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Cellular FLIP Long Form Augments Caspase Activity and Death of T Cells through Heterodimerization with and Activation of Caspase-8

Austin Dohrman,* Jennifer Q. Russell,* Solange Cuenin,*† Karen Fortner,* Jürg Tschopp, † and Ralph C. Budd2*†

Caspase activity is required not only for the death of T cells, but also for their activation. A delicate balance of caspase activity is thus required during T cell activation at a level that will not drive cell death. How caspase activity is initiated and regulated during T cell activation is not known. One logical candidate for this process is cellular FLIP long form (c-FLIPL), because it can block caspase-8 recruitment after Fas (CD95) ligation as well as directly heterodimerize with and activate caspase-8. The current findings demonstrate that after T cell activation, caspase-8 and c-FLIPL associate in a complex enriched for active caspases. This occurs coincidently with the cleavage of two known caspase-8 substrates, c-FLIPL and receptor interacting protein 1. Caspase activity is higher in wild-type CD8+ than CD4+ effector T cells. Increased expression of c-FLIPL results in augmented caspase activity in resting and effector T cells to levels that provoke cell death, especially of the CD8 subset. c-FLIPL is thus not only an inhibitor of cell death by Fas, it can also act as a principal activator of caspases independently of Fas. The Journal of Immunology, 2005, 175: 311–318.

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Abbreviations used in this paper: c-FLIPL, cellular FLIP long form; c-FLIPS, short form of c-FLIP; FasL, Fas ligand; NLC, normal littermate control; Tg, transgenic; FADD, Fas-associated death domain protein; RIP1, receptor interacting protein 1.

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Materials and Methods

Mice

c-FLIPL was expressed transgenically in the T cell compartment as previously reported (14). Briefly, FLAG-tagged mouse FLIPL cDNA was
inserted into a target vector containing the β-globin promoter and a downstream human CD2 locus enhancer element. Transgenic mice were screened by PCR of ear DNA using the following primers: 5’- primer, 5’-GGAAGGACGGGCTGGGACAATAAA-3’; and 3’ primer, 5’-GACT CACCCCTGAATGTCCTAGGATCC-3’. Immunoblot using anti-FLIP mAb (Dare-2; Apotech) also confirmed expression of the transgene. The c-FLIPL-Tg mouse strain has been backcrossed to C57BL/6 mice (The Jackson Laboratory) for nine generations.

OT-I mice bear a transgenic TCR that recognizes chicken OVA peptide 257–264 (SIINFEKL) restricted to class I MHC, Kb and were provided by Drs. F. Carbone and M. Bevan (15). OT-I mice were maintained by breeding TCR transgenic male mice to normal C57BL/6 females. Offspring were screened for the clonotype TCR using anti-Vα2 mAb. c-FLIPL-Tg mouse strain has been backcrossed to C57BL/6 mice (The Jackson Laboratory) for nine generations.

FIGURE 1. Effector T cells manifest caspase activity, which is increased by c-FLIPL. A, Total purified T cells from B6 normal littermate control mice (NLC; ○) or from B6 c-FLIPL-Tg mice (●), freshly isolated on day 0 (upper panel) or day 3 after activation with anti-CD3/CD28 plus IL-2 (50 U/ml; middle panel), were assayed for caspase activity using the DEVD-rhodamine cleavage assay. The lower panel compares the levels of caspase activity in day 3 effector T cells with the same cells after treatment with FasL for 2 h or pretreatment for 15 min with 50 μM z-VAD.

B, Immunoblot analysis of caspase-8, c-FLIP, and RIP1 in T cell lysates from NLC or c-FLIPL-Tg mice at the times indicated after stimulation with anti-CD3/CD28 plus IL-2. Similar results were obtained in two additional studies. C, Immunoblot of c-FLIP and RIP1 from day 3 c-FLIPL-Tg effector T cells that were incubated during the last 24 h in the absence or the presence of 50 μM z-VAD-fmk.

Abs and flow cytometry

Anti-murine CD8α mAb conjugated to Red613 was purchased from Invitrogen Life Technologies. Anti-murine CD4 mAb conjugated to Tricolor or PE was purchased from Caltag Laboratories. Anti-murine Vα2 mAb conjugated to PE was purchased from BD Biosciences.

For flow cytometry, 750,000 cells were incubated in 0.1 ml of PBS containing 0.5% BSA fraction V, 0.001% (w/v) sodium azide (PBS-azide; Sigma-Aldrich), and the appropriate Abs at 4°C for 30 min. After washing with PBS-azide, cells were fixed in 1% methanol-free formaldehyde (Ted Pella) in PBS-azide. Samples were stored at 4°C until being analyzed with an Elite flow cytometer (Coulter).

For TUNEL assay of apoptosis, surface staining was first completed as described above, except that cells were fixed on ice for 15 min using 2% formaldehyde in PBS. Cells were then washed twice with PBS, fixed in 70% ice-cold ethanol for 15 min, and washed twice with PBS. Nicked DNA was labeled by incubating cells with TdT buffer (2.5 mM CoCl₂, 1 U TdT, and 0.5 nmol of biotin-dUTP (Roche)) in a total volume of 50 μl at 37°C for 1 h. Cells were washed twice with 1% BSA in PBS and subsequently incubated with streptavidin-Tricolor for 30 min. Samples were then washed twice with 1% BSA in PBS and fixed using 1% formaldehyde in PBS.

Caspase activity assay

Total cellular caspase activity was quantitated using Apo-ONE Caspase Assay (Promega) according to the manufacturer’s protocol. In brief, viable cells were isolated using centrifugation over Lympholyte M (Cedarlane Laboratories), then titrated at the concentrations indicated in 100 μl of culture medium, and an equal volume of DEVD-rhodamine was added to the cells according to the manufacturer’s protocol. Because the DEVD substrate is cleaved by caspases, rhodamine was released and measured by a fluorescent spectrophotometer at 2 h. Positive and negative controls of caspase activity were obtained using, respectively, day 3 effector T cells treated with 100 ng/ml cross-linked soluble Fas ligand (FasL; Apotech) for 2 h or the addition of 50 μM of the pan-caspase blocker z-VAD-fmk (MP Biomedicals) for 15 min before the addition of DEVD-rhodamine.
Immunoblot blot analysis

Cells were washed once in ice-cold PBS and solubilized in lysis buffer (0.2% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, sodium orthovanadate, 10% glycerol, and complete protease inhibitor (Roche)). Postnuclear lysate proteins (40 μg/lane) were separated by 12.5% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad), and blots were blocked and probed with the indicated Abs in 4% nonfat milk in TBS/0.1% Tween 20. Immunoreactive proteins were visualized using HRP-labeled conjugates (Southern Biotechnology Associates) and developed using LumiGLO (Kirkegaard & Perry Laboratories). The Abs used were specific for caspase-8 and c-FLIP (Apotech), caspase-3 (gift from Dr. Y. Lazebnik, Cold Spring Harbor Laboratories, Plainview, NY), and receptor interacting protein 1 (RIP1) (BD Biosciences).

FIGURE 2. Association of active caspase-8 with c-FLIP, in effector, but not resting, T cells. A, Lysates were prepared from FLAG-tagged c-FLIP, Tg T cells, either freshly isolated or after day 4 of activation by anti-CD3/CD28. Lysates were then subjected to immunoprecipitation using anti-FLAG M2 Ab and were examined by immunoblot for c-FLIP and coprecipitating caspase-8. B, Freshly isolated or day 3 effector T cells from c-FLIP, Tg mice were incubated at 37°C for 15 min with biotin-VAD-fmk and then lysed. Lysates were pre-cleared with Sepharose, then active caspases were precipitated using avidin-Sepharose. Precipitates were analyzed by immunoblot for active caspase-8, caspase-3, and associating c-FLIP. C, Immunoblots of biotin-VAD precipitates of day 4 effector T cells from normal littermate control (NLC) or c-FLIP, Tg mice analyzed for active caspase-8 and caspase-3. All findings were consistent in at least three studies each.

FIGURE 3. Selective decrease in CD8 T cells in c-FLIP, Tg mice. A, Flow cytometric profiles of LN cells from c-FLIP, Tg mice and normal littermate control (NLC) mice. B, Cell numbers of the CD4 and CD8 subsets from spleens and lymph nodes of six age- and sex-matched NLC and c-FLIP, Tg mice. *, p < 0.05, by unpaired t test.
Biotin-VAD-fmk caspase precipitation assay

Viable T cells (freshly isolated or day 4 T cell blasts) were incubated with 10 μM biotin-VAD-fmk (MP Biomedicals). Cell membranes were disrupted using lysis buffer containing 20 μM biotin-VAD. Protein lysate (600 μg) was then precleared by rocking with 40 μl of Sepharose 6B agarose beads (Sigma-Aldrich) at 4°C for 2 h. Supernatant was incubated with 30 μl of strepavidin-Sepharose beads (Zymed Laboratories) on a rocker at 4°C overnight. Beads were washed five times in lysis buffer without complete protease inhibitor, boiled in loading buffer, and removed by centrifugation, and immunoblot analysis was performed on the supernatants.

In vivo measurement of T cell death

OT-I and OT-I/c-FLIPL-Tg mice received a 150-μl i.p. injection of 100 μM OVAp (SIINFEKL) and lymph node and liver V2+CD8+ lymphocytes were analyzed 2 days later for the proportion of apoptotic cells by TUNEL assay and the percentage of V2+CD8+ cells. As an alternative, Thy1.1+ C57BL/6 mice received injections on day 0 of 20 × 10^6 CD8+V2+ cells from either OT-I or OT-I/c-FLIPL-Tg mice (both Thy1.2+). On the days indicated, lymph node spleen cells from three mice per group were analyzed for the proportion of Thy1.2+V2+ cells.

Results

Caspase activity of T cells parallels levels of c-FLIP_L expression

Because caspase activity is required for both initiation of T cell proliferation as well as cell death (10–13, 16, 17), we examined the levels of caspase activity by three independent assays before and after activation of resting T cells and assessed potential substrates of such caspase activity. Resting murine wild-type T cells manifested essentially undetectable levels of caspase activity, as quantitated using a DEVD-rhodamine detection substrate (Fig. 1A, upper panel). By day 3, however, caspase activity was readily detectable in viable effector T cells (Fig. 1A, middle panel). The specificity of the assay was confirmed by its inhibition after 15-min pretreatment of effector T cells with the pan-caspase blocker, z-VAD-fmk (Fig. 1A, lower panel). In addition, the level of caspase activity in day 3 effector T cells was substantially less than...
that seen with the same cells after a 2-h treatment with FasL (Fig. IA, lower panel). The caspase activity of effector T cells was not merely the result of dead cells in the culture, because these were removed before analysis in each case through Ficoll-Hypaque centrifugation.

These findings were corroborated by the absence in resting wild-type T cells of detectable cleavage of caspase-8 or two of its known substrates, c-FLIP<sub>L</sub> and RIP1, by immunoblot analysis (Fig. 1B). After activation with anti-CD3/CD28, however, evidence of caspase-8 cleavage was observed with the appearance of a p43/41 fragment, corresponding to the cleavage of a C-terminal p10/12 fragment of caspase-8 at Asp<sup>74</sup> and Asp<sup>85</sup>, respectively. This was apparent in wild-type cells on day 1 as shown in Fig. 1B, but was also detectable as early as 4 h after activation (data not shown). Caspase activity increased further over days 2 and 3, as reflected by additional cleavage fragments of caspase-8 and the appearance of cleavage of c-FLIP<sub>L</sub> to p43FLIP, and of RIP1 to p38RIP1, each corresponding to known caspase cleavage products. Another demonstration that the appearance of p27FLIP and p38RIP1 was due to caspase-dependent cleavage is shown by inhibition of these products in day 3 effector c-FLIP<sub>L</sub>-Tg T cells that were treated for 24 h with z-VAD-fmk (Fig. 1C).

If c-FLIP<sub>L</sub> were responsible for the activation of caspase-8 through mutual heterodimerization, then the two molecules would be expected to be found in association after T cell activation. As shown in Fig. 2A, caspase-8 indeed coimmunoprecipitated with c-FLIP<sub>L</sub> in cycling T cells, although it was barely detectable in resting T cells. In agreement with the findings reported by Micheau et al. (5), the addition of the pan-caspase blocker, z-VAD-fmk, stabilized the interaction of caspase-8 with c-FLIP<sub>L</sub> and augmented the amount of caspase-8 that coimmunoprecipitated with c-FLIP<sub>L</sub>. Conceivably, cleavage of caspase-8 or c-FLIP<sub>L</sub> might reduce their mutual affinity and decrease the amount of protein coimmunoprecipitated. This would be consistent with the observation that the form of caspase-8 that associated with c-FLIP<sub>L</sub> was primarily full-length caspase-8, although a small amount of p43 caspase-8 was also detected (Fig. 2A).

The association of c-FLIP<sub>L</sub> with caspase-8 was also verified using biotin-VAD-fmk to selectively bind active caspases in effector T cells. Precipitates from resting T cells made with avidin-Sepharose demonstrated very little active caspase-8, caspase-3, or associating c-FLIP, but from day 4 effector T cells there was readily detectable active caspase-8, caspase-3, and associating c-FLIP (Fig. 2B). Addition of unlabeled z-VAD-fmk at the time of incubation with biotin-VAD nearly completely blocked the ability to precipitate caspase-8 with avidin-Sepharose (19). Of particular note is that biotin-VAD bound to full-length active caspase-8, consistent with the model proposed by Micheau et al. (5) and similar to activation of caspase-9 by Apaf-1 and cytochrome c (20). By contrast, biotin-VAD bound almost exclusively to cleaved p12/10 caspase-3, and not to full-length caspase-3, even though cleaved caspase-3 represented only a small proportion of the total caspase-3 in whole cell lysates. This is also consistent with the structural model that caspase-3 becomes active only after its cleavage (21). Of additional interest was that the form of c-FLIP in association with active caspases was highly enriched for cleaved p43FLIP over full-length c-FLIP<sub>L</sub>, compared with the proportions in whole cell lysates from the same cells (Fig. 2B). This was also observed for endogenous c-FLIP in wild-type T cells. Consistent with the other assays of caspase activity, the amounts of active caspase-8 and caspase-3 precipitated by biotin-VAD were increased in c-FLIP<sub>L</sub>-Tg effector T cells compared with control mice (Fig. 2C).

**Effect CD8<sup>+</sup> T cells manifest greater caspase activity than CD4<sup>+</sup> effectors and, hence, more sensitivity to caspase-mediated cell death**

c-FLIP<sub>L</sub>-Tg mice contain relatively normal numbers of total T cells in lymph nodes and spleen, but there is a selective loss in the proportion and absolute numbers of CD8<sup>+</sup> T cells by ~30–50% (Fig. 3). Because thymocyte development in c-FLIP<sub>L</sub>-Tg mice is normal, including the number of single-positive CD8<sup>+</sup> T cells (14), we considered that CD8<sup>+</sup> T cells might either generate more caspase activity upon activation or be more sensitive to a given
level of caspase activity than CD8^+ T cells. Both wild-type and c-FLIPL-Tg CD8^+ day 3 effector T cells revealed greater caspase activity than their equivalent CD4^+ effector T cells (Fig. 4, A and B). In fact, the level of caspase activity in c-FLIPL-Tg CD8^+ T cells was nearly equivalent to the level in wild-type CD8^+ effector T cells after treatment with FasL (Fig. 4C). These findings were reflected in the cleavage of caspase-8 and caspase-3 (Fig. 4D) as well as the amount of active caspase-3 that bound biotin-VAD-fmk (Fig. 4E). CD4^+ T cells manifested less cleavage of caspase-8 or caspase-3 than CD8^+ T cells from the same mice (Fig. 4D) and greater active caspase-3 in c-FLIPL-Tg effector T cell subsets than the equivalent subsets from normal littermate controls (Fig. 4E). Because the levels of c-FLIP and caspase-8 do not differ between the equivalent subsets from normal littermate controls (Fig. 4D and data not shown), it is not apparent what initiates the increased caspase activity in the CD8^+ subset.

In parallel with their increased caspase activity, CD8^+ T cells also manifested greater cell death in vitro than CD4^+ T cells, both spontaneously (Fig. 5A) and after CD3/CD28 stimulation (Fig. 5B). As anticipated, the level of death was more prominent in the equivalent T cell subsets from c-FLIPL-Tg mice than in those from non-Tg littermate control mice. Cell death was largely blocked by z-VAD-fmk, substantiating that the death was caspase dependent (Fig. 5B, right panel).

The cell death observed in vitro was also seen after in vivo activation of T cells. For these studies, c-FLIPL-Tg mice were crossed with OT-I mice that express a transgenic TCR (Vα2 Vβ5) responsive to OVA peptide SIINFEKL (OVAp) restricted to H-2K^b (15). Two days after administration of OVAp, Vα2^+CD8^+ lymph node T cells from OT-I mice contained ~25% TUNEL^+ cells, whereas those from OT-I × c-FLIPL-Tg mice manifested more than twice the proportion of dead CD8^+ cells (Fig. 6A, upper panel). This corresponded with fewer Vα2^+CD8^+ cells in OT-I × c-FLIPL-Tg mice than OT-I mice after OVAp administration (Fig. 6A, lower panel). Activated CD8^+ T cells are known to migrate to the liver, and hence, differences in the proportions of dying lymph node T cells might reflect different trafficking patterns of dying cells in these mice. However, a similar relationship of TUNEL^+Vα2^+CD8^+ T cells was observed in the liver, with those from OT-I × c-FLIPL-Tg mice containing a higher proportion of dead cells and resulting in fewer CD8^+ cells than from the livers of similarly treated OT-I mice (Fig. 6A). A similar pattern of decreased survival of OT-I × c-FLIPL-Tg T cells was observed after adoptive transfer to B6 Thy1.1^+ recipients and administration of OVAp (Fig. 6B). The limited expansion of OT-I × c-FLIPL-Tg T cells compared with OT-I T cells was not due to decreased proliferation, because we have previously shown by in vivo BrdU labeling that after OVAp administration, OT-I × c-FLIPL-Tg T cells cycle nearly twice as rapidly as OT-I T cells (14). Thus, the blunted expansion of OT-I × c-FLIPL-Tg T cells is consistent with increased cell death.

Increased caspase activity and death of CD8^+ T cells from c-FLIPL_Tg mice does not require Fas

We have previously observed that T cell blasts from c-FLIPL-Tg mice are resistant to FasL-induced death (14). Thus, it was of interest to determine whether the increased death of CD8^+ T cells in c-FLIPL-Tg mice was independent of Fas. B6 lpr mice were crossed with B6 FLIPL_Tg mice, and the degree of adenopathy and composition of the peripheral T cells were measured at different

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**FIGURE 7.** Decreased numbers of CD8^+ cells in c-FLIPL_Tg mice do not require Fas. Lymph node and spleen cells from B6lpr and B6/lprFLIP mice (age and sex matched, six per group) were analyzed at 15 and 27 wk of age for numbers of T cell subsets. A, Representative FACS profile of lymph node cells for expression of CD4 and CD8. B, Summary of spleen (upper panels) and lymph node (lower panels) cell numbers from all mice analyzed. *, p < 0.05, by unpaired t test.
ages. Thymuses of lpr mice were of normal cell number and composition compared with age- and sex-matched wild-type mice, and this was also true of lprFLIP thymuses (data not shown). Peripheral lymph nodes and spleens of lprFLIP mice contained, on the average, ~10–15% more cells than those from littermate-matched lpr mice, but the composition was considerably modified. As shown in Fig. 7A, whereas B6 lpr mice at 3 mo of age contained, on the average, 8% CD8+ and 11% CD4+ lymph node T cells; age- and sex-matched lprFLIP mice had only 3% CD8+ T cells and a consequently increased proportion of CD4+ T cells to 38%. This was also reflected in the absolute numbers of each subset in spleens and lymph nodes (Fig. 7B), with lprFLIP mice bearing ~2-fold more CD4+ T cells and half the number of CD8+ T cells compared with lpr mice.

An interesting additional observation was a decreased proportion of CD4−CD8− TCRαβ cells with age in lprFLIP mice despite the slightly increased number of lymphoid tissues in these mice (Fig. 7B). CD4−CD8− TCRαβ cells in lpr mice are believed to derive from CD8+ precursors, as evidenced by the complete demethylation of the CD8α gene in lpr CD4−CD8− TCRαβ cells (22) as well as their near-complete absence in lpr mice lacking β2-microglobulin (23, 24). Because the CD8+ subset was considerably reduced in lprFLIP mice, this may explain the decreased proportion of CD4−CD8− TCRαβ cells from these mice.

In parallel with the decreased proportion of CD8+ T cells in lprFLIP mice, CD8+ T cells from these mice also manifested both greater caspase activity (Fig. 8A) as well as greater cell death (Fig. 8B) after CD3/CD28 stimulation than the same subset from lpr mice. These findings were consistent with the observation that active caspase-8 and associating c-FLIPL could also be identified in lpr effector T cells (data not shown), as was seen with wild-type effector T cells. Thus, Fas is dispensable for the association of caspase-8 with c-FLIPL and caspase activation in effector T cells.

**Discussion**

The current findings demonstrate that caspase activity increases after T cell activation and that a delicate balance is maintained to provide sufficient caspase activity to initiate cell cycling, but not to a level that promotes cell death. In this regard, c-FLIPL is probably an important regulator of caspase activity. The results are consistent with the view that c-FLIPL not only inhibits apoptosis induced by Fas, but can also directly promote caspase-8 activation independently of Fas (5, 6). As c-FLIPL levels increase in T cells, the resulting increased caspase activity reaches a level that promotes cell death, particularly in the CD8+ subset.

c-FLIPL was originally identified as a caspase-8 homologue that competes with caspase-8 for recruitment to FADD during Fas-induced death (1, 2, 25). As such, c-FLIPL inhibits Fas-mediated apoptosis. However, some of the original reports of c-FLIPL noted that it actually promoted cell death (26). This dilemma remained unresolved until recent nuclear magnetic resonance modeling studies revealed that c-FLIPL contains an activation loop that binds and opens the enzymatic pocket of caspase-8 when the two molecules heterodimerize (5, 6). In this capacity, c-FLIPL not only can block Fas-induced death, but also can directly activate caspase-8. This suggests that the level of c-FLIPL may greatly influence cell survival independently of death receptor ligation. Because caspase-8 activation is critical to initiate T cells cycling (11–13), a major question has been what molecule is responsible for the activation of caspase-8. We also observed in wild-type effector T cells that c-FLIPL was in association with active caspase-8. Thus, our findings do not merely reflect increased levels of c-FLIPL expression. They would also predict that in the absence of c-FLIPL in T cells, T cell activation would be diminished, as it is for caspase-8-deficient T cells (12).

The association of c-FLIPL with caspase-8 was apparent in actively proliferating T cells, but not in resting T cells, and was partly enhanced by the addition of z-VAD-fmk. This implies that the heterodimer formation may be reduced by persistent caspase activity. The ability of caspase-8 to cleave c-FLIPL at a known cleavage site (Asp376) to p43FLIP might reduce the mutual affinity of the two molecules. Cleavage of c-FLIPL by caspase-8 may represent a natural means to prevent excessive activation of caspase-8. This may also suggest a function for the known alternatively spliced short form of c-FLIP (c-FLIPs) (27). Because c-FLIPs lacks the activation loop found in c-FLIPL, but retains the death effector domains that bind FADD, it does not directly activate caspase-8, but it still retains the ability to inhibit Fas-induced recruitment of caspase-8 to FADD. In this regard it is of interest that the viral form of FLIP is only c-FLIPs and not full-length c-FLIPL (2).

It is presently not clear why wild-type effector CD8+ T cells manifest more caspase activity than CD4+ T cells. There are no apparent differences in the levels of either caspase-8 or c-FLIPL in resting wild-type CD4+ vs CD8+ cells. However, the naturally higher caspase activity of wild-type CD8+ T cells renders this subset particularly sensitive to increased c-FLIPL expression, resulting in selective loss of CD8+ T cells in c-FLIPL- Tg mice due to excess activation of caspase-8.

The ability of c-FLIPL to activate caspase-8 and the initiation of caspase activity by TCR ligation are both independent of Fas expression. The lprFLIP mice manifest the same selective loss of CD8+ T cells as c-FLIPL- Tg mice. LprFLIP T cells also hyperproliferate to TCR ligation, as seen with c-FLIPL- Tg T cells (A. Dohrman, unpublished observations). This suggests a dual function of c-FLIPL. In one situation, c-FLIPL can inhibit Fas-induced cell death by competing with recruitment of caspase-8 to FADD. In another context, c-FLIPL is able to heterodimerize with and activate caspase-8 independently of Fas. As the level of c-FLIPL increases, caspase-8 activation is also enhanced, eventually leading to cell death.
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Disclosures
The authors have no financial conflict of interest.

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