Up-regulation of c-FLIP<sub>short</sub> by NFAT contributes to apoptosis resistance of short-term activated T cells

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Upon encounter with pathogens, T cells activate several defense mechanisms, one of which is the up-regulation of CD95 ligand (CD95L/FasL) which induces apoptosis in sensitive target cells. Despite expression of the CD95 receptor, however, recently activated T cells are resistant to CD95L, presumably due to an increased expression of antia apoptotic molecules. We show here that, in contrast to naive or long-term activated T cells, short-term activated T cells strongly up-regulate the caspase-8 inhibitor, cellular FLICE-inhibitory protein (c-FLIP). Intriguingly, upon activation, T cells highly induced the short splice variant c-FLIP<sub>short</sub>, whereas expression of c-FLIP<sub>long</sub> was only marginally modulated. In contrast to the general view that c-FLIP transcription is controlled predominantly by nuclear factor-κB (NF-κB), induction of c-FLIP<sub>short</sub> in T cells was primarily mediated by the calcineurin-nuclear factor of activated T cells (NFAT) pathway. Importantly, blockage of NFAT-mediated c-FLIP expression by RNA interference or inhibition of calcineurin rendered T cells sensitive toward CD95L, as well as activation-induced apoptosis. Thus, the resistance of recently activated T cells depends crucially on induction of c-FLIP expression by the calcineurin/NFAT pathway. Our findings imply that preventing autocrine CD95L signaling by c-FLIP facilitates T-cell effector function and an efficient immune response. (Blood. 2008;112:690-698)

Introduction

Activation of naive peripheral T cells by their cognate antigen results in induction of their proliferation and differentiation into T effector cells. These comprise CD4<sup>+</sup> helper cells and CD8<sup>+</sup> cytotoxic T cells, which in turn can induce immune responses by the secretion of cytokines, cell-cell interactions, or their ability to directly kill target cells. Importantly, despite high expression of death receptors such as CD95, effector T cells display an antia apoptotic phenotype allowing immune defense function and clearance of invading antigens.¹,² Once the infection has been efficiently contained, these T cells become apoptosis-sensitive, leading to deletion of most cells and survival of a few memory T cells.³,⁴ Apoptosis thus represents a tightly regulated process by which lymphocyte homeostasis is controlled and the emergence of autoreactive cells is prevented.

Upon activation, T cells show a rapid up-regulation of antiapoptotic proteins. In addition to Bcl-x<sub>L</sub>, a strong increase in FLICE-inhibitory proteins (FLIPs) is most prominent.⁵⁻⁷ FLIPs inhibit death-receptor-mediated apoptosis by preventing the cleavage of death-inducing signaling complex (DISC)–associated procaspase-8 and −10 to the mature active enzymes, thereby blocking initiation of the extrinsic apoptosis cascade.⁸,⁹ Three different isoforms of the cellular FLIP (c-FLIP) have been reported so far, namely c-FLIP<sub>long</sub>, c-FLIP<sub>short</sub>, and c-FLIP<sub>R</sub>, which are generated by alternative splicing.⁵,⁶,⁹ All 3 isoforms comprise a tandem death effector motif (DED) that is crucial for their recruitment into the DISC.¹⁰ Additionally, c-FLIP<sub>long</sub> contains an inactive caspase-like domain, whereas each of the 2 short isoforms, c-FLIP<sub>short</sub> and c-FLIP<sub>R</sub>, possesses a unique truncated C-terminus. For the latter ones only antiapoptotic functions have been described so far, whereas the role of c-FLIP<sub>R</sub> remains controversial. Next to its antiapoptotic abilities, low levels of c-FLIP<sub>long</sub> have also been shown to promote cell death by increasing the enzymatic activity of caspase-8.¹¹⁻¹³ In vitro studies with primary human T cells have shown that FLIP proteins, especially c-FLIP<sub>short</sub>, are highly up-regulated upon T-cell activation, which correlates with a protection against CD95-mediated apoptosis.⁷,¹⁴ Conversely, treatment of activated T cells with the protein translation inhibitor cycloheximide reduced c-FLIP expression, without affecting Bcl-x<sub>L</sub>, expression, and rendered the cells apoptosis sensitive.⁷ Therefore, c-FLIP proteins are thought to play a central role in the protection of activated T cells.

How c-FLIP expression in T cells is regulated at the molecular level is a matter of active debate. Inducible c-FLIP expression in peripheral T cells has primarily been linked to the nuclear factor-κB (NF-κB) signaling pathway;¹⁵⁻¹⁸ however, other transcription factor–binding sites do exist in the FLIP promoter. In addition, studies in various tumor cell lines have indicated a regulation of c-FLIP expression by the PI3K/Akt and Erk/MAP kinase pathway.¹⁹ To elucidate the regulation of c-FLIP expression in T lymphocytes, we investigated several signaling pathways for their involvement in activation-induced up-regulation of c-FLIP. Surprisingly, we found that c-FLIP proteins, and especially c-FLIP<sub>short</sub> expression, were mainly regulated via the calcineurin/nuclear factor of activated T cells (NFAT) pathway. Analysis of the human c-FLIP promoter revealed 3 putative NFAT binding sites in front of exon 1, of which 2 are actively used in T cells for induction of c-FLIP expression. Furthermore, down-regulation of c-FLIP expression by RNA interference or the calcineurin inhibitors cyclosporine A or FK506 sensitized T cells to apoptosis, supporting a central...

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role of NFAT-mediated c-FLIP expression for the protection of activated T cells.

Methods

Cell culture and stable transfections

Approval for these studies was obtained from the institutional review board of Heinrich-Heine-University Düsseldorf’s medical faculty. Informed consent was obtained in accordance with the Declaration of Helsinki.

The human T-cell lines HuT78 and CEM were cultured in RPMI 1640 (PAA Laboratories, Cölbe, Germany) supplemented with 10% fetal calf serum (BioWest, Frickenhausen, Germany) and 50 μg/mL each of penicillin and streptomycin (Invitrogen, Karlsruhe, Germany). Human embryonic kidney cells (293T) were cultured in Dulbecco modified Eagle medium (DMEM high glucose; PAA Laboratories) supplemented with 10% fetal calf serum and 50 μg/mL each of penicillin and streptomycin. HeLa human cervix carcinoma cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 50 μM β-mercaptoethanol, and 50 μg/mL each of penicillin and streptomycin.

Preparation and activation of human peripheral T cells

Human peripheral T cells were isolated by Ficoll-Hypaque density gradient centrifugation (Biochrom, Berlin, Germany) from buffy coat material obtained from the Institute of Hemostasis and Transfusion Medicine of the University Hospital Düsseldorf. Monocytes were depleted by incubation of peripheral blood lymphocytes (PBLs) for 1 hour in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μg/mL each of penicillin and streptomycin in 175-cm² cell culture flasks. Nonadherent cells were collected and B lymphocytes were removed by magnetic-activated cell sorting (MACS) via CD20⁺ MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified T lymphocytes were incubated with 5 μg/mL Leucagglutinin (PHA-L) (Sigma, Deisenhofen, Germany) overnight if not stated otherwise. For further cultivation for up to 6 days, T cells were washed 3 times in phosphate-buffered saline (PBS) and supplemented with 25 U/mL interleukin-2 (IL-2; Tebu-bio, Offenbach, Germany). Human embryonic kidney cells (293T) were cultured in Dulbecco modified Eagle medium (DMEM high glucose; PAA Laboratories, Coëble, Germany) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 μg/mL each of penicillin and streptomycin.

Transient transfections and luciferase assays

Transient transfection of 293T cells was performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocols. For luciferase assays, 4 μg of a pG3L reporter construct containing nucleotides −501 to +104 relative to the proposed transcriptional start site of CFLAR (c-FLIP) exon 1 were cotransfected with 0.4 μg of a control reporter construct containing the Rous sarcoma virus promoter in front the β-galactosidase (lacZ) gene. The c-FLIP promoter construct was kindly provided by Dr Wafik El-Deiry (Philadelphia, PA). In addition to the luciferase constructs, 2 μg of an NFATc1 or NFATc2 plasmid were cotransfected with 2 μg of a plasmid encoding a constitutively active form of calcineurin. The plasmids encoding NFATc1, NFATc2, and calcineurin were a kind gift of Dr Edgar Serfling (Würzburg, Germany). After a 24-hour transfection, cells were lysed in 100 μL of 25 mM glycylglycine/pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 1 mM dithiothreitol, and 1% Triton X-100 and were centrifuged at 20 000g at 4°C for 5 minutes. Then, 100 μL reaction buffer (25 mM glycylglycine/pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 1 mM dithiothreitol, 2 mM ATP, 15 mM potassium phosphate, pH 7.8) was added to 30 μL supernatant and luciferase activity was measured after injection of 100 μL luciferin (0.3 mg/mL) using a microplate luminometer (Berthold Technologies, Bad Wildbad, Germany). For normalization, β-galactosidase assay was performed using the Galacto Light system (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. FLIP promoter mutants were generated by using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and verified by DNA sequencing.

Lentiviral infection of cells

c-FLIP MISSION TRC shRNA Target Set clone into the lentiviral vector pLKO.1 was purchased from Sigma. c-FLIPshort shRNA oligonucleotides (see Document S1, available on the Blood website; see the Supplemental Materials link at the top of the online article) were annealed and cloned into the lentiviral vector pLKO.1. Lentiviral vectors were cotransfected with the envelope vector pMD2.G (Addgene no. 12259) and the gag-pol expression plasmid pCMV_dr8.2dvr (Addgene no. 8455) into 293T cells as described in “Transient transfections and luciferase assays.” Lentiviruses (LVs) were harvested 36 hours and 60 hours after transfection. Crude virus was filtered through 0.45-μm PVDF filters (Millipore, Billerica, MA), concentrated by ultracentrifugation at 100 000g for 60 minutes at 4°C and stored at −80°C until further use. Titers were determined by infection of HeLa cells with serial dilutions of LV stocks and 5 μg/mL polybrene (Sigma). Specific knockdown of the various c-FLIP isoforms was verified by Western blot analysis. CEM cells were infected by adding LVs and 5 μg/mL polybrene to 2 × 10⁶ cells. Cells were centrifuged for 1 hour at 860g and then incubated overnight. At 48 hours after infection, cells were cultured for 2 weeks in selection medium containing 2 μg/mL puromycin (Sigma). Successful integration of LVs was confirmed by Western blot analysis.

RNA isolation, RT-PCR, and quantitative real-time PCR

Total RNA was isolated from 5 × 10⁶ to 10 × 10⁶ cells with the RNeasy kit (Qiagen, Hilden, Germany). Total mRNA (1 μg) was used for cDNA synthesis using the SuperScript III First-Strand reverse transcriptase–polymerase chain reaction (RT-PCR) kit of Invitrogen. Real-time PCR was carried out on an Applied Biosystems 7300 Real-Time PCR system using the Quantitect SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer’s instructions. Measurements were run in triplicate and normalized to GAPDH values.

FACS assays

Cell-surface staining and cytotoxicity assays were performed as described previously. For assaying apoptosis, 5 × 10⁵ cells were stimulated for 16 hours in 24-well plates (if not stated otherwise) with CD95L, or left untreated for the indicated times. CD69, CD25, and CD3 antibodies used for cell-surface staining were purchased from BD Biosciences (Heidelberg, Germany). CD95 surface staining was performed with 2R2 antibody, which was purified and FITC-conjugated in house. For blocking activation-induced apoptosis, neutralizing anti-CD95L (clone 5G51; BioCheck, Münster, Germany) was added at 5 μg/mL to cell cultures. Specific apoptosis was calculated as follows: (% experimental apoptosis − % spontaneous apoptosis) / (100 − % spontaneous apoptosis) × 100.

Western blot analysis

Western blot analysis was performed as described previously. Quantification of protein expression was carried out using a LAS 3000 CCD camera (Fujifilm, Düsseldorf, Germany) and AIDA software (Raytest, Sprockhövel, Germany).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) using nuclear extracts of primary peripheral T lymphocytes or HuT78 cells was performed according to standard procedures. A detailed protocol can be found in the supplementary information.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using a ChIP kit from Upstate Biotechnology (Lake Placid, NY). For each precipitation
2.5 × 10^6 CEM cells were fixed for 25 minutes at 37°C by adding formaldehyde directly to the culture medium at a final concentration of 1%. Cells were washed twice in ice-cold PBS with protease inhibitors (1 mM PMSF and 5 μg/mL each of aprotinin, leupeptin, pepstatin A, and chymostatin) and lysed in sodium dodecyl sulfate (SDS) solution (1% SDS, 10 mM Tris/HCl, pH 8.0) for 10 minutes at 4°C. Lysates were sonicated (Sonoplus; Bandelin Electronics, Berlin, Germany) 3 times (30% power, 0.5 seconds) to shear genomic DNA into fragments of 200 bp to 1200 bp. After 10-fold dilution in a solution of 0.01% SDS, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl (pH 8.0), lysates were precleared with 75 μL salmon sperm DNA-saturated protein A agarose (50% vol/vol) for 30 minutes at 4°C. The agarose was removed by centrifugation and each sample was incubated with 10 μg anti-NFATc1 (sc-13033-X; Santa Cruz Biotechnologies, Santa Cruz, CA), anti-NFATc2 (4G6-G5; Santa Cruz Biotechnologies), rabbit IgG, or mouse control IgG overnight at 4°C. Protein/DNA complexes were harvested and washed according to the manufacturer's protocol. After reversal of the cross-linking by incubation at 65°C for 4 hours, DNA was isolated with a PCR purification kit (Qiagen) and amplified using primers specific for both NFAT binding sites.

Results

We have previously reported that primary T cells up-regulate c-FLIPshort after 1 day of T-cell receptor (TCR) stimulation.7,14 For a more detailed analysis, we stimulated freshly isolated primary human T cells (d0) with the lectin PHA or anti-CD3ε antibodies overnight to trigger TCR activation and analyzed the cells directly (d1) or after culture for an additional 2 days (d3) or 5 days (d6) with IL-2. IL-2 was added because of its role as a survival and growth factor as well as its ability to sensitize T cells for activation-induced cell death.14 Both PHA and anti-CD3ε strongly induced the expression of c-FLIPshort, which was absent in freshly isolated T cells (Figure 1A). Importantly, induction of c-FLIPshort expression was only transient and declined upon longer cultivation. In contrast, expression of c-FLIPlong and Bcl-xL was detectable under all culture conditions and was only slightly up-regulated on day 1 (Figure 1A). The expression of XIAP and Bcl-2, 2 other antiapoptotic proteins, and the MAP kinase (MAPK) Erk2 remained unchanged in the course of T-cell activation (Figure 1A).

To investigate the mechanism of c-FLIPshort up-regulation, we first used pharmacologic inhibitors of several signaling pathways. Inhibition of the 3 classic MAPK pathways (Erk, p38MAPK, and JNK) by PD098059, SB203580, and JNK-II, respectively, had no effect on c-FLIPshort expression (Figure 1B). Even when used at higher micromolar concentrations, c-FLIP expression remained unaffected by MAPK inhibitors (Figure S1A). Similarly, the inhibitors wortmannin (PI3K), rapamycin (mTOR), and SB415286 (GSK3) also showed no effect (Figure S1B). However, expression of c-FLIPshort was significantly reduced in d1 T cells treated with the immunosuppressant cyclosporine A (CsA) (Figure 1B). Interestingly, CsA had only a minor effect on the expression of c-FLIPlong, which was similar to that observed in d0 T cells. Of note, CsA reduced but did not abolish T-cell activation, as determined by the expression of the activation markers CD25 and CD69 on the surface of d1 T cells (Figure 1C). In contrast, the cdk2 inhibitor roscovitine suppressed c-FLIP up-regulation most likely by a complete inhibition of T-cell activation (Figure S1B, data not shown).
Figure 2. Block of c-FLIP up-regulation in short-term activated T cells by inhibition of calcineurin.
(A) Human peripheral T cells were lysed immediately (d0) or stimulated for 16 hours (d1) with 5 µg/mL PHA-L in the presence or absence of 1 µM cyclosporine A (CsA), 100 nM FK506, 200 nM rocaiglamide, or 10 µM VIVIT peptide. c-FLIP expression was analyzed by Western blotting. Tubulin or β-actin served as a loading control. (B) Inhibition of c-FLIPlong and c-FLIPshort up-regulation in short-term activated T cells by CsA and FK506 was analyzed by quantitative Western blotting. Protein expression levels are represented as the means plus or minus the standard deviation (SD) of at least 4 independent experiments. (C,D) Quantification of c-FLIP mRNA levels in unstimulated versus PHA-L–, PHA-L/CsA–, and PHA-L/FK506–treated peripheral T cells of 5 different blood donors by real-time PCR. Panel D displays the percentage of inhibition of c-FLIP mRNA up-regulation in CsA- and FK506-treated cells.

CsA inhibits the phosphatase calcineurin, which dephosphorylates and thereby activates transcription factors of the NFAT family.22 As NFAT plays a major role in T-cell biology, we wanted to confirm that CsA inhibits c-FLIP expression by interfering with calcineurin-mediated NFAT activation. Therefore, we used additional inhibitors of the NFAT pathway, such as FK506,23,24 rocaiglamide,25 and VIVIT peptide, which blocks calcineurin-NFAT interaction.20 As shown in Figure 2A, FK506 and the VIVIT peptide inhibited up-regulation of c-FLIPshort to an extent similar to CsA, whereas c-FLIPlong expression was again only marginally affected. Interestingly, rocaiglamide strongly reduced expression of both c-FLIP isoforms, which might be explained by its ability to inhibit both NFAT and NF-κB.20 Taken together, the potent induction of c-FLIPshort in T cells relies strongly on the calcineurin/NFAT pathway.

We then performed quantitative Western blot analysis to determine the extent to which the calcineurin/NFAT pathway contributes to c-FLIPshort induction. Upon T-cell activation, c-FLIPshort was up-regulated approximately 5-fold compared with freshly isolated T cells (Figure 2B left panel). Addition of CsA or FK506 reduced the expression to a 2-fold induction compared with unstimulated control cells (Figure 2B top panel). In contrast, c-FLIPlong was induced upon TCR stimulation at most 2-fold, and inhibition of calcineurin by CsA or FK506 had only marginal effects (Figure 2B bottom panel). Next, we asked whether induction of c-FLIPshort was indeed regulated at the transcriptional level. To this end, we performed real-time RT-PCR experiments with mRNAs from different blood donors and analyzed c-FLIPshort and c-FLIPlong transcript levels in freshly isolated T cells (d0), PHA-activated T cells (d1), and d1 T cells either treated with either CsA or FK506. The mRNA of c-FLIPlong was induced at most 3-fold upon activation with PHA (Figure 2C bottom panel). Addition of CsA or FK506 attenuated c-FLIPlong induction only slightly. In contrast, PHA up-regulated c-FLIPshort mRNA 5-fold to 27-fold depending on the donor (Figure 2C top panel), whereas addition of CsA or FK506 reduced expression by 40% to 60% (Figure 2C,D), suggesting that c-FLIPshort is regulated by NFAT at the transcriptional level in primary human T cells.

To assess whether the c-FLIP gene (CFLAR, gene ID 8837) is a direct target of NFAT transcription factors, we initially undertook a promoter analysis of the human c-FLIP (CFLAR) promoter. CFLAR is located on human chromosome 2 between the genes NDUFB3 (encoding NADH dehydrogenase [ubiquinone] 1β subcomplex 3; ending at position 201,658,710) and CASP10 (encoding caspase-10; starting at position 201,756,100). As a first step, we used the gene2promoter software (Genomatix, Munich, Germany) to determine possible promoter sequences within this chromosomal region. The analysis suggested 2 potential promoter regions, one directly upstream of exon 1 comprising part of the 5′ UTR and another adjacent to exon 3 containing the start codon (Figure 3A). We then analyzed the 2 potential promoters using the MatInspector (Genomatix) software for the prediction of potential NFAT-binding sites.27 The first promoter region upstream of exon 1 comprises 3 potential NFAT-binding sites (Figure 3B), whereas in the second putative promoter no NFAT-binding sites could be identified (data not shown). Notably, the first promoter also contained one NF-κB site (Figure 3B), which may explain the NF-κB–dependent expression of c-FLIP in certain cell types.

To investigate whether T-cell activation induces binding of NFAT to these putative sites in the c-FLIP promoter region 1, we performed electrophoretic mobility shift assays (EMSA) using nuclear extracts of the human T-cell line HuT78. Similar to primary T cells, activation of HuT78 cells by Phorbol 12-myristate 13-acetate (PMA) and ionomycin triggered a strong up-regulation of c-FLIPshort that was efficiently blocked by CsA (Figure 4A). For EMSAs, oligonucleotides were used comprising 1 of the 3 potential binding sites in promoter region 1, termed sites 1, 2, and 3 (Figure 3B). T-cell activation rapidly induced DNA-binding activity to site 1 and 2 probes (Figure 4B), but not to an oligonucleotide comprising the binding site 3 (data not shown). Interestingly, binding to the first putative NFAT site occurred more rapidly with the strongest shift after 1 hour, whereas DNA binding to the second site was slightly delayed, peaking after 2 hours of cell activation. Binding to the radioactive NFAT consensus oligonucleotide was efficiently competed by an excess of unlabeled site 1 or site 2 oligonucleotides, resulting in a concentration-dependent decrease of the shifted complex, whereas unlabeled site 1 or site 2 probes...
mutated in the NFAT recognition sequence had no effect on protein-DNA complex formation (Figure 4C).

In order to determine which of the NFAT proteins bind to the FLIP promoter, we performed supershift experiments with antibodies specific for NFATc1, NFATc2, and NFATc3. Supershift or disappearance of the NFAT-DNA complex could be detected by incubation of nuclear extracts with antibodies against NFATc2 and NFATc1, but not NFATc3 (Figure 4D). Because transcription factor AP-1 can associate with NFAT under certain conditions,28 we also included a c-Jun antibody in the supershift analyses. Interestingly, the c-Jun antibody did not reduce or supershift the NFAT-DNA complex, suggesting that AP-1 was not coupled to NFAT at the 2 sites of c-FLIP promoter (Figure 4D). Furthermore, we prepared nuclear extracts from freshly isolated, primary human T lymphocytes which also demonstrated an activation-dependent and CsA-inhibitable NFAT binding to site 1 and site 2 (Figure 4E,F).

To analyze the transcriptional activation of the human c-FLIP promoter by NFAT, luciferase assays were performed using a c-FLIP reporter construct (pGL3-c-FLIP) comprising the
promoter region 1 with both NFAT-binding sites (Figure 5A). For this purpose, we transiently expressed NFATc1 or NFATc2 together with a constitutively active form of calcineurin. Calcineurin was cotransfected because NFAT transcription factors are readily phosphorylated and thereby inactivated upon expression. Indeed, overexpression of NFATc1 as well as of NFATc2 in 293T cells resulted in an increased luciferase activity (Figure 5B). Moreover, mutation of the proposed NFAT binding sites strongly reduced luciferase expression, indicating that induction of the c-FLIP promoter by NFAT was indeed regulated by the suggested binding sites (Figure 5B). Next, we tested direct binding of NFAT transcription factors to the endogenous c-FLIP promoter by chromatin immunoprecipitation (ChIP). A stimulation-dependent binding of NFATc1 and NFATc2 to the first binding site was observed in CEM cells, confirming that c-FLIP was indeed a direct transcriptional target of NFAT (Figure 5C). Unfortunately, we were unable to analyze site 2 by ChIP, most likely because the surrounding A-T-rich sequences prevented an efficient PCR amplification. Nevertheless, these experiments clearly show that c-FLIP is a bona fide NFAT target gene.

FLIP proteins are strongly up-regulated in activated T cells and inhibit apoptosis by preventing the cleavage of initiator caspases in the DISC. To further determine the importance of the c-FLIP isoforms, we treated the human T-cell lines HuT78 and CEM with the CD95L to trigger the extrinsic apoptosis cascade. Both cell lines were highly apoptosis-sensitive, as detected by a CD95L-induced increase of DNA fragmentation (Figure 6). CEM cells, like HuT78 cells, strongly up-regulated FLIP proteins upon activation, which was blocked by the addition of CsA (Figure S2A). Accordingly, T-cell activation led to a certain degree of apoptosis, which was almost completely prevented by a neutralizing CD95L antibody (Figure 7B). In line with the reduced c-FLIP expression, CEM cells expressing either of the c-FLIP shRNAs displayed increased apoptosis sensitivity upon activation, in comparison to the respective control cells. Interestingly, cells with a c-FLIPshort knock-down (c-FLIP or c-FLIPS/L shRNA) were more sensitive than cells expressing an shRNA targeting specifically c-FLIPlong (Figure 7B). Stimulation of knock-down cells with increasing concentrations of CD95L led to strongly elevated apoptosis (Figure 7C). Similarly to untransfected cells (Figure 6), activation with PMA/ionomycin rescued control cells from apoptosis, whereas c-FLIP knock-down cells remained sensitive toward CD95L

In order to delineate the relative contributions of the short and long isoforms to this protection, we next used RNA interference and stably infected CEM cells with lentiviral shRNA constructs targeting c-FLIPlong, c-FLIPshort, or both isoforms (Figure 7A). The knock-down of c-FLIPlong was less efficient than that of c-FLIPshort, which may be explained by the different half-life of the 2 proteins.7–29 Activation of CEM cells by PMA/ionomycin induced an up-regulation of CD95L and a certain degree of apoptosis, which was almost completely prevented by a neutralizing CD95L antibody (Figure 7B). In line with the reduced c-FLIP expression, CEM cells expressing either of the c-FLIP shRNAs displayed increased apoptosis sensitivity upon activation, in comparison to the respective control cells. Interestingly, cells with a c-FLIPshort knock-down (c-FLIP or c-FLIPS/L shRNA) were more sensitive than cells expressing an shRNA targeting specifically c-FLIPlong (Figure 7B). Stimulation of knock-down cells with increasing concentrations of CD95L led to strongly elevated apoptosis (Figure 7C). Similarly to untransfected cells (Figure 6), activation with PMA/ionomycin rescued control cells from apoptosis, whereas c-FLIP knock-down cells remained sensitive toward CD95L.

Figure 5. The human c-FLIP promoter is induced upon NFAT activation. (A) Schematic representation of the luciferase reporter construct comprising the human c-FLIP promoter 1 (in gray). The reporter construct contains both putative NFAT binding sites indicated as . (B) Luciferase assays were performed in 293T cells transiently transfected with the c-FLIP reporter construct (pGL3-FLIP), an empty vector control (pGL3), or, as a positive control, a reporter construct comprising 3 tandem repeats of the NFAT binding site of the IL-2 promoter (NFAT). Expression plasmids encoding either NFATc1 or NFATc2 and constitutively active calcineurin were cotransfected. At 24 hours after transfection, luciferase activity was quantified. β-galactosidase activity was used for normalization. Results are displayed as the mean plus or minus SD of 3 independent experiments. (C) ChIP analysis of the c-FLIP promoter in CEM cells stimulated for the indicated time points with 20 ng/mL PMA and 1 μM ionomycin. ChIP procedure was done as described in “Chromatin immunoprecipitation.” NFAT-chromatin complexes were immunoprecipitated with NFATc2 and NFATc1 antibody, respectively. Rabbit IgG (rIgG) and mouse IgG (mIgG) were used as negative controls. PCR was performed with primers specific for the NFAT binding site 1 of the c-FLIP promoter and analyzed by agarose gel electrophoresis. Genomic DNA (input) was used as a positive control in the PCR analysis.

Figure 6. Cyclosporine A abolishes activation-induced resistance to apoptosis. (A) HuT78 and (B) CEM cells were either left untreated or stimulated for 16 hours with 20 ng/mL PMA and 1 μM ionomycin (Iono) with or without 1 μM CsA. Cells were then treated for an additional 8 hours with the indicated concentrations of CD95L. Apoptosis was determined by measuring DNA fragmentation and cell nuclei with hypodiploid DNA.
Cells expressing shRNAs against both c-FLIP isoforms were highly sensitive to apoptosis and significantly more susceptible than isoform-specific knock-down cells. Of note, while both single knock-down cells showed a similar apoptosis sensitivity upon addition of CD95L, c-FLIPshort single knock-down cells displayed a higher overall apoptosis rate due to the increased level of activation-induced apoptosis (Figure 7D).

To analyze whether c-FLIP is the major antiapoptotic target of the calcineurin/NFAT pathway, we tested whether CsA can still sensitize the different knock-down cell lines. CsA sensitized control shRNA-expressing cells toward apoptosis, whereas cells in which c-FLIPshort or both isoforms were knocked down showed only a marginal PMA/ionomycin-mediated protection against apoptosis and a strongly reduced sensitization by CsA (Figure 7D). Interestingly, c-FLIPshort single knock-down cells were still markedly sensitized by CsA, which was most likely due to the CsA-induced inhibition of c-FLIPshort expression. Altogether, these data suggest that c-FLIP plays a central role in the protection against CD95-mediated apoptosis upon T-cell activation. Furthermore, apoptosis inhibition in activated T cells appears to be predominantly regulated by the short rather than the long c-FLIP isoform.

**Discussion**

Previous reports have shown that c-FLIP and particularly the truncated isoform c-FLIPshort is up-regulated upon T-cell activation.7,9,14,30,31 The signaling mechanisms leading to c-FLIP gene induction in short-term activated T cells, however, have not directly been investigated. We show here by various means including pharmacologic inhibition, reporter gene assays as well as EMSA and ChIP analyses, that c-FLIP is a bona fide NFAT target gene in T cells. Moreover, we demonstrate that induction of c-FLIP in recently activated T cells is important to prevent activation-induced suicide mediated by a concomitant up-regulation of the CD95L. In this context, it is important to note that treatment with CsA almost exclusively affected c-FLIPshort expression and that CsA-treated T cells were as sensitive toward CD95L as T cells with a knock-down of both c-FLIP isoforms. Moreover, isoform-specific knock-down of c-FLIP splice variants revealed that particularly c-FLIPshort-deficient cells were sensitive toward activation-induced apoptosis. Therefore, c-FLIPshort rather than c-FLIPlong may be a crucial factor determining the resistance of recently activated T cells.
Transcription of the c-FLIP gene has been shown to be induced by NF-κB and the androgen receptor and to be repressed by c-Myc, E2F1, FOXO3a, and AP-1. Interestingly, p53 had opposite effects on c-FLIP expression in different cellular systems, as one report showed induction and another study repression of c-FLIP by p53. Given this context-specific regulation of c-FLIP expression by p53, it is conceivable that distinct transcription factors are involved in c-FLIP expression in different cell types. As most of the previous studies have been performed in nonhematopoietic cell lines, we therefore wished to analyze signaling pathways involved in the regulation of the c-FLIP gene in T cells. We demonstrate that in primary T cells and in T-cell lines, the calcineurin/NFAT pathway is particularly important for c-FLIPshort transcription. Interestingly, NFATc1/A, an inducible isoform with a truncated C-terminal domain, is strongly up-regulated in activated T cells by an NFAT-dependent autoregulatory loop. In contrast to other NFAT proteins, NFATc1/A is unable to promote anti-CD3-induced apoptosis. It is therefore assumed that different NFAT isoforms have distinct target gene preferences and differentially regulate pro- and antiapoptotic genes in effector T cells. As we found a role for both NFATc1 and NFATc2 in c-FLIP promoter activity, it is thus tempting to speculate that NFATc1/A or threshold levels of individual NFAT proteins control c-FLIPshort expression and thereby regulate T-cell effector function.

In contrast to previous reports, we did not find any evidence that the MAPK or PI3K/Akt pathways were essential for expression of c-FLIP in T cells. This may be explained by differences in the experimental systems, as we analyzed protein instead of RNA expression and investigated primary human T cells instead of transfected cell lines. Although no direct binding to the promoter has been shown, c-FLIP is generally considered an NF-κB target gene. This view is supported by the finding that activators of NF-κB (including TNFα, LPS, or anti-CD40) induce c-FLIP expression, whereas cells deficient in NF-κB activation show reduced c-FLIP induction. Accordingly, high expression of c-FLIP is generally observed in chronic inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, and Behçet disease. It was, however, also reported that NF-κB RelA-deficient mouse embryonic fibroblasts do not differ in tumor necrosis factor (TNF)-induced c-FLIP expression compared with wild-type cells, suggesting that c-FLIP expression can occur independently of NF-κB.

In contrast to CsA, we found that rocaclamide, which has been shown to inhibit NF-κB as well as NFAT transcription factors, impaired the expression of both c-FLIPlong and c-FLIPshort, suggesting that NF-κB indeed contributes to c-FLIP gene regulation in T cells. It is therefore conceivable that NF-κB acts as a general activator of the c-FLIP gene during T-cell activation, whereas the calcineurin/NFAT pathway might more specifically enhance expression of the c-FLIPshort isoform. Thus, our data demonstrate for the first time that a particular c-FLIP splice variant is induced by a certain transcription factor. Whether NFAT is involved in a transcription-coupled splicing event is an intriguing possibility that should be investigated in future studies.

Upon encounter with pathogens, T cells become activated and initiate effector mechanisms, one of which is the killing of infected target cells by CD95L. Surprisingly, although recently activated T cells highly up-regulate CD95 receptor as well as its ligand, they do not commit suicide or induce fratricide in the initial immune response. Therefore, T cells must have various cell-survival mechanisms to prevent their elimination during activation. One of these is the up-regulation of antiapoptotic proteins such as Bcl-xL upon T-cell activation (Figure 1). Moreover, we have previously shown that short-term activated T cells have a reduced capacity for DISC formation. Here we demonstrate that in primary human T cells c-FLIPshort is highly up-regulated at the mRNA and protein level, while expression of c-FLIPlong is only marginally modulated. The induction of c-FLIP, especially the c-FLIPshort isoform, in recently activated T cells plays an important functional role in protection from CD95-mediated apoptosis, as an RNAi-mediated knock-down of c-FLIP sensitized T cells for CD95L-induced as well as activation-induced apoptosis. Strikingly, the cells, especially those in which c-FLIPshort had been knocked down, displayed a highly elevated sensitivity toward activation-induced apoptosis. Therefore, c-FLIPshort is an important mediator of resistance acquired by an activated T cell to protect itself from death receptor-mediated apoptosis.

CsA and FK506 are immunosuppressive drugs commonly used in transplantation medicine to induce immunologic tolerance. Sensitization to CD95-mediated apoptosis may contribute to the tolerance-promoting effects of these drugs. Supporting this hypothesis is a study showing that CsA sensitized CD28-costimulated rat T cells toward CD95-mediated apoptosis by up-regulating caspase-3 expression. We observed impaired c-FLIPshort expression in CsA-treated activated T cells, providing an alternative molecular mechanism for tolerance induction by regulating apoptosis sensitivity. Interestingly, accumulating evidence suggests that calcineurin and NFAT transcription factors not only play an essential role in development and the immune system, but also in tumor development. Moreover, c-FLIP is highly expressed in certain tumors, suggesting it as a potential therapeutic target. In line with these observations, NFAT and c-FLIP were shown to support angiogenesis, an essential process for tumor growth. In addition, it was recently demonstrated that inhibition of calcineurin by CsA or FK506 in vivo led to clearance of malignant cells and a better survival in two mouse models of leukemia. Nevertheless, due to the various functions of calcineurin, long-term treatment with CsA is associated with severe side effects. Thus, direct interference with the expression of NFAT target genes could be an alternative strategy for immunosuppression. Our data suggest that c-FLIP might be such a target gene involved in tumorigenesis and immunosuppression.

Acknowledgments

We thank Daniel Scholtyssik for expert technical assistance and Dr Bernhard Reis for scientific advice. In addition, we would like to thank Drs Wafik S. El-Deiry, Peter H. Krammer, David Root, Edgar Serfling, Gudrun Totzke, Didier Trono, Bob Weinberg, and Harald Wajant for various reagents.

This work was supported by grants from the Deutsche Krebshilfe (Bonn, Germany) and the Deutsche Forschungsgemeinschaft (Bonn, Germany: GK1033, SFB575, SFB612).

Authorship

Contribution: N.U. designed and performed research and wrote the paper; M.S. and E.K. performed research; K.S.O. contributed vital reagents and wrote the paper; and I.S. designed research, wrote the paper; M.S. and E.K. performed research; K.S.O. contributed vital reagents.

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References


Up-regulation of c-FLIP\textsubscript{Short} by NFAT contributes to apoptosis resistance of short-term activated T cells

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