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Resistance of Short Term Activated T Cells to CD95-Mediated Apoptosis Correlates with De Novo Protein Synthesis of c-FLIP\textsubscript{short}\textsuperscript{1}

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In the early phase of an immune response, T cells are activated and acquire effector functions. Whereas these short term activated T cells are resistant to CD95-mediated apoptosis, activated T cells in prolonged culture are readily sensitive, leading to activation-induced cell death and termination of the immune response. The translation inhibitor, cycloheximide, partially overcomes the apoptosis resistance of short term activated primary human T cells. Using this model we show in this study that sensitization of T cells to apoptosis occurs upstream of mitochondria. Neither death-inducing signaling complex formation nor expression of Bcl-2 proteins is altered in sensitized T cells. Although the caspase-8 inhibitor c-FLIP\textsubscript{long} was only slightly down-regulated in sensitized T cells, c-FLIP\textsubscript{short} became almost undetectable. This correlated with caspase-8 activation and apoptosis. These data suggest that c-FLIP\textsubscript{short}, rather than c-FLIP\textsubscript{long}, confers resistance of T cells to CD95-mediated apoptosis in the context of immune responses. The Journal of Immunology, 2004, 172: 2194–2200.

The elimination of damaged or potentially dangerous cells by apoptosis is of pivotal importance for the organism. The role of apoptosis mediated by the death receptor CD95 (APO-1/Fas) has been investigated intensively, especially in the immune system (1, 2). Triggering of CD95 by either agonistic Abs or CD95L leads to oligomerization of CD95 receptors. Subsequently, the adapter molecule Fas-associated death domain (FADD),\textsuperscript{5} procaspase-8, and procaspase-10 are recruited to oligomerized CD95, forming a death-inducing signaling complex (DISC) (3). In the DISC, procaspase-8 and -10 are autoproteolytically cleaved and form the active enzymes (1, 2).

Two signaling pathways of CD95 were identified in different cell types (4). In type I cells, apoptosis is initiated by activation of large amounts of caspase-8 at the DISC, followed by rapid cleavage of caspase-3 before the loss of mitochondrial transmembrane potential ($\Delta \Psi_{M}$). In contrast, in type II cells, DISC formation is reduced, and the mitochondrial apoptosis pathway plays a dominant role. Prominent caspase activation occurs in type II cells only after the loss of $\Delta \Psi_{M}$ (4), indicating that mitochondria amplify the executionary apoptosis caspase cascade in these cells. Activation of mitochondria by death receptors is mediated by the Bcl-2 family member Bid. Bid is cleaved by caspase-8, and truncated Bid then translocates to the mitochondria and induces the release of apoptogenic factors, such as cytochrome c, SMAC/Diablo, Omi/HtrA2, and endonuclease G (5). In the cytoplasm, cytochrome c binds to Aapaf1, leading to procaspase-9 recruitment. At this protein complex, called apoptosome, caspase-9 is activated (5), which, in turn, activates executioner caspases, such as caspase-3 and caspase-7. Accordingly, Bcl-2 overexpression blocks activation of caspases downstream of mitochondria and subsequent apoptosis only in type II cells (4).

Death receptor-induced apoptosis can be counteracted by c-FLIP, also known as FLAME-1/I-FLICE/CaspertMRIT/CLARP/Usurpin (6, 7). c-FLIP occurs in two isoforms, a short form and a long form (c-FLIP\textsubscript{short} and c-FLIP\textsubscript{long}), which both can be recruited to the DISC. c-FLIP\textsubscript{long} has similar domains as caspase-8, but an inactive enzymatic site. When stably overexpressed, c-FLIP\textsubscript{long} interferes with generation of the large active subunit of caspase-8 at the DISC level in both CD95 type I and type II cells (8–10). However, although c-FLIP has been previously considered mainly to be a caspase-8 inhibitor, recent reports indicate that at low and physiological concentrations c-FLIP\textsubscript{long} may be required for efficient caspase-8 activation (11, 12). This is also reflected by c-FLIP-deficient mice, which are characterized by heart failure and have a strikingly similar phenotype as caspase-8-deficient mice. In contrast to c-FLIP\textsubscript{long}, the splice variant c-FLIP\textsubscript{short} structurally resembles viral FLIP (v-FLIP) and contains two death effector domains, but no caspase-like domain. High levels of c-FLIP\textsubscript{short} completely abolish cleavage of caspase-8 at the DISC (8). The role of c-FLIP proteins in T cells is controversial. c-FLIP\textsubscript{short} clearly contributes to the resistant phenotype of CD3-resimulated and CD28-costimulated T cells (13, 14). In contrast, although some studies show down-regulation of c-FLIP\textsubscript{long} during in vitro culture of primary T cells (15, 16), others did not find changes in c-FLIP\textsubscript{long} expression (9). Therefore, the exact roles of...
c-FLIP and its different isoforms in resistance of short term activated T cells remain to be determined.

Stimulation of resting peripheral T cells (referred to as day 0 T cells) during an immune response leads to their activation (day 1 T cells), resulting in increased expression of several genes, including cytokines and CD95 (2). However, despite high CD95 surface expression, day 1 T cells are resistant to CD95-mediated apoptosis (17, 18). Stimulation of previously activated T cells results in activation-induced cell death, which has been shown to involve the CD95 receptor/ligand system (1, 2). After prolonged culture in the presence of IL-2 (day 6 T cells), activated T cells develop an apoptosis-sensitive phenotype (17, 18). As CD95 surface expression changes only slightly between day 1 and day 6 T cells, the apoptosis-resistant phenotype of day 1 T cells must be caused by a block in CD95 signal transduction. Our laboratory previously showed that DISC formation is impaired in these resistant T cells, whereas the antiapoptotic protein Bcl-xL is highly up-regulated (9, 19). Moreover, resistant day 1 T cells can be sensitized to CD95-mediated apoptosis by treatment with the translation inhibitor cycloheximide (CHX) or the transcription inhibitor actinomycin D, indicating that de novo protein synthesis is required for activated T cells to maintain a resistant phenotype (17). We now show that inhibition of protein synthesis does not influence either DISC formation or the expression of antiapoptotic Bcl-2 family members. However, c-FLIP protein and, importantly, mainly the c-FLIP(s) variant were down-regulated, suggesting that not only the expression levels but also the differential degradation of the c-FLIP isoforms might be involved in the resistant phenotype of short term activated T cells.

Materials and Methods

Preparation of primary T cells and cell culture

Human peripheral T cells were prepared as described previously (17). For activation, resting T cells (day 0) were cultured at 2 × 10^6 cells/ml with 1 μg/ml PHA for 16 h (day 1). T cells were then washed three times and cultured for additional 5 days in the presence of 25 U/ml IL-2 (day 6). The human B lymphoblastoid cell line SKW6.4 was cultured in RPMI 1640 supplemented with 10% FCS, 50 μg/ml gentamicin, and 5 mM HEPES.

Abs and reagents

The mAbs against FADD and the polyclonal Abs against Bcl-xL were purchased from Transduction Laboratories (Lexington, KY). The anti-Bax Ab was obtained from Upstate Biotechnology (Lake Placid, NY), the anti-Bcl-2 Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the anti-caspase-10 Ab was obtained from MBL (Watertown, MA). The C15 mAb (mouse IgG2b) recognizes the p18 subunit of caspase-8 (20). The anti-c-FLIP mAb NF6 (mouse IgGl) was generated against GST-N-c-FLIP as previously described (9). Anti-APO-1 (anti-CD95) is an agonistic mAb IgG3, κ recognizing an epitope on the extracellular part of APO-1 (CD95/Fas) (21). The HRP-conjugated goat anti-rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated goat anti-mouse IgGl and IgG2b were obtained from Southern Biotechnology Associates (Birmingham, AL). Leucine zipper-tagged CD95 ligand (LZ-CD95) was produced as described previously (22). Cycloheximide was purchased from Sigma-Aldrich (St. Louis, MO) and used at a concentration of 10 μg/ml for the indicated time periods. All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich.

Immunoprecipitation and Western blot

Immunoprecipitation of CD95 DISC was conducted as previously described (3, 20). Briefly, 2 × 10^6 cells, either unstimulated or treated with 1 μg/ml anti-APO-1 for 5 min, were lysed in buffer containing 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM PMSF, and 1 μg/ml leupeptin, antipain, chymostatin, and pepstatin A) for 15 min on ice and centrifuged (15 min, 14,000 × g). Subsequently, DISC was precipitated with protein A beads (Sigma-Aldrich) for 4 h at 4°C. For Western blot analysis, postnuclear supernatant equivalents of 10^6 cells or 30 μg of protein, as determined by the bicinchoninic acid method (Pierce, Rockford, IL), were separated by 12% SDS-PAGE, blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Arlington Heights, IL), and blocked with 5% nonfat dry milk in PBS/Tween (0.05% Tween 20 in PBS). After washing with PBS/Tween the blots were incubated overnight with NF6 anti-c-FLIP, C15 anti-caspase-8, anti-FADD, or anti-Bcl-xL Abs at 4°C. Blots were washed again with PBS/Tween, incubated with HRP-coupled antibody-specific secondary Abs (1/20,000) for 1 h at room temperature, washed again, and developed with a chemiluminescence reagent (NEN, Boston, MA). For stripping, blots were incubated for 30 min in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-ME at 60°C. The blots were washed six times for 10 min each time in PBS/Tween and blocked again in 5% nonfat dry milk.

Surface staining

Cells (5 × 10^5) were incubated with 1 μg/ml anti-APO-1 for 15 min at 4°C, washed with PBS, incubated another 15 min with PE-labeled goat-anti-mouse Abs (Dianova, Hamburg, Germany), and after further washing analyzed in a FACScan cytometer (BD Biosciences, Mountain View, CA).

Cytotoxicity assay

For assaying apoptosis 10^6 cells were stimulated in 24-well plates with 1 μg/ml anti-APO-1 and 10 ng/ml protein A or were left untreated for 16 h at 37°C. Cells were centrifuged in a minifuge (Heraeus, New York, NY) at 4000 rpm for 5 min, washed once with PBS, and resuspended in a buffer containing 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100, and 50 μg/ml propidium iodide (Sigma-Aldrich). After incubation at 4°C in the dark for at least 16 h, apoptotic nuclei were quantified by FACScan (BD Biosciences). Specific apoptosis was calculated as follows: (% experimental apoptosis − % spontaneous apoptosis)/100 × 100.

Determination of mitochondrial membrane potential

To measure ΔΨM, anti-CD95 (1 μg/ml)-treated or untreated cells (5 × 10^6/ml) were incubated with 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; 5 μg/ml - FL-1; Molecular Probes, Eugene, OR) for 20 min at room temperature in the dark, followed by analysis on a flow cytometer (FACScan).

Results

Cycloheximide sensitizes short term activated T cells by affecting CD95 signaling upstream of mitochondria

Short term activated primary human T cells (day 1) are resistant to CD95-mediated apoptosis. However, after prolonged culture, these T cells (day 6) become sensitive to CD95-triggered apoptosis (17). Day 1 T cells can be sensitized to CD95-mediated apoptosis by CHX, an inhibitor of translation, suggesting that resistance to CD95-mediated apoptosis is dependent on protein biosynthesis (Fig. 1A). In contrast, CHX did not increase the sensitivity of resting T cells (day 0) or augment that of activated day 6 T cells. To exclude the possibility that CHX treatment simply up-regulates CD95 surface expression, resulting in higher sensitivity, we performed FACS analysis of untreated and CHX-treated T cells. CD95 was expressed in low amounts on day 0 T cells, but was strongly up-regulated on the cell surface of activated day 1 and day 6 T cells. The mean fluorescence intensities (MFI) are 10.7 for day 0, 69.6 for day 1, and 72.9 for day 6 T cells. Treatment with CHX led to a slightly decreased expression of CD95 on T cells regardless of their activation state, with MFI of 12.6 for day 0, 48.6 for day 1, and 49.1 for day 6 CHX-treated T cells (Fig. 1B). Thus, CHX apparently affected an intracellular factor in the signaling cascade of CD95.

Mitochondria have been shown to play a central role in apoptosis induction by many stimuli (5). To address at which level CHX sensitizes in the apoptotic signaling cascade, we measured the ΔΨM. In agreement with their resistant phenotype, the ΔΨM of anti-APO-1-treated day 1 T cells remained unchanged (Fig. 2). However, in the presence of CHX, the ΔΨM of anti-APO-1-treated cells was significantly decreased, although CHX alone had almost
Materials and Methods

As described in Materials and Methods, all experiments were performed in accordance with institutional guidelines for the care and use of laboratory animals. All animals were housed in a specific pathogen-free facility. The experimental protocol was approved by the local animal ethics committee.

Cultured overnight with or without 10 μg/ml CHX and subsequently cultured in IL-2-containing medium (d6) toward CD95-mediated apoptosis was determined. The sensitivity of resting day 0 (d0), PHA-activated (d1), and T cells subsequent cultured in IL-2-containing medium (d6) toward CD95-mediated apoptosis was determined in the presence or the absence of 10 μg/ml CHX as described in Materials and Methods. The d0, d1, and d6 T cells were cultured overnight with or without 10 μg/ml CHX. Subsequently, the surface expression of CD95 was analyzed by FACS staining. The data shown in A and B are representative for three independent experiments.

CHX affects the expression of c-FLIP, but not that of Bcl-2 family proteins

Next we addressed which apoptotic relevant molecules were affected by CHX treatment. Upstream of changes in ΔΨm are DISC components such as FADD, caspase-8, caspase-10, and c-FLIP as well as Bcl-2 family proteins such as Bcl-2, Bcl-xL, and Bax. CHX-induced sensitization of day 1 T cells could be associated with up-regulation of proapoptotic molecules or down-regulation of antiapoptotic molecules. To investigate the expression levels of DISC components and Bcl-2 family proteins, lysates of untreated day 0, day 1, and day 6 T cells as well as CHX-treated day 1 T cells were prepared and analyzed by Western blot. First, the expression of c-FLIP proteins, which are the most upstream regulators in the signaling cascade of CD95-mediated apoptosis, was analyzed. c-FLIPlong was similarly expressed in day 0 and day 1 T cells. Day 0, day 1, and day 6 T cells as well as CHX-treated day 1 T cells were incubated for another 2 h with LZ-CD95L in the presence or the absence of CHX (resulting in total time of treatment 1 h or were left untreated. After CHX pretreatment, 1 μg/ml anti-APO-1 was added, and the cells were cultured for an additional 2 h. Subsequently, cells were stained with the mitochondrial membrane potential-sensitive dye JC-1 and analyzed by FACS. The data shown are representative of three independent experiments.

6 T cells showed an ~2-fold reduction in protein expression. CHX treatment reduced c-FLIPlong expression of day 1 T cells to levels detected in day 6 T cells (Fig. 3A). In contrast, c-FLIPshort was only expressed in day 1 T cells, not in day 0 or day 6 T cells, and expression was substantially reduced by CHX (Fig. 3A). Thus, the expression of c-FLIPshort correlates with resistance to CD95-mediated apoptosis. Caspase-8, one of the initiator caspases in death receptor signaling, showed similar expression in day 0, day 1, and day 6 T cells and was only marginally reduced by CHX treatment. Therefore, caspase-8 may serve as a loading control (Fig. 3A). The expression of the other initiator caspase, caspase-10, and the proapoptotic Bcl-2 family member, Bax, was only slightly decreased by CHX, as was the loading control β-actin (Fig. 3B). FADD expression was decreased in a more pronounced manner, suggesting that FADD protein turnover is faster than that of other proapoptotic molecules. The expression of the antiapoptotic molecules Bcl-2 and Bcl-xL was slightly decreased comparable to that of the β-actin control. Although the ratio between both c-FLIP isoforms sometimes varied depending on the blood donor (compare, e.g., Fig. 3, A and B), analysis of the kinetics showed that c-FLIPshort was down-regulated faster than c-FLIPlong (Fig. 3C). Taken together, these results imply that loss of c-FLIPshort expression was most likely responsible for sensitization of day 1 T cells to CD95-mediated apoptosis.

Down-regulation of c-FLIPshort correlates with loss of ΔΨm

As prominent activation of caspase-8 takes place directly at the DISC of type I cells, but relies on a mitochondrial amplification loop in type II cells (4), we next analyzed the correlation between c-FLIP down-regulation and the downstream effects of CHX on ΔΨm in a time-course experiment. Day 1 T cells were pretreated for different time periods (0–3 h) with CHX. Subsequently, the cells were incubated for another 2 h with LZ-CD95L in the presence or the absence of CHX (resulting in total time of treatment with CHX of 0–5 h). Some of these cells were then tested for ΔΨm by JC-1 staining, whereas the remaining cells were lysed and subjected to Western blot analysis. As shown in Fig. 4A, the expression of c-FLIPlong and its p43 cleavage product decreased only
slightly after 4 h of CHX treatment and 5-fold when CHX was administered for 24 h. In contrast, the decrease in c-FLIP<sub>short</sub> expression was much more rapid after CHX treatment. After 2-3 h of CHX treatment, the expression of c-FLIP<sub>short</sub> was reduced more than 2-fold, and it was barely detectable by Western blotting after longer treatment. This is consistent with Fig. 3C. Moreover, loss of c-FLIP<sub>short</sub> expression correlated with the decrease in Δψ<sub>M</sub> in these cells (Fig. 4B). The loss of Δψ<sub>M</sub> was highest when c-FLIP<sub>short</sub> expression was virtually absent. Taken together, these results strongly suggest that the major factor responsible for sensitization of day 1 T cells is c-FLIP<sub>short</sub>, rather than-FLIP<sub>long</sub>.

CHX does not influence DISC formation

As c-FLIP proteins regulate CD95-mediated apoptosis by inhibiting caspase-8 activation at the DISC, we analyzed the DISC composition in day 1 and day 6 T cells in the presence or the absence of CHX. As a control, the DISC of the type I cell line SKW6.4 was immunoprecipitated in parallel. As previously reported (9), in day 1 T cells only weak DISC formation could be detected by Western blot analysis, as indicated by the detection of p43 c-FLIP<sub>long</sub> and c-FLIP<sub>short</sub>. In contrast, day 6 T cells formed a DISC comparable to the SKW6.4 cells, as detected by the presence of FADD and the intermediate p43/p41 cleavage fragments of caspase-8 (Fig. 5). Interestingly, full-length caspase-8 was hardly detectable in the DISCs of primary T cells, implying a more efficient cleavage in these cells. Taken together, these data suggest that, in terms of DISC formation, day 1 T cells behave like type II cells, whereas day 6 T cells behave like type I cells. Down-regulation of c-FLIP proteins by CHX led to reduced recruitment to the DISC. However, CHX treatment had no detectable effect on DISC formation, suggesting that CHX acts downstream of DISC formation.

Increased caspase-8 activation in the presence of CHX

Next we addressed activation of caspase-8 in day 1 T cells after CD95 engagement. Therefore, day 1 T cells were left untreated or were incubated with CHX for 4 h, a condition under which the expression of c-FLIP<sub>long</sub> and c-FLIP<sub>short</sub> is reduced and abolished, respectively. Subsequently, these cells were incubated with LZ-CD95L for different time periods. The activation of caspase-8 in
FIGURE 5. CHX does not influence DISC formation. PHA-activated (d1) and day 6-activated T cells (2 × 10^6) were treated for 4 h with 10 μg/ml CHX and subsequently stimulated with 2 μg/ml anti-CD95 (clone anti-AP0-1) for 5 min at 37°C or were left untreated. After lysis of the cells, unstimulated CD95 and DISC were immunoprecipitated by anti-CD95 Ab (clone anti-APO-1) and analyzed by Western blotting using anti-caspase-8, anti-c-FLIP, and anti-FADD mAbs. The positions of anti-APO-1 IgG H chain, procaspase-8, c-FLIP proteins, FADD, and the respective cleavage fragments are indicated. As a control, DISC formation of 10^7 SKW6.4 cells, which were stimulated with anti-APO-1 or were left untreated, was analyzed in parallel. The data shown are representative of two independent experiments.

Discussion

The immune response can be divided into three phases, namely activation, effector, and down phases. Upon Ag encounter, T cells are activated, proliferate, and subsequently exert their effector functions, e.g., elimination of invading pathogens. During these phases of the immune response, T cells are resistant to induction of CD95-mediated apoptosis (1, 2). After clearance of the Ag, most of the effector T cells die, and only a few survive as memory cells. Death by apoptosis in the down-phase of an immune response has been linked to limitations in cytokine availability (23, 24) as well as to activation-induced cell death, which is mainly mediated by the CD95 system (2). In vitro, short term activated T cells (day 1) are resistant to CD95-mediated apoptosis, whereas T cells during prolonged culture become sensitive (day 6) (17). We show in this study that short term activated T cells can be sensitized to apoptosis by the translation inhibitor CHX. This sensitization is not due to enhanced CD95 surface expression, differential expression of Bcl-2 family members, or increased DISC formation. However, sensitization correlated with a reduction of c-FLIP expression, in particular of the short form, c-FLIPshort. Loss of c-FLIPshort expression also correlated with a reduction of Δψm and increased formation of the active subunit p18 of caspase-8.

Although the expression of c-FLIPshort was completely abolished by CHX treatment, only a fraction of day 1 T cells became susceptible to CD95-mediated apoptosis. Thus, it appears that the resistance and sensitivity of short term activated T cells are regulated at multiple levels. c-FLIPshort seems responsible for a resistance mechanism that depends on de novo protein synthesis. In addition, CD95 signaling is regulated by the capability of DISC formation (9) and the expression of Bcl-xL (19, 25). The fact that day 1 T cells do not efficiently form a DISC (Fig. 5) (9) indicates that these cells behave like type II cells, which are dependent on mitochondrial events to execute apoptosis (4). However, mitochondria are protected in short term activated T cells by the expression of Bcl-xL (19, 25). This resembles the situation in long term activated T cells upon costimulation where inducible expression of c-FLIPshort and Bcl-xL confers resistance to CD95-mediated apoptosis by inhibiting DISC activity and by protection of mitochondria, respectively (14, 26). A third level of regulation is mediated by CD28-induced inhibition of CD95 ligand expression (14). Without costimulation, long term activated T cells acquire the ability to efficiently form a DISC and loose expression of Bcl-xL, and thereby are sensitized to CD95-mediated apoptosis. This switch from protected type II cells to sensitive type I cells is dependent on the presence of IL-2 (25). This complex regulation of CD95 resistance and sensitivity is important to facilitate an efficient immune response and to prevent the accumulation of activated T cells after Ag clearance.

Apoptosis sensitivity of T cells might be further controlled by the complex regulation of the relative expression levels of c-FLIPlong and c-FLIPshort. Although both c-FLIP isoforms had been previously regarded solely as apoptosis inhibitors, recent results have challenged this view by showing that c-FLIPlong might even promote caspase-8 activation and apoptosis, which presumably depends on the expression level (11, 12). The proapoptotic activity of c-FLIPlong is mediated by its heterodimerization with caspase-8 through the caspase-like domain that is present in c-FLIPlong, but not c-FLIPshort. It has been demonstrated that the affinity of the caspase-8/c-FLIPlong heterodimer to FADD is considerably higher than that of the caspase-8 homodimer (11). Thus, c-FLIPlong, in contrast to c-FLIPshort, might allosterically modulate caspase-8 activation and might act as an apoptosis inhibitor only at high concentrations, achievable mostly after ectopic expression. Of note, in primary T cells c-FLIPlong levels are ~100 times lower than those of caspase-8 (9), which also suggests that c-FLIPlong is not a critical mediator of T cell resistance. In our experiments the expression of c-FLIPlong was only slightly reduced, but could still be observed after CHX treatment for several hours (Figs. 3B and 4A). This together with a constant expression of c-FLIPlong in T cells of different activation stages (9) strongly argue against a central role of c-FLIPlong in resistance of T cells to CD95-mediated apoptosis. The present and previously published data of our laboratory suggest that c-FLIPshort rather than c-FLIPlong is the prime inhibitor of CD95-mediated apoptosis in T cells.
Apoptosis sensitivity has been correlated with c-FLIP expression in several other cell types, including dendritic cells (27, 28), keratinocytes (29), vascular smooth muscle cells (30), and endothelial cells (31, 32). Recent studies reveal that FLIP proteins might also play a role in tumorigenesis. In EBV-transformed B cells, resistance to CD95-mediated death correlates with the ratio between c-FLIP and caspase-8 (33, 34). In addition, the sensitivity of non-Hodgkin B cell lymphomas correlated with the expression of c-FLIP proteins (35). In view of the newly described dual function of c-FLIP as an inhibitor as well as an activator of caspase-8 activation, the impact of individual c-FLIP isoforms on apoptosis sensitivity has to be re-examined in the different cell types.

The rapid decrease in c-FLIP expression indicates that FLIP proteins have a short half-life, probably regulated by proteolytic degradation. Our results suggest that not only the expression levels and post-transcriptional mechanisms, such as gene splicing, but also the differential degradation of the c-FLIP isoforms might be important determinants of apoptosis sensitivity. In addition to primary T cells, down-regulation of c-FLIP by CHX has been observed in other cellular systems (28, 36–38). However, the kinetics of c-FLIP degradation differ in the cellular systems analyzed. In some cases CHX treatment did not lead to reduced c-FLIP expression (39–41). Moreover, inhibition of the proteasome was shown to cause elevated or reduced c-FLIP levels, respectively (42, 43). Thus, the molecular mechanisms of c-FLIP degradation may vary in different cell types. In most cell types, mainly the expression and degradation of c-FLIP short have been examined. This might be due to the preferential expression of this isoform or the use of Abs that could not detect c-FLIP short. Further insight into the regulation of c-FLIP expression and its degradation might be important for understanding apoptosis regulation and also promote the development of specific c-FLIP inhibitors useful for the treatment of lymphoid disorders.

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