of β-galactosidase activity. Values shown are averages for representative experiments in which each transfection was carried out in duplicate. Expression of FLIP proteins in the cell extracts is shown in the lower panel. **(b)** Dose-response of the activation of NF-κB by FLIP(L). NF-κB activation by the IL-1 receptor adaptor protein MyD88 [36] is shown as a positive control. **(c)** Inhibition of FLIP-induced NF-κB activation by dominant negative (DN)-IKKβ and DN-TRAF1. 293T cells were cotransfected with 100 ng FLIP(L) and 0.5 μg DN-IKKβ, DN-TRAF1, DN-TRAF2, or control DN-IRAK1 expression vectors. Samples were analyzed for luciferase activity as in (a). **(d)** 293T cells were cotransfected with an expression vector for vesicular stomatitis virus (VSV)-tagged FLIP(L) and for FLAG-tagged TRAF1–6, as indicated, and anti-FLAG immunoprecipitates (IP) were analyzed for the presence of FLIP(L) by western blotting (WB) using anti-FLIP antibody. Expression of the FLAG-tagged TRAFs and VSV-tagged FLIP(L) in the cell extracts is shown below. **(e)** The interaction of haemagglutinin (HA)–TRAF-1 with FLIP was mapped in 293T cells using FLAG-tagged FLIP constructs. **(f)** 293T cells were cotransfected with expression vectors for FLAG-tagged FLIP constructs as indicated as well as for RIP, and anti-FLAG immunoprecipitates were analyzed for the presence of RIP. Equal loading is shown by the IgG band (arrowhead). **(g)** 293T cells were cotransfected with an expression vector for VSV-tagged FLIP(L) and for FLAG-tagged Raf-1. Anti-FLAG immunoprecipitates were analyzed for the presence of FLIP(L) by western blotting using an anti-FLIP antibody. Expression of the FLAG-tagged Raf-1 and VSV-tagged FLIP(L) in the cell extracts is shown below.

domain. Moreover, through the action of caspase-8, DISC-associated FLIP(L) was completely processed into the 43 kDa form [20–22]. Only the cleaved form of FLIP(L) was observed in the DISC, despite the presence of similar amounts of the full-length and the 43 kDa cleaved forms in the cytoplasm. Incorporation of RIP and TRAF2 into the DISC was also observed. Recruitment of TRAF2 and RIP to Fas depended on FLIP, because it was substantially increased in cells overexpressing FLIP.

To examine whether the interaction between TRAF1 and FLIP found on overexpression of the protein also occurred at physiological protein levels, we analyzed DISC assembly in Raji B cells, which, in contrast to Jurkat T cells, express TRAF1 (Figure 3b). In addition to TRAF2, TRAF1 was also detected in the DISC of Raji cells, whereas TRAF6 (and TRAF3, which is only weakly expressed; data not shown) was not detectable. There were also notable other differences in the DISC isolated from the two cell types. In Raji cells, recruitment of processed FLIP was very efficient; it was detected even in wild-type cells that express barely detectable amounts of endogenous FLIP. The inverse was true for RIP, recruitment of which was difficult to detect in Raji cells. Moreover, Raf-1 incorporation into the DISC was very inefficient, suggesting that binding was substoichiometric or indirect. We therefore studied the time course of DISC assembly (Figure 3c). Recruitment of Fas, FADD and caspase-8 by FasL was immediate and detectable as early as 2 minutes after ligand addition. Maximum DISC formation occurred

Figure 3



FLIP recruits TRAF1, TRAF2, RIP and Raf-1 to the DISC of Fas. **(a)** Wild-type Jurkat cells and Jurkat cells stably transfected with VSV-FLIP(L) were treated with 2 μg/ml FasL in the presence (+) and absence (–) of a cross-linking anti-FLAG antibody (2 μg/ml) for 15 min and then lysed. The DISC was then immunoprecipitated using protein-A–Sepharose. The coimmunoprecipitated Fas, FADD, caspase-8, FLIP and TRAFs were detected by western blot analysis. Note that endogenous FLIP is recruited to the DISC although it is not detectable in the cytoplasm. **(b)** As (a) except that Raji cells rather than Jurkat cells were analyzed. Two antibodies to caspase-8 were used: caspase-8 (N) detects an epitope in the two death effector domains and recognizes the pro-form and the processed caspase-8 (p43); caspase-8 (C) directed against the small caspase subunit detects only the pro-form. The right panels show the analysis of cellular lysates. **(c)** As (b) but DISC assembly was analyzed after the indicated time periods.

10–15 minutes after stimulation. Raf-1 (and RIP) recruitment was clearly discernible in the signaling complex of Raji cells that express exogenous FLIP, whereas in mock-transfected cells no association was detectable. The kinetics of RIP and Raf-1 recruitment resembled that of FADD. Taken together, these results suggest a model whereby FLIP recruitment mediated by Fas and FADD leads to the further recruitment of proteins that are important in signaling pathways activated during Fas-mediated costimulation of T cells (Figure 1).

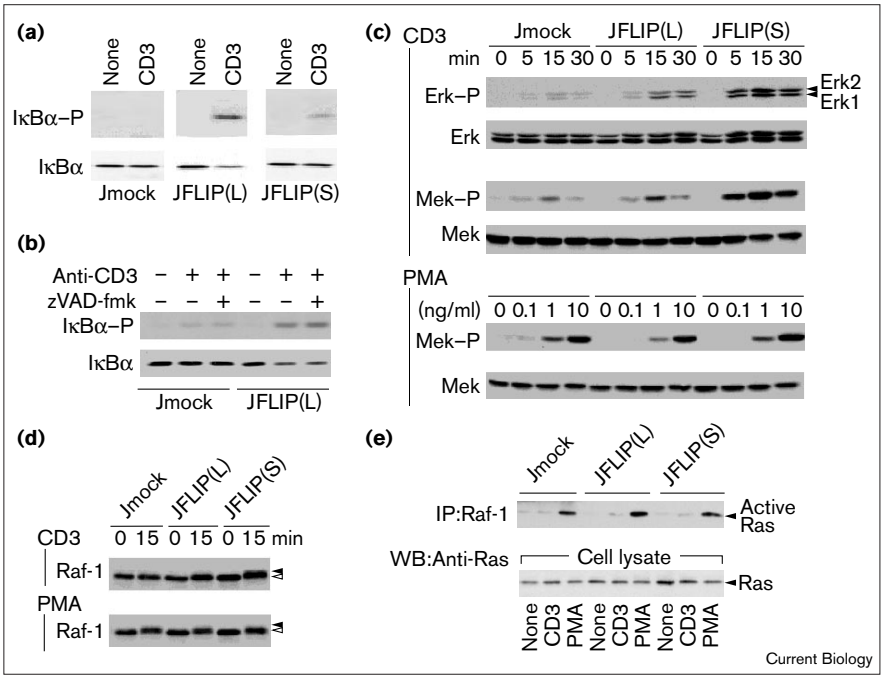
T cells overexpressing FLIP show increased Erk and NF-κB activation

Given that overexpression of FLIP results in NF-κB and Erk activation, we investigated whether these two signaling pathways can also be activated through natural stimuli in a FLIP-dependent manner. We examined activation of these signaling pathways in Jurkat cell lines that stably expressed increased levels of either FLIP(L) or FLIP(S) [20] after TCR and Fas stimulation. Levels of caspase-8, surface CD3, and Fas were similar in mock-transfected Jurkat cells (Jmock) and the FLIP transfectants (data not shown). After activation by CD3, there was rapid and intense phosphorylation of IκBα within 15 minutes in FLIP(L)-transfected Jurkat cells, whereas the response was only moderate in Jurkat FLIP(S) cells and negligible in control Jurkat cells (Figure 4a). IκBα phosphorylation was not affected by the caspase inhibitor z-VAD-fmk (Figure 4b). In contrast to primary T cells (Figure 1b), no striking further increase in signal strength was observed when FasL was added (data not shown).

After activation of Jurkat cells via CD3, there was also rapid phosphorylation of Erk in FLIP-transfected Jurkat cells, whereas the response in control Jmock cells was insignificant (Figure 4c). Again, the addition of FasL had no enhancing effect, perhaps because Jurkat cells are already actively cycling, or because the rapid surface expression of FasL (or of another death ligand) upon TCR stimulation is already optimal [13]. Erk activation was transient, with maximal activation detectable 15 minutes after stimulation. There were quantitative differences in the capacities of FLIP(L) and FLIP(S) to activate the various signaling pathways; FLIP(L) preferentially activated NF-κB (Figures 4a,2b), but FLIP(S) more strongly activated Erk.

Erk activation is most frequently a result of the Ras-initiated membrane recruitment of the MAP kinase kinase kinase Raf-1, which leads to the activation of the MAP kinase kinase Mek and then Erk. In FLIP-transfected Jurkat T cells, TCR stimulation also resulted in the activation of the upstream kinases Mek and Raf-1 (Figure 4c,d).

Figure 4



High levels of FLIP mediate activation of the NF-κB and the Erk pathways in T cells. **(a)** FLIP(L) and FLIP(S) Jurkat clones, and a mock transfectant (Jmock) were stimulated with immobilized anti-CD3 and anti-CD28 antibodies for 15 min, and NF-κB activation was assayed by probing for phosphorylated (–P) IκBα and degradation of IκBα. **(b)** As (a) but the experiment was performed in the presence of 100 μM z-VAD-fmk where indicated. **(c)** FLIP-expressing Jurkat clones were stimulated with immobilized anti-CD3 antibody (1 μg/ml) for the times indicated. Erk and Mek activation was determined by western blotting using antibodies specifically detecting the active phosphorylated (P) kinases. Expression of total Erk and Mek proteins was determined using anti-Erk or anti-Mek antibody, respectively. No increase in active phosphorylated PKB (Akt) was discernible (data not shown). In the lower two panels, Mek activation induced by PMA is shown. **(d)** Raf-1 activation in FLIP-expressing Jurkat cells was assessed 15 min after stimulation by determining the fraction of phosphorylated protein that showed lower electrophoretic mobility (closed arrowhead) compared with the dephosphorylated form (open arrowhead). Positive control is Raf-1 activated by PMA (10 ng/ml). **(e)** Activation of Ras was determined using recombinant Raf-1 coupled to beads to immunoprecipitate activated Ras [37]. Positive control are cells treated with PMA (10 ng/ml).

The presence of FLIP had no influence on Mek activation induced by phorbolester (PMA) (Figure 4c), indicating that the effect of FLIP on Mek activation was linked to TCR engagement. Since no increased Ras activity was detectable in FLIP-transfected Jurkat cells with CD3 activation (Figure 4e), FLIP is likely to be connected to the Erk pathway at the level of Raf-1. This notion is consistent with the observed FLIP–Raf-1 interaction (Figure 2g).

To ensure that signals in non-transformed cells were similarly affected by FLIP, transgenic mice were generated that show increased FLIP expression in T lymphocytes under the control of the T cell-specific human CD2 enhancer element [28] (Figure 5a). Stimulation of T-cell blasts from FLIP transgenic mice via CD3 resulted in the potent activation of Mek and Erk within 15 minutes (Figure 5b). By contrast, little activation of the Erk pathway was detected in cells from non-transgenic littermates. Interestingly, the presence of increased FLIP expression lead to the spontaneous loss of IκBα, reflecting activation of NF-κB, without further enhancement on TCR stimulation (Figure 5b). A detailed analysis of FLIP transgenic mice is underway.

Increased interleukin-2 production in cells with increased FLIP levels

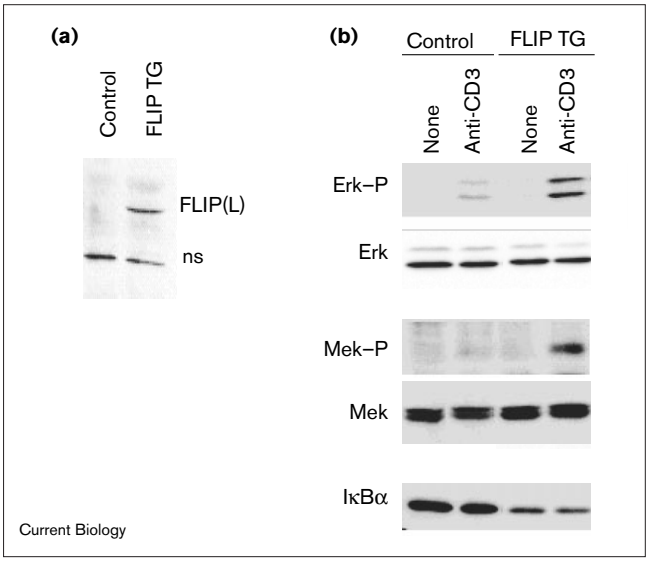
Because Erk/AP-1 and NF-κB, which are activated during FasL-induced costimulation, are extensively involved in transcriptional regulation of the T-cell growth factor interleukin-2 (IL-2), production of IL-2 was examined in T cells derived from FLIP transgenic mice. 24 hours after activation by either CD3 or CD3 plus CD28, a threefold increase in IL-2 secretion was measured in stimulated T cells from FLIP transgenic mice composed with T cells from non-transgenic littermates (Figure 6a). Similarly, IL-2 production was consistently increased by sixfold in Jurkat cells expressing either the FLIP(S) or FLIP(L) compared with mock-transfected Jurkat cells (Figure 6b). FLIP thus appears to potentiate TCR signaling pathways that are required for T-cell proliferation and IL-2 production, most likely through its capacity to augment NF-κB and Erk signaling pathways.

Discussion

These findings indicate that increased levels of FLIP can lead to the activation of the Erk and NF-κB signaling pathways. In T cells, these signals are known to contribute to IL-2 transcription; indeed, TCR stimulation of activated T cells bearing increased FLIP levels leads to increased IL-2 production. Augmented triggering of the Erk and NF-κB signaling pathways is also detectable in TCR-stimulated T cells on costimulation with Fas.

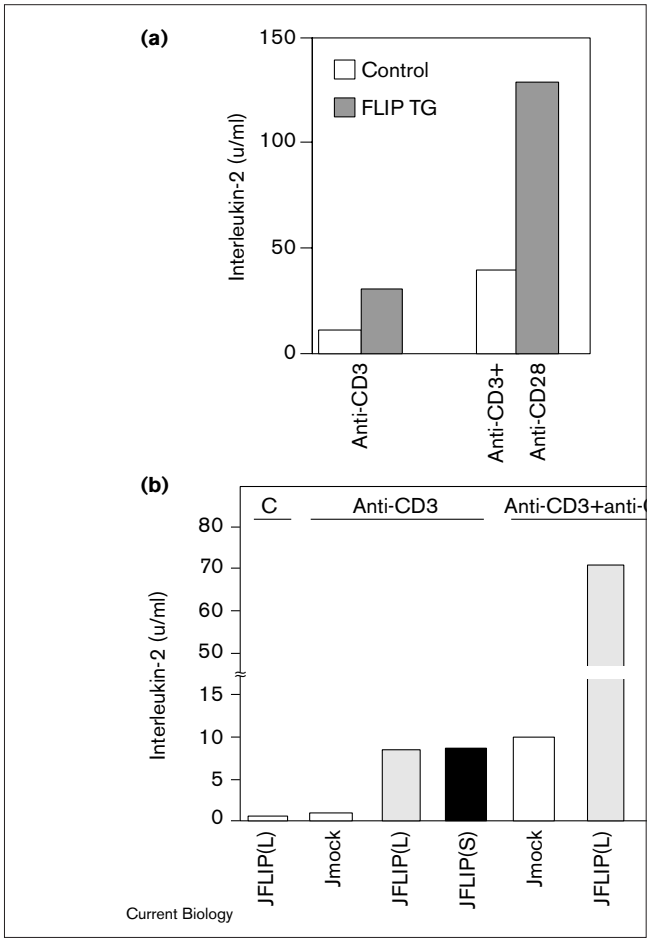
How TCR activation leads to FLIP-mediated Erk and NF-κB signals is currently uncertain. A likely circuit might

Figure 5



Primary T cells from FLIP transgenic mice show increased Erk and NF-κB activation. **(a)** Levels of FLAG-tagged transgenic murine FLIP in splenic T cells from one mouse line was determined with the anti-FLAG antibody. **(b)** Anti-CD3-mediated Mek and Erk activation in T cells from transgenic mice overexpressing FLIP. Purified splenic T cell blasts from transgenic mice (FLIP TG) and non-transgenic littermates (control) were stimulated with anti-CD3 antibody for 15 min and the amount of total and phosphorylated (–P) Mek and Erk, and total IκBα determined by western blot analysis as in Figure 4.

Figure 6



Increased IL-2 production in cells with increased FLIP expression. Increased IL-2 production by **(a)** FLIP transgenic T cell blasts and **(b)** FLIP-transfected Jurkat clones. Cells were stimulated as indicated for 24 h and secreted IL-2 in supernatants determined by the CTLL bioassay [31].

be through the known TCR-induced rapid membrane exposure of preformed FasL [29] or biosynthesis of FasL (or of another death ligand), which could then ligate surface Fas (or other death receptors) and lead to recruitment of FADD and FLIP. This mechanism would be consistent with our observations and those of others that exogenous FasL costimulates proliferation with suboptimal doses of anti-CD3 antibody, and that CD3-induced proliferation (which at higher doses becomes FasL-independent) is blocked by FasL inhibitors [30]. However, other death ligands such as TRAIL may also contribute, since in Jurkat cells, TCR-triggered signal initiation was only partly blocked by Fas-Fc (data not shown). This may also indicate differences between primary cells and tumor cell lines. In any case, activation of Erk and NF-κB signals in T cells was always dependent on signals emanating from the TCR and was never observed upon the addition of FasL alone, even though FasL alone was able to induce assembly of a complex that included proteins involved in the two signaling pathways. This suggests a complex cross-talk of signals.

Since Erk and NF-κB signals are modulated by FLIP, levels of this protein may determine the outcome of Fas-triggered signals. Depending on the ratio of caspase-8 and FLIP levels, Fas-FADD complexes might recruit either caspase-8 homo-complexes or caspase-8–FLIP hetero-complexes. In the latter case, FLIP could stop apoptotic signaling events downstream of caspase-8 activation. FLIP in turn would act as a platform to recruit TRAF1, TRAF2, RIP and Raf-1, leading to the simultaneous activation of the Erk and NF-κB pathways. Thus, FLIP can be considered as a multifunctional protein, capable of switching Fas signaling pathways, by blocking progression of caspase-8 activation and by recruiting adaptor proteins and kinases that are crucial in the initiation of signaling pathways leading to proliferation and or differentiation. This model is similar to what is known about signals triggered by TNF-R1. Addition of TNF can lead to cell death via TRADD–FADD–caspase-8 or, alternatively, to activation of NF-κB via TRADD–RIP–TRAF1–TRAF2. FLIP-induced Erk and NF-κB signals may also help to explain the observation that the absence of functional FADD leads to both defective apoptosis and defective T-cell proliferation [9–12], and is also in agreement with

the known decreased IL-2 production by mature T cells from Fas-deficient *lpr* mice [31].

The switch from Fas–FADD-triggered apoptosis to proliferative signals may be more broadly applicable to other cell types. Fibroblasts and B cells show increased proliferation with anti-Fas antibodies or FasL [15,32]. FLIP expression is increased in many tumor cells [7], suggesting that FasL expressed on attacking cytolytic T cells may in some circumstances stimulate tumor cell growth. This hypothesis is in agreement with recent experiments that show that FLIP-transfected tumor cell lines escape immune surveillance [33]. Mice deficient in FADD or caspase-8 die at about day 11 of embryogenesis, presumably as a consequence of abnormal heart development [11,12,34]. Since FLIP is highly expressed in cardiac myocytes [7,35], and was found to be deficient in myocardial infarcts [35], FLIP signals may also be crucial in heart development and function. Conceivably, through its activation of NF-κB and Erk, FLIP may be pivotal in turning signals for cell death into those for cell survival.

Materials and methods

Cell preparation and proliferation

Human peripheral blood lymphocytes were prepared by Ficoll-Hypaque centrifugation, and T cells were purified by E-rosetting. Cells were cultured in 96-well plates at 5×10^4 cells per well and stimulated with the indicated concentrations of anti-CD3 antibody (TR66), IgM anti-CD28 antibody (28/34), or soluble recombinant FasL (Alexis) with or without anti-FLAG antibody (M2, Sigma) at 1 μg/ml. Proliferation was measured by ^{3}H-thymidine incorporation after 3 days. Murine T cells were prepared by nylon wool purification from mice transgenic for the DNA binding sites of AP-1 or NF-κB linked to a luciferase gene reporter [18,19]. T cells were activated with either plastic immobilized anti-CD3 antibody (145-2C11) at the indicated concentration, alone or with anti-Fas (Jo2, 5 μg/ml) or anti-CD28 antibody (37.51, 3 μg/ml). Luciferase activity was measured after 48 h from 2×10^6 cells. Proliferation to the same stimuli was measured after 3 days in parallel cultures using T cells from the same mice (5×10^4 cells per well). Splenic T cell blasts from wild-type or FLIP-transgenic mice were generated via stimulation by plate-bound anti-CD3 antibody (0.1 μg/ml) and anti-CD28 antibody (0.3 μg/ml) for 3 days. Supernatants for IL-2 production were taken from Jurkat T cells (10^6/ml) or murine splenic T cell blasts (10^6 /ml) that were stimulated for 24 h with immobilized anti-CD3 antibody and, when indicated, with anti-CD28 antibody.

Transfection, immunoprecipitation, Erk and NF-κB activation assays

FLIP expression clones used in this study have been described before [20,36]. Stimulated or transfected cells were washed once with PBS, and lysed in lysis buffer (50 mM Tris–HCl pH 7.5, 1% Triton X-100, 2 mM DTT, 2 mM sodium vanadate, and protease inhibitor cocktail (Complete, Boehringer Mannheim)), followed by centrifugation. Post-nuclear lysates (30 μg protein) were separated by SDS–PAGE and analyzed by western blotting. Antibodies were used to the following molecules: phospho-specific p44/42 MAP kinase (Erk1/Erk2), total P44/42 MAP kinase (Erk1/Erk2), phospho-specific Mek1/2, total Mek1/2, phospho-specific Jun N-terminal kinase (JNK), phospho-specific IκBα, total IκBα (all New England Biolabs), FLAG (Sigma), VSV (Babco), Fas (Alexis). Raf-1 beads were purchased from UBI.

DISC analysis

Purified human T cells and FLIP- and mock-transfected Raji and Jurkat T cells [20] were treated with 2 μg/ml FasL in the presence or absence of the cross-linking anti-FLAG M2 antibody (2 μg/ml) for the indicated times (in the negative control, the cross-linking antibody was added after lysis). In the case of T cells, cells were prestimulated for 1 h with anti-CD3 antibody (TR66) and FasL (50 ng/ml) as described above. Cells were quickly cooled down by adding 5 volumes of ice-cold PBS, then lysed with 0.2% NP-40, 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM sodium vanadate, 10% glycerol, and the protease inhibitor cocktail. Cytosolic fractions were precleared with Sepharose 6B for 90 min and then incubated with protein-A- or protein-G-coupled Sepharose beads for 3 h. Beads were washed four times with the lysis buffer. Proteins were separated on 10% SDS–PAGE, and blotted onto nitrocellulose filters. In control experiments, the anti-FLAG antibody was added after lysis. The following antibodies were used for western blotting: anti-Fas (Santa Cruz), anti-FADD (Transduction Labs), anti-caspase 8 (PharMingen, MBL), anti-FLIP AL148 and Dave-2 (Alexis), anti-caspase-3 (Transduction Laboratories), anti-Raf-1 (PharMingen), anti-RIP (Transduction Labs and PharMingen), anti-TRAF1, 2, 6 (Santa Cruz).

Generation of FLIP transgenic mice

A FLAG-tagged mouse FLIP(L) cDNA sequence was inserted into a targeting vector that contains the β-globin promoter and the CD2 downstream locus enhancer element [28]. The resulting construct was injected into (BALB/c × C57BL/6) embryos, and transgenic founders were screened by PCR of tail DNA using oligonucleotide primers JT 766 (5′-GGAGCCAGGGCTGGGCATAAAA-3′) and JT767 (5′-GACTCACCCTGAAGTTCTCAGGATCC-3′). Expression of the transgene was further confirmed by western blotting using anti-FLAG antibodies.

Acknowledgements

This work was supported by grants from the Swiss National Science Foundation (to J.T.), the European Molecular Biology Organization (to M.T.), and the National Institutes of Health (AI36333 and F06 TW02294 to R.C.B.). T.K. was on leave from Department of Bioengineering, Tokyo Institute of Technology, Japan. We thank Salvatore Valitutti for the anti-CD3 antibody and Pedro Romero for the anti-CD28 antibody. Expression plasmids related to the MAPK signaling pathways were a kind gift of C. Widmann, University of Lausanne.

References

1. Wallach D: **Cell death induction by TNF: a matter of self control.** *Trends Biochem Sci* 1997, **22:**107-109.
2. Ashkenazi A, Dixit VM: **Death receptors: signaling and modulation.** *Science* 1998, **281:**1305-1308.
3. Nagata S: **Apoptosis by death factor.** *Cell* 1997, **88:**355-365.
4. Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME: **Apoptosis signaling by death receptors.** *Eur J Biochem* 1998, **254:**439-459.
5. Thome M, *et al.*: **Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors.** *Nature* 1997, **386:**517-521.
6. Bertin J, *et al.*: **Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis.** *Proc Natl Acad Sci USA* 1997, **94:**1172-1176.
7. Tschopp J, Irmler M, Thome M: **Inhibition of Fas death signals by FLIPs.** *Curr Opin Immunol* 1998, **10:**552-558.
8. Wallach D: **Apoptosis: placing death under control.** *Nature* 1997, **388:**123.
9. Newton K, Harris AW, Bath ML, Smith KGC, Strasser A: **A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes.** *EMBO J* 1998, **17:**706-718.
10. Walsh CM, Wen BG, Chinnaiyan AM, O'Rourke K, Dixit VM, Hedrick SM: **A role for FADD in T cell activation and development.** *Immunity* 1998, **8:**439-449.
11. Zhang J, Cado D, Chen A, Kabra NH, Winoto A: **Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1.** *Nature* 1998, **392:**296-300.
12. Yeh WC, *et al.*: **FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis.** *Science* 1998, **279:**1954-1958.
13. Anel A, Buferne M, Boyer C, Schmitt VA, Golstein P: **T cell receptor-induced Fas ligand expression in cytotoxic T lymphocyte clones is blocked by protein tyrosine kinase inhibitors and cyclosporin A.** *Eur J Immunol* 1994, **24:**2469-2476.

14. Alderson MR, Armitage RJ, Maraskovsky E, Tough TW, Roux E, Schooley K, *et al.*: **Fas transduces activation signals in normal human T lymphocytes.** *J Exp Med* 1993, **178:**2231-2235.
15. Rathmell JC, Townsend SE, Xu JC, Flavell RA, Goodnow CC: **Expansion or elimination of B cells *in vivo*: dual roles for CD40- and Fas (CD95)-ligands modulated by the B cell antigen receptor.** *Cell* 1996, **87:**319-329.
16. Brunner T, *et al.*: **Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas.** *Nature* 1995, **373:**441-444.
17. Schneider P, Holler N, Bodmer JL, Hahne M, Frei K, Fontana A, Tschopp J: **Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity.** *J Exp Med* 1998, **187:**1205-1213.
18. Rincon M, Flavell RA: **AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes.** *EMBO J* 1994, **13:**4370-4381.
19. Rincon M, Flavell RA: **Transcription mediated by NFAT is highly inducible in effector CD4+ T helper 2 (Th2) cells but not in Th1 cells.** *Mol Cell Biol* 1997, **17:**1522-1534.
20. Irmler M, *et al.*: **Inhibition of death receptor signals by cellular FLIP.** *Nature* 1997, **388:**190-195.
21. Scaffidi C, Schmitz I, Krammer PH, Peter ME: **The role of c-FLIP in modulation of CD95-induced apoptosis.** *J Biol Chem* 1999, **274:**1541-1548.
22. Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME: **Cytotoxicity-dependent Apo-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor.** *EMBO J* 1995, **14:**5579-5588.
23. Cohen L, Henzel WJ, Baeuerle PA: **IKAP is a scaffold protein of the IkappaB kinase complex.** *Nature* 1998, **395:**292-296.
24. Mercurio F, *et al.*: **IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation.** *Science* 1997, **278:**860-866.
25. Cao Z, Henzel WJ, Gao X: **IRAK: a kinase associated with the interleukin-1 receptor.** *Science* 1996, **271:**1128-1131.
26. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P: **The death domain kinase RIP mediates the TNF-induced NF-kappaB signal.** *Immunity* 1998, **8:**297-303.
27. Shu HB, Halpin DR, Goeddel DV: **Casper is a FADD- and caspase-related inducer of apoptosis.** *Immunity* 1997, **6:**751-763.
28. Lang G, Wotton D, Owen MJ, Sewell WA, Brown MH, Mason DY, *et al.*: **The structure of the human CD2 gene and its expression in transgenic mice.** *EMBO J* 1988, **7:**1675-1682.
29. Bossi G, Griffiths GM: **Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells.** *Nat Med* 1999, **5:**90-96.
30. Kennedy NJ, Kataoka T, Tschopp J, Budd RC: **Caspase activation is required for T cell proliferation.** *J Exp Med* 1999, **190:**1891-1896.
31. Budd RC, Schumacher JH, Winslow G, Mosmann TR: **Elevated production of interferon-gamma and interleukin 4 by mature T cells from autoimmune lpr mice correlates with Pgp-1 (CD44) expression.** *Eur J Immunol* 1991, **21:**1081-1084.
32. Aggarwal BB, Singh S, LaPushin R, Totpal K: **Fas antigen signals proliferation of normal human diploid fibroblast and its mechanism is different from tumor necrosis factor receptor.** *FEBS Lett* 1995, **364:**5-8.
33. Medema JP, de Jong J, van Hall T, Melief CJ, Offringa R: **Immune escape of tumors In vivo by expression of cellular FLICE-inhibitory protein.** *J Exp Med* 1999, **190:**1033-1038.
34. Varfolomeev EE, *et al.*: **Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally.** *Immunity* 1998, **9:**267-276.
35. Rasper D, Vaillancourt J, Hadano S, Houtzager V, Seiden I, Keen L, *et al.*: **Cell death attenuation by 'Usurpin', a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD95 (Fas/Apo-1) receptor complex.** *Cell Death Differ* 1998, **5:**271-288.
36. Burns K, Martinon F, Esslinger C, Pahl H, Schneider P, Bodmer JL, *et al*: **MyD88, an adapter protein involved in interleukin-1 signaling.** *J Biol Chem* 1998, **273:**12203-12209.
37. de Rooij J, Bos JL: **Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras.** *Oncogene* 1997, **14:**623-625.

Because *Current Biology* operates a 'Continuous Publication System' for Research Papers, this paper has been published on the internet before being printed. The paper can be accessed from http://biomednet.com/cbiology/cub – for further information, see the explanation on the contents page.