c-FLIP Mediates Resistance of Hodgkin/Reed–Sternberg Cells to Death Receptor–induced Apoptosis

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Abstract

Resistance to death receptor–mediated apoptosis is supposed to be important for the deregulated growth of B cell lymphoma. Hodgkin/Reed-Sternberg (HRS) cells, the malignant cells of classical Hodgkin's lymphoma (cHL), resist CD95-induced apoptosis. Therefore, we analyzed death receptor signaling, in particular the CD95 pathway, in these cells. High level CD95 expression allowed a rapid formation of the death-inducing signaling complex (DISC) containing Fas-associated death domain–containing protein (FADD), caspase-8, caspase-10, and most importantly, cellular FADD-like interleukin 1β–converting enzyme-inhibitory protein (c-FLIP). The immunohistochemical analysis of the DISC members revealed a strong expression of CD95 and c-FLIP overexpression in 55 out of 59 cases of cHL. FADD overexpression was detectable in several cases. Triggering of the CD95 pathway in HRS cells is indicated by the presence of CD95L in cells surrounding them as well as confocal microscopy showing c-FLIP predominantly localized at the cell membrane. Elevated c-FLIP expression in HRS cells depends on nuclear factor (NF)-κB. Despite expression of other NF-κB–dependent antiapoptotic proteins, the selective down-regulation of c-FLIP by small interfering RNA oligoribonucleotides was sufficient to sensitize HRS cells to CD95 and tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis. Therefore, c-FLIP is a key regulator of death receptor resistance in HRS cells.

Key words: lymphoma • CD95 antigen • TRAIL protein • NF-κB • siRNA

Introduction

Death receptors are members of the TNFR superfamily, which transmit death-inducing signals via the so-called "death domain" (1). After activation and recruitment of intracellular adapter proteins, they initiate a signaling cascade that activates caspases and finally executes cell death. Regarding this signaling cascade, CD95 (Fas) is the best characterized receptor (2). CD95 oligomerizes upon ligation, thereafter the Fas-associated death domain–containing protein (FADD) is recruited to CD95, and subsequently FADD recruits caspase-8 (FLICE; C-8). The CD95 receptor and these associated proteins form the death-inducing signaling complex (DISC). Usually, C-8 becomes autocatalytically processed in the DISC and initiates a caspase cascade leading to

the execution of the apoptotic program. A second group of death receptors are the TNF-related apoptosis–inducing ligand (TRAIL)-Rs (for review see reference 3). Stimulation of TRAIL-Rs is supposed to kill predominantly transformed but not normal cells, which is the reason why soluble TRAIL might be of interest for cancer therapy (3). To avoid cellular self-destruction, death receptor systems have to be tightly controlled. For example, one of the most proximal steps of death receptor signaling, which is the autoproteolytic processing of C-8, is inhibited by FLICE-inhibitory proteins (FLIPs; 4, 5). FLIPs structurally resemble caspases, but lack proteolytic activity. Both known isoforms of cellular FLIP (c-FLIP), c-FLIP long (c-FLIP$_L$) and c-FLIP short (c-FLIP$_S$), can be incorporated into the DISC and associate with C-8 (6). Consequently, c-FLIPs were proposed to function as antiapoptotic proteins because activation of C-8 is blocked.

The death receptor system is an essential component in the regulation of homeostasis of the lymphoid system (7–10). It is assumed that the negative selection process of B as well as T cells in the germinal center (GC) and thymus, respectively, depends on the CD95 system. Several lines of evidence indicate the importance of this system for the balance between B cell proliferation and apoptosis. (a) Mice lacking functional CD95 expression suffer from lymphadenopathy, autoimmunity, and higher incidence of B cell lymphomas (11, 12). (b) Purified GC B cells rapidly undergo apoptotic cell death due to CD95 activation (13) and the CD95 system has been reported to drive negative selection of B cells in vivo (7). (c) Patients with autoimmune lymphoproliferative syndrome (ALPS) carry germline mutations of proteins involved in lymphocyte apoptosis (14, 15). These mutations have the impairment of CD95 function in common, which predisposes these patients to autoimmune disorders and lymphoma development. (d) Clonal deleterious somatic mutations of the CD95 gene were identified in lymphomas, in particular those deriving from GC B cells, suggesting a role in lymphomagenesis (for review see reference 16).

Classical Hodgkin's lymphoma (cHL), a common human lymphoma, has been proposed to be derived from GC B cells in the majority of cases (17). Currently, the molecular pathogenesis of cHL remains unclear. Our group identified the transcription factors nuclear factor (NF)-κB (for review see reference 18) and activator protein 1 (AP-1; for review see reference 19) as important regulators of Hodgkin/Reed-Sternberg (HRS) cell biology (20–22). Most of the known proteins aberrantly expressed in HRS cells were shown to depend on NF-κB, some of them being coregulated by NF-κB and AP-1 (21–23). NF-κB and AP-1 support proliferation of HRS cells, and NF-κB is a key survival factor for these cells. Recently described genomic alterations of the NF-κB system in the malignant HRS cells support the idea that at least NF-κB plays a central role in the pathogenesis of cHL (17, 24).

Two characteristics of cHL are unique among human lymphomas. First, among tumor-forming cells the malignant HRS cells are rare. Usually they represent <1% of cells in affected lymph nodes. They are surrounded by benign cells, which consist, among others, of an abundant number of T cells. Second, HRS cells have lost their B cell phenotype, including Ig expression (25). In accordance with the current concept of B cell ontogenesis, in contrast to HRS cells, cells with nonfunctional Ig expression usually die from apoptosis. These characteristics of cHL raise two questions. How do HRS cells evade the control of the immune system, especially the cytotoxic T cell response? And, how do they survive despite the absence of Ig expression? A common answer for both questions might be the evasion from death receptor–induced apoptosis. In particular, the CD95 system is involved in both processes, the CTL-mediated target cell lysis including the control of tumor growth (26–28) as well as the negative selection in the GC (7, 9, 13). Indeed, despite expression of WT CD95 in most cases of cHL, HRS cells do not undergo apoptotic cell death after CD95 stimulation (29, 30). Although c-FLIP expression (29, 31) and alteration of C-8 expression (32) were described in HRS cells, the functional significance of these data is unclear.

The experiments described in this work were performed to functionally analyze the CD95 system in HRS cells. We report that in contrast to many other tumor entities, the CD95 system on HRS cells is rather up-regulated than down-regulated. This is evident from CD95 overexpression in HRS cell lines and from the immunohistochemical analysis of CD95, FADD, and c-FLIP on 59 cases of cHL. Upon CD95 stimulation, a strong DISC formation is observed in HRS cell lines. c-FLIP proteins, the expression of which strictly depends on NF-κB, are rapidly incorporated into the DISC. Most importantly, although several other NF-κB–dependent antiapoptotic proteins are up-regulated in HRS cells, the specific down-regulation of c-FLIP proteins by small interfering RNA oligoribonucleotides (si-RNAs) is sufficient to render HRS cell lines sensitive to CD95 and TRAIL stimulation.

Materials and Methods

Cell Lines and Culture Conditions. HRS (L428, L1236, KM-H2, HDLM-2, L540, L540Cy [provided by A. Engert, University Hospital, Cologne, Germany], L591, HD-My-Z), ALCL (K299, SU-DHL-1), Reh (pro-B lymphoblastic leukemia), Namalwa, Daudi (both Burkitt's lymphoma), U266 and INA-6 (both myeloma), Jurkat WT cells, a FADD and a C-8–deficient mutant thereof (provided by J. Blenis, Harvard Medical School, Boston, MA), H9 (provided by P. Krammer, DKFZ, Heidelberg, Germany), and HEK293 cells were cultured as previously described (21). Cells were treated with 25 μg/ml cycloheximide (CHX; Sigma-Aldrich), 500 ng/ml CH-11 (Immunotech), or an IgM control (Qbiogene) antibody, where indicated. TRAIL-mediated apoptosis was induced by use of the TRAIL Soluble (human recombinant) Kit (Qbiogene). Cells were incubated with 500 ng/ml sTRAIL together with 2 μg/ml enhancer. Electroporation was performed using a Gene-Pulser II (Bio-Rad Laboratories) with 960 μF and 0.18 kV. Transfection efficiency of L428 cells was 30–40% and of L540Cy cells it was 70–80%. Transfection-associated cell death was negligible. Before electroporation

of siRNA molecules, cells were washed twice with serum-free RPMI 1640 medium. Cells were transfected with 600 nM control (siK3; specific for nucleotides 2610–2630 of neomycin phosphotransferase mRNA; sequence data are available from GenBank/EMBL/DDBJ under accession no. U55763) or c-FLIP$_{S/L}$–specific siRNAs (F1; siFLIP$_{S/L}$; specific for nucleotides 452–472 of c-FLIP$_L$ mRNA; sequence data are available from GenBank/EMBL/DDBJ under accession no. U97074; both provided by Ribopharma AG; reference 33), 10 μg pEGFP-N3 (CLONTECH Laboratories, Inc.), or 20 μg c-FLIP$_S$ or c-FLIP$_L$ expression plasmids (provided by J. Tschopp, University of Lausanne, Epalinges, Switzerland), where indicated. For reconstitution experiments, L540Cy cells were transfected with 600 nM siRNA along with 15 μg mock plasmid or mutated c-FLIP$_S$ cDNA. Transfection efficiency was determined by FACS® analysis of green fluorescent protein (GFP)$^+$ cells. Enrichment of L428 cells was performed by FACS® sorting of GFP$^+$ cells 24 h after transfection using FACS Vantage™ and CELLQuest™ software (Becton Dickinson). Adenoviral infection of L428 and HDLM-2 cells was performed as previously described (22, 23). A mutated c-FLIP$_S$ cDNA, which cannot be recognized by the siFLIP, was generated by PCR amplification using c-FLIP$_S$ expression plasmid PL296 (provided by J. Tschopp) as template and primers sense 5′-CCCGGGAATCAAAACATGGATTACAAAG-3′ containing a SmaI site and antisense (generating seven silent mutations at positions 452, 457, 460, 463, 466, 469, and 472 [sequence data are available from GenBank/EMBL/DDBJ under accession no. U97074], marked in italics) 5′-AGGTCCCTGA-CATTAGGTGGAACCACATC*A*AT*G*GC*C*AC*G*TC*G*CG*A*C ACA*G*AAAGAGCAGC-3′ containing an ECO0109I site. The amplified mutated fragment was cloned into pGEMT easy and sequenced. Thereafter, the WT 5′ SmaI ECO0109I fragment of plasmid PL296 was replaced with the mutated sequence to generate the mutated c-FLIP$_S$ cDNA with FLAG at the NH$_2$ terminus.

Western Blotting. The preparation of whole cell extracts and SDS-PAGE has been described (21). For Western blot analysis the following primary antibodies were used: monoclonal anti–c-FLIP$_{S/L}$ (Dave-2; Qbiogene and G-11; Santa Cruz Biotechnology, Inc.), polyclonal anti-IκBα (C-21), polyclonal anti-CD95 (C-20), polyclonal anti-p65 (A; all from Santa Cruz Biotechnology, Inc.), monoclonal anti-FADD (Clone-1 and Clone A66-2; BD Biosciences), monoclonal anti–tubulin α (MCA78A; Serotec), monoclonal anti–C-8 (C-15; provided by P. Krammer), and polyclonal anti–Bcl-x$_L$ (Transduction Laboratories). Filters were incubated with horseradish peroxidase–conjugated secondary antibodies. Bands were visualized using the enhanced chemiluminescence system (Amersham Biosciences).

Analysis of the CD95 DISC by Immunoprecipitation. DISC immunoprecipitation was essentially performed as described by Scaffidi et al. (34). In brief, cells were left untreated or treated with 2 μg/ml anti–APO-1 antibody (IgG3; provided by P. Krammer). 10 min after the addition of the antibody, unbound anti–APO-1 antibody was removed by washing the cells once with ice-cold PBS. Cells were incubated in lysis buffer (30 mM Tris-HCL, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% [vol/vol] glycerol, and a protease inhibitor cocktail; Complete, Mini; Roche). As control, untreated cells were lysed and the lysate was supplemented with the anti–APO-1 antibody. 25 μl protein A–Sepharose beads were added to 700 μg of each lysate. Immunoprecipitation was performed for 1.5 h while rotating at 4°C. Protein A–Sepharose beads were washed five times with ice-cold lysis buffer and boiled for 5 min in SDS loading buffer. The supernatant was separated by SDS-PAGE and analyzed by Western blot analy-

sis using monoclonal anti–C-8 (C-15, IgG2b; provided by P. Krammer), monoclonal anti-FADD (IgG1; BD Biosciences), and monoclonal anti–c-FLIP$_{S/L}$ (IgG1; Santa Cruz Biotechnology, Inc.) as primary antibodies followed by incubation with isotype-specific, horseradish peroxidase–conjugated secondary antibodies (all from Southern Biotechnology Associates, Inc.).

Analysis of Apoptosis. Cells were stained with 5 μg/ml acridine orange (Sigma-Aldrich) and analyzed by fluorescence microscopy. The number of cells with fragmented or condensed nuclei was counted and the percentages were determined. All assays were performed in triplicate. Alternatively, cells were stained with FITC-conjugated annexin V and propidium iodide (Bender MedSystems™; Biozol) according to the manufacturer's recommendations. Samples were analyzed using a FACSCalibur™ flow cytometer and CELLQuest™ software (Becton Dickinson).

Immunohistology. All cases were drawn from the files of the Consultation and Reference Center for Haematopathology at the Institute of Pathology, Benjamin Franklin Clinic, Free University of Berlin. Final diagnosis had been established after extensive immunohistological analysis according to the criteria of the World Health Organization classification (35). Representative areas of the lymph node specimens containing diagnostic HRS cells were sampled out in the form of a cylinder and arrayed on one recipient paraffin block (generated by S. Joos, DKFZ, Heidelberg, Germany). The 59 available specimens originated from 28 cases of nodular sclerosing and 31 cases of mixed cellularity cHL subtypes. The specificity of the CD95 antibody C-20 (Santa Cruz Biotechnology, Inc.) as well as the CD95L antibody G247-4 (BD Biosciences) has been described (36, 37). Specificity of the FADD antibody A66-2 (BD Biosciences), the c-FLIP antibody G-11 (Santa Cruz Biotechnology, Inc.), and the C-8 antibody C-15 (provided by P. Krammer) was controlled as described below (see Fig. S2, available at http://www.jem.org/cgi/content/full/ jem.20031080/DC1). All antibodies required a heat-induced antigen-demasking procedure before their application. Specifically bound antibodies were demonstrated using a streptavidin alkaline phosphatase method and FastRed as chromogen. For confocal microscopy, representative cHL cases were stained with the anti-CD30 antibody Ber-H2 (DakoCytomation), c-FLIP G-11 antibody (Santa Cruz Biotechnology, Inc.), or the respective IgG1 isotype control (BD Biosciences). Specifically bound antibodies were detected using anti–mouse Cy3-conjugated secondary antibodies. Nuclei were stained with DAPI. Immunofluorescence microscopy was performed on an LSM510 inverted laser scanning microscope (Carl Zeiss MicroImaging, Inc.). Cy3 and DAPI signals were recorded with λ_{exc} = 543 nm (HeNe), LP λ_{em} = 560 nm and λ_{exc} = 364 nm (Argon laser), and BP λ_{em} = 390–465 nm wavelengths, respectively.

Online Supplemental Material. In Fig. S1, total RNA was prepared using RNeasy kit (QIAGEN). 10 μg total RNA was subjected to gel electrophoresis and transferred to a nylon membrane (Appligene). The membrane was hybridized (ExpressHyb solution; CLONTECH Laboratories, Inc.) with [α-^{32}P]dCTP–labeled random prime–labeled DNA probes (c-FLIP, GAPDH). Fig. S2 shows specificity controls of antibodies used for immunohistochemistry. Specificity of the FADD antibodies Clone-1 and Clone A66-2 (both BD Biosciences) as well as NCL-FADD (Novocastra) was analyzed by the staining of paraffin-embedded Jurkat cells and a mutant thereof lacking FADD expression. Only Clone A66-2 recognized FADD under these conditions. The other two antibodies failed to detect any specific signal. The c-FLIP antibodies Dave-2 (Qbiogene), G-11 and C-19 (both from Santa Cruz Biotechnology, Inc.), and c-FLIP$_L$ (Sigma-Aldrich)

were tested on paraffin-embedded HEK293 cells transfected with c-FLIP$_S$ or c-FLIP$_L$ expression plasmids. Only antibody G-11 specifically stained the c-FLIP–transfected cells. The specificity of the C-8 antibody C-15 was controlled by the staining of paraffin-embedded Jurkat cells and a mutant thereof lacking C-8. Figs. S1 and S2 are available at http://www.jem.org/cgi/content/full/jem.20031080/DC1.

Results

Strong Expression of CD95 in HRS Cell Lines: FADD, C-8, Caspase-10 (C-10), and c-FLIP$_S$ and c-FLIP$_L$ Are Rapidly Recruited into the DISC. DISC formation is a prerequisite for CD95-induced signaling, at least in type I cells, which are characterized by rapid and strong DISC formation (38). Therefore, in HRS cell lines we analyzed the protein expression of CD95, FADD, C-8, and C-10, which together can form a functional DISC (Fig. 1 A). All investigated HRS cell lines expressed FADD and C-8 to a similar extent as observed in nonHodgkin cell lines (Fig. 1 A). Furthermore, C-10, which was recently described to be incor-

porated into the DISC (39), was detectable in the cell lines, with the exception of Namalwa and L540Cy. In contrast to the other cell lines tested, HRS cell lines strongly expressed CD95 as demonstrated by Western blot (Fig. 1 A) or FACS® analysis (not depicted), which is in agreement with previously published data (40, 41).

To determine whether CD95-associated signaling is functional in HRS cells, we investigated the DISC formation. The CD95-associated proteins in the T cell leukemia cell line H9, which served as positive control, and in HRS cell lines (Fig. 1 B) were immunoprecipitated after the addition of the CD95-agonistic antibody anti–APO-1. FADD, C-8 (procaspases and p43/41 cleavage products), and C-10 (procaspases and p43/p47 cleavage products) coimmunoprecipitated with CD95 in H9 and HRS cells. C-10 was not detectable in L540Cy immunoprecipitates, which confirms the lack of expression observed in whole cell lysates of this cell line (Fig. 1 A). After the addition of the anti–APO-1 antibody to the lysate of unstimulated cells, which served as control, no coprecipitation of any of the proteins was observed (not depicted). In L1236 cells DISC induction was only very weak (not depicted). The DISC formation in
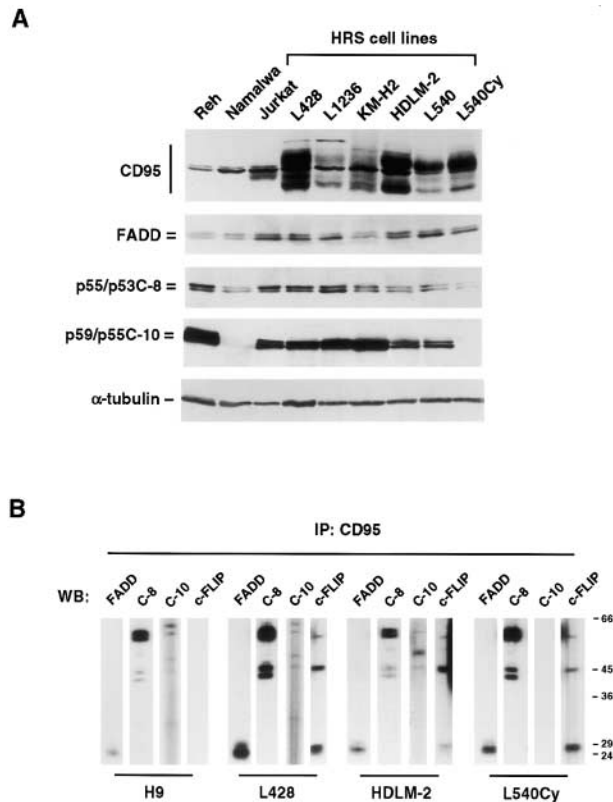

Figure 1. DISC analysis in HRS cell lines. (A) Whole cell lysates of different nonHodgkin and Hodgkin cell lines were analyzed for expression of CD95, FADD, C-8 (p55/p53C-8), and C-10 (p59/p55C-10) by Western blot. As control, α-tubulin expression is shown. (B) DISC analysis in H9 cells and HRS cell lines by CD95 immunoprecipitation. The indicated cell lines were incubated for 10 min with the agonistic anti-CD95 mAb APO-1. Thereafter, cells were lysed and immunoprecipitation was performed. The precipitates were washed, subjected to 10% SDS-PAGE, and analyzed by Western blot for coimmunoprecipitation of FADD, C-8, C-10, and c-FLIP, as indicated.
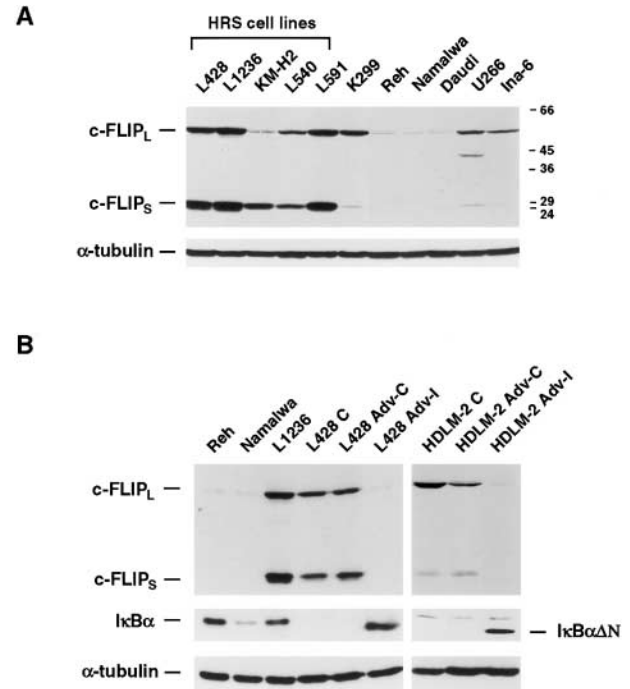
Figure 2. c-FLIP expression and regulation in HRS cell lines. (A) Elevated c-FLIP protein expression in HRS compared with nonHodgkin cell lines. Whole cell lysates of the indicated cell lines were analyzed by Western blot for expression of c-FLIP$_L$ and c-FLIP$_S$ (mAb Dave-2). As control, the Western blot was reprobed with an α-tubulin antibody. (B) c-FLIP expression in HRS cell lines depends on NF-κB. Whole cell extracts of Reh, Namalwa, L1236, uninfected L428 (L428 C), and HDLM-2 (HDLM-2 C) cells, and L428 or HDLM-2 cells infected for 24 h with a control adenovirus (L428 Adv-C, HDLM-2 Adv-C) or an adenovirus encoding for the NF-κB superrepressor IκBαΔN (L428 Adv-I, HDLM-2 Adv-I) were analyzed by Western blot analysis for expression of c-FLIP, IκBα, IκBαΔN, and as control, α-tubulin. Note that L428 cells lack endogenous IκBα expression.
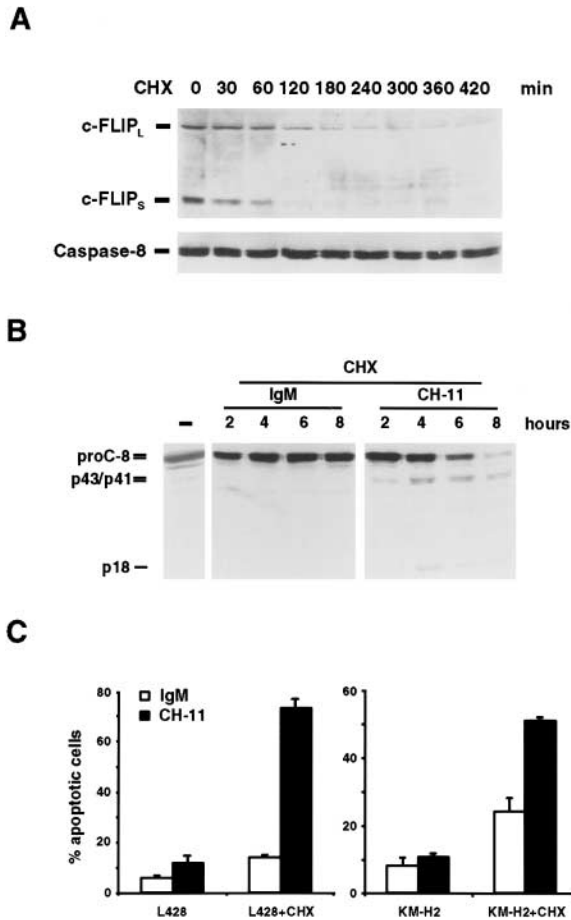
Figure 3. Treatment with CHX down-regulates c-FLIP and sensitizes HRS cells to CD95-induced apoptosis. (A) L428 cells were treated with CHX. At the indicated times, whole cell lysates were prepared and analyzed for expression of c-FLIP$_L$ and c-FLIP$_S$ and C-8 by Western blot. (B) Treatment with CHX allows CD95-induced activation of C-8. L428 cells were left untreated ($-$) or incubated with CHX. After 2 h, the agonistic anti-CD95 antibody CH-11 or an isotype control (IgM) were added to the CHX-treated cells. At the indicated times, whole cell lysates were prepared and analyzed for expression of C-8 by Western blot. Positions of procaspase-8 (proC-8) and cleavage products (p43/p41, p18) are indicated. (C) Treatment of HRS cell lines with CH-11 induces apoptosis in the presence of CHX. L428 and KM-H2 cells were treated for 14 h with CHX together with the agonistic anti-CD95 antibody CH-11 or an IgM control. The percentage of apoptotic cells was determined by acridine orange staining. Error bars denote SDs.

the other HRS cell lines with a composition that usually allows death induction suggested a functional inhibition of the death-inducing signaling cascade. This could be mediated by incorporation of c-FLIP proteins into the DISC (6). Indeed, in contrast to H9 cells, c-FLIP$_S$ and c-FLIP$_L$ and the p43 cleavage product of c-FLIP$_L$ were incorporated in the DISC of HRS cells after CD95 stimulation (Fig. 1 B).

c-FLIP$_S$ and c-FLIP$_L$ Are Up-regulated in HRS Cells; c-FLIP Expression is NF-κB Dependent. Because of the high amount of c-FLIP proteins detectable in DISC immunoprecipitates of HRS cell lines, we analyzed mRNA and protein levels of c-FLIP proteins in HRS and nonHodgkin cell lines.

mRNA overexpression, most evident for two splice variants presumably corresponding to c-FLIP$_S$ (4), was found in HRS compared with nonHodgkin cell lines by Northern blot analysis (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20031080/DC1). As suggested by these data, we observed c-FLIP protein overexpression in HRS cell lines (Fig. 2 A). Remarkably, the robust c-FLIP$_S$ expression was restricted to HRS cell lines.

Transient activation of NF-κB up-regulates expression of c-FLIP, a key mediator of the inducible resistance to death receptor–induced apoptosis (42). Therefore, we investigated the contribution of constitutive NF-κB activity in HRS cells (22) to c-FLIP expression. L428 cells, which lack endogenous expression of the NF-κB inhibitory protein IκBα (23), and HDLM-2 HRS cells were infected with an adenovirus encoding for the NF-κB superrepressor IκBαΔN, a stabilized form of IκBα. As previously described (22, 23), 24 h after infection the NF-κB activity decreased dramatically (not depicted). Concomitantly, we observed a strong reduction of c-FLIP$_{S/L}$ mRNA and protein expression levels (Fig. 2 B and not depicted). 24 h after infection, c-FLIP expression had disappeared nearly completely, which identified, compared with other known NF-κB–dependent genes in HRS cells, c-FLIP as one of the most strongly NF-κB–regulated genes.

Treatment of HRS Cells with CHX Rapidly Down-regulates c-FLIP and Allows Activation of C-8 and Induction of Apoptosis by CD95 Stimulation. The rapid loss of c-FLIP expression after NF-κB inhibition prompted us to investigate the stability of c-FLIP proteins in HRS cell lines. Treatment of L428, HDLM-2, and L1236 HRS cells with CHX revealed that c-FLIP$_S$ and c-FLIP$_L$ are indeed short-lived proteins. Thus, as soon as 30–60 min after CHX treatment a dramatic decrease of protein expression was detectable (Fig. 3 A and not depicted). In contrast, even after 420 min of CHS treatment, no decrease of C-8, FADD, Bcl-x$_L$, or NF-κB-p65 protein expression level was observed (Fig. 3 A and not depicted).

The B cell–derived HRS cell lines L428, KM-H2, and L1236 were resistant to death receptor–induced apoptosis, and no cleavage of the initiator C-8 could be observed after activation of the CD95 or TRAIL death receptor pathway (not depicted). Therefore, we asked whether C-8 could be activated after CHX induced c-FLIP down-regulation. As a model system for death receptor–induced apoptosis, we investigated CD95-induced cell death. No activation of C-8 was observed in L428 cells incubated with or without CHX and the IgM control antibody. Furthermore, after stimulation of untreated cells with the agonistic anti-CD95 antibody CH-11, only very weak cleavage of C-8 to p43/p41 was observed (not depicted; also refer to Fig. 1 B). In contrast, upon treatment with CHX and CH-11, C-8 was strongly activated (Fig. 3 B). C-8 p43/p41 cleavage products as well as active C-8 (p18) were rapidly detectable and procaspase-8 was cleaved after 8 h. In line with these data, CHX treatment sensitized the HRS cell lines L428 and KM-H2 toward CD95-induced apoptosis (Fig. 3 C). In L1236 cells, sensitization for induction of apoptosis was
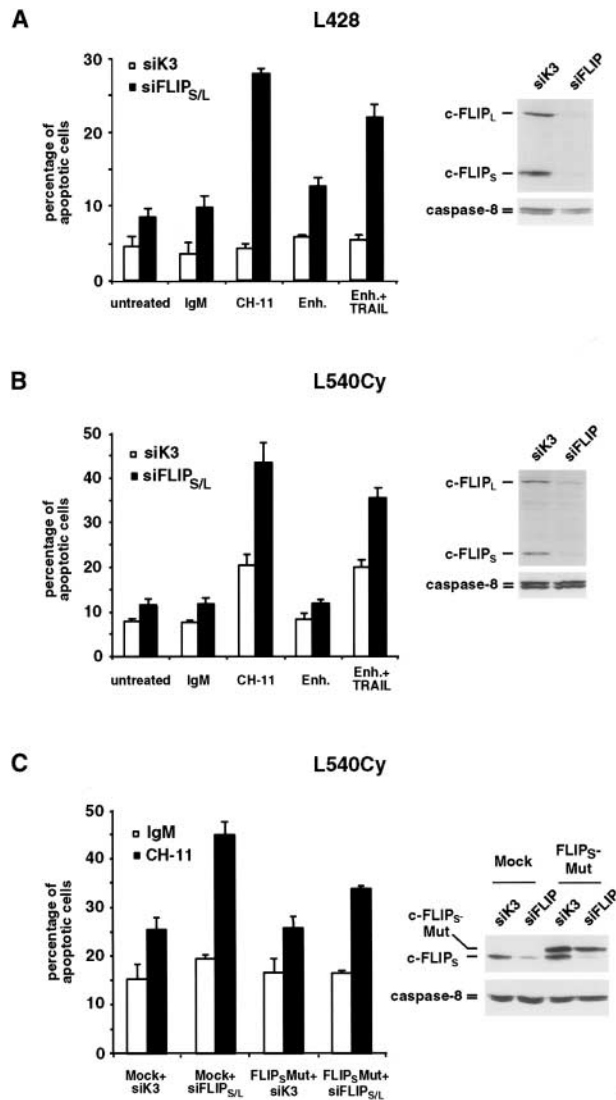
Figure 4. Specific down-regulation of c-FLIP sensitizes HRS cell lines to CD95- and TRAIL-induced apoptosis. (A) L428 cells were transfected with control (siK3) or siFLIP$_{S/L}$ along with pEGFP-N3. 24 h after transfection, GFP$^+$ cells were FACS® sorted, analyzed for expression of c-FLIP by Western blot (right; as control, analysis of C-8 is shown), and left untreated or were incubated for 14 h with an IgM control (IgM) or the agonistic anti-CD95 antibody CH-11, cross-linking antibody for TRAIL alone (enhancer, Enh.), or with enhancer and recombinant human soluble TRAIL (Enh. + TRAIL). Thereafter, the percentage of apoptotic cells was determined by acridine orange staining (left). Error bars denote SDs. (B) L540Cy cells were transfected and analyzed for c-FLIP and C-8 expression (right) as described in A. 24 h after transfection, cells were left untreated or treated as described in A. Thereafter, the percentage of apoptotic cells was determined by annexin V–FITC and propidium iodide staining and subsequent FACS® analysis (left). The combined results of four independent experiments are shown. Error bars denote SDs. (C) L540Cy cells were transfected with control (siK3) or siFLIP$_{S/L}$ along with mock plasmid (Mock) or a mutated c-FLIP$_S$ cDNA (FLIP$_S$-Mut), which contains seven silent mutations and cannot be recognized by siFLIP. 24 h after transfection, cells were analyzed for endogenous (c-FLIP$_S$) or ectopically expressed c-FLIP (c-FLIP$_S$-Mut) and C-8 by Western blot (right), and further processed as described in B. The combined results of three independent experiments are shown (left). Error bars denote SDs.

moderate (not depicted). We concluded that a protein responding to CHX treatment, most likely c-FLIP, inhibits CD95-mediated apoptosis in HRS cells.

Specific Down-regulation of c-FLIP by siRNA Sensitizes HRS Cell Lines to CD95- and TRAIL-induced Apoptosis. To directly analyze the function of c-FLIP proteins in death receptor–induced apoptosis of HRS cells, we specifically down-regulated c-FLIP by siRNAs, which were recently shown to strongly down-regulate c-FLIP expression (33). Experiments were performed with the cell lines L428 and L540Cy, which were the only HRS cell lines sufficiently transfectable for functional assays. 24 h after transfection, cells were analyzed for c-FLIP$_{S/L}$ expression and incubated with the anti-CD95 antibody CH-11 or recombinant human TRAIL (rhTRAIL), respectively (Fig. 4). Transfection of both cell lines with the siFLIP rapidly down-regulated endogenous c-FLIP expression (Fig. 4, A and B, right).

In L428 cells (Fig. 4 A), neither the CD95-agonistic antibody CH-11 nor rhTRAIL significantly induced apoptosis in cells transfected with a control siRNA (siK3). Untreated siFLIP-transfected cells reproducibly showed a slight increase of the percentage of apoptotic cells. In contrast, incubation of siFLIP-transfected cells with CH-11 induced apoptosis in 25–30% of cells. Similarly, an increase of apoptosis was observed after activation of the TRAIL system (Fig. 4 A, left). In L540Cy cells (Fig. 4 B), which are not completely resistant to stimulation of the CD95 or TRAIL death receptor system, a slight increase of apoptosis was again observed in siFLIP compared with siK3-transfected cells. After treatment with CH-11 or rhTRAIL, ∼15–20% of siK3-transfected cells underwent apoptotic cell death. However, in siFLIP-transfected cells the percentage of apoptotic cells was at least doubled. CH-11 or rhTRAIL induced apoptosis in 40–50 and 30–40% of cells, respectively. To prove that the observed increase in apoptosis is indeed caused by c-FLIP down-regulation, we transfected L540Cy cells with a mutated c-FLIP$_S$-cDNA, which cannot be recognized by siFLIP. Despite siRNA mediated down-regulation of endogenous c-FLIP, this construct should maintain a c-FLIP expression level sufficient to block apoptosis sensitization. Indeed, transfection of the cells with the mutated cDNA resulted in a c-FLIP expression level comparable to endogenous c-FLIP expression despite cotransfection of siFLIP (Fig. 4 C, right). In contrast to endogenous c-FLIP, the mutated c-FLIP mRNA is not degraded by siFLIP, which is reflected by the decrease of the endogenous WT but not the ectopically expressed c-FLIP$_S$ protein transcribed from the mutated cDNA. Importantly, c-FLIP reconstitution reverted to a large extent the siFLIP-mediated sensitization for apoptosis induction by the CD95 system (Fig. 4 C, left). In summary, down-regulation of endogenous c-FLIP expression sensitized L428 and L540Cy cells for CD95 or TRAIL-R–induced apoptosis. These data indicate that c-FLIP proteins play a key role in the protection of HRS cells from death receptor–induced apoptosis.

Immunohistochemical Analysis of CD95, FADD, C-8, c-FLIP, and CD95L in cHL. To draw valid conclusions
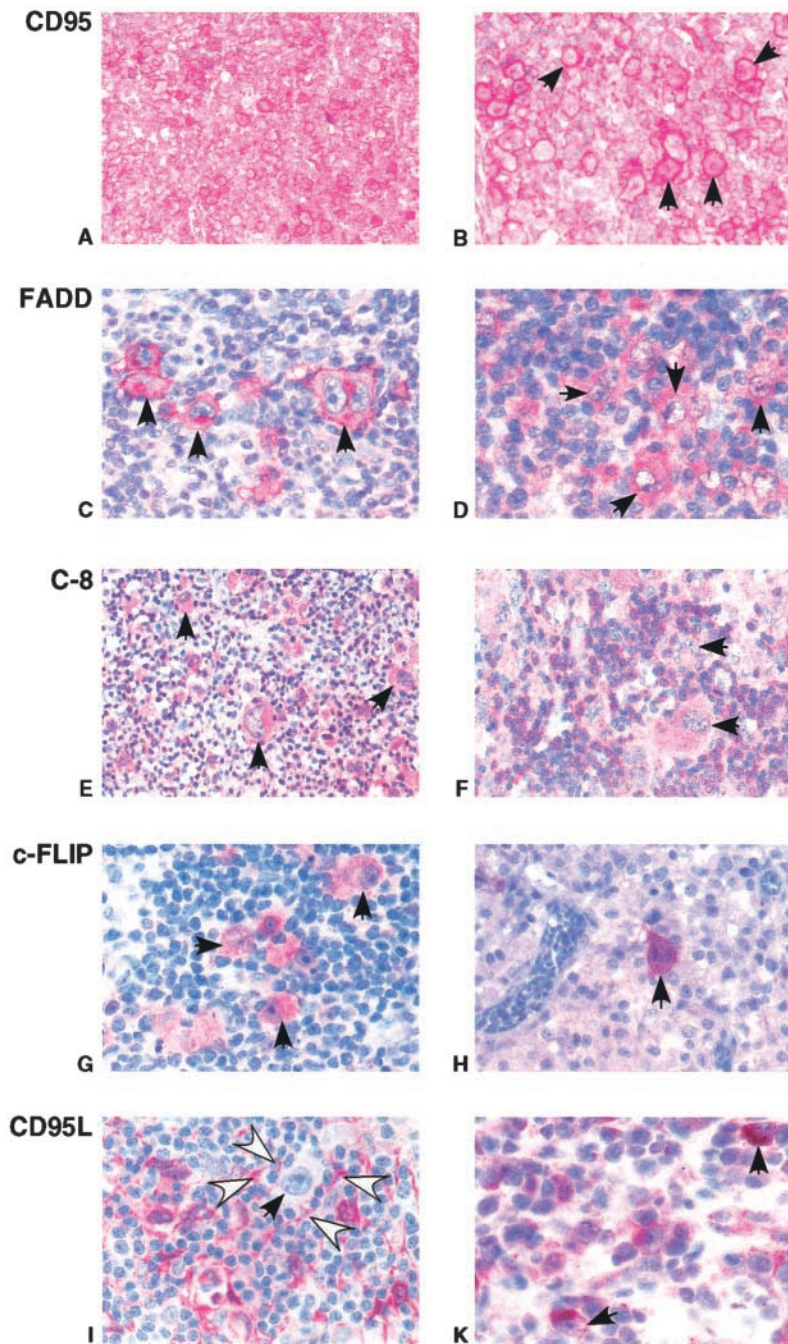
Figure 5. Expression patterns of CD95, FADD, C-8, c-FLIP, and CD95L in HRS cells of cHL. Immunohistochemistry of representative biopsy specimen. (A and B) Strong CD95 expression in HRS cells (exemplarily marked by arrows). (C) Strong FADD expression in HRS (arrows), but not in surrounding cells. (D) Moderate FADD expression in HRS cells (arrows). (E and F) C-8 expression of two representative cases. (E) HRS cells (arrows) stain at least as strong as surrounding cells for C-8. (F) HRS cells stain in a less intense fashion than surrounding cells. (G and H) Strong c-FLIP expression in HRS cells (arrows), but not surrounding cells. (I and K) CD95L expression in cHL. (I) In the majority of cases, HRS cells (solid arrow) do not stain for CD95L. In contrast, surrounding cells (open arrows) stain for CD95L. (K) In one case, strong expression of CD95L was detectable in HRS cells (arrows).

from our in vitro experiments, we analyzed expression of CD95, FADD, C-8, c-FLIP, and CD95L on 59 samples of patients with cHL arrayed on a single paraffin block by immunohistochemistry (Fig. 5). Controls for antibody specificity were performed as we have described (refer to Material and Methods; Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20031080/DC1). We included the analysis of c-FLIP in this work because the c-FLIP$_L$ antibody used in previous studies (29, 31) gave no specific signal in our experiments, neither in a Western blot nor by immunohistochemical analysis (not depicted). In HRS cells of all

except one case we detected a strong expression of CD95 (Fig. 5, A and B). Because staining was performed with a COOH-terminal antibody, these data confirm that CD95 mutations leading to a loss of the death domain are rare events in cHL (29). FADD expression was found in all cases (Fig. 5, C and D) and some cases presented FADD overexpression in HRS compared with surrounding cells. This is surprising because ectopic FADD overexpression was reported to induce apoptosis in other cell types (43). C-8 was detected in HRS cells of all cases, even though in many cases (32 out of 59) only very weak staining was observed

(Fig. 5, E and F). c-FLIP expression was analyzed by an antibody recognizing c-FLIP$_S$ and c-FLIP$_L$ (Fig. 5, G and H). As suggested by the cell line data, we observed in all except four cases a c-FLIP overexpression in HRS compared with surrounding cells, which is in accordance with previously published data (29, 31). Finally, we analyzed CD95L expression in cHL. Only one case presented a strong staining of CD95L in HRS cells (Fig. 5 K), whereas HRS cells of the other cases did not stain for CD95L (Fig. 5 I). However, in all except three cases we detected CD95L in plasma cells and endothelia surrounding the HRS cells (Fig. 5 I). Thus, CD95L is produced by cells in the microenvironment of HRS cells. Taken together, all components of the CD95 system are expressed in primary HRS cells and regarding CD95, FADD, and c-FLIP, this system is rather up-regulated than down-regulated.

To analyze a DISC formation in primary HRS cells, we performed confocal microscopy of c-FLIP in representative cHL cases (Fig. 6). If the CD95 system would be stimulated in primary HRS cells, a significant percentage of the cellular c-FLIP protein pool should be attracted to the membrane and incorporated into the DISC. In our final analysis of eight cases of cHL we detected in three cases a signal for c-FLIP and CD30, which was performed as a control staining. In the other cases none of the antibodies showed a specific signal, so that the material was apparently not suitable for immunofluorescence analysis. However, in the three cases CD30 showed a distinct staining pattern of the membrane as well as a perinuclear staining, most probably derived from the staining of CD30 in the Golgi area (Fig. 6 A and not depicted). In these cases, the strongest signal for c-FLIP in HRS cells was detected at the membrane (Fig. 6 B and not depicted). In contrast, staining with the isotype control did not show a similar staining pattern (Fig. 6 C and not depicted). Although shown in a limited number of cases, these data ar-
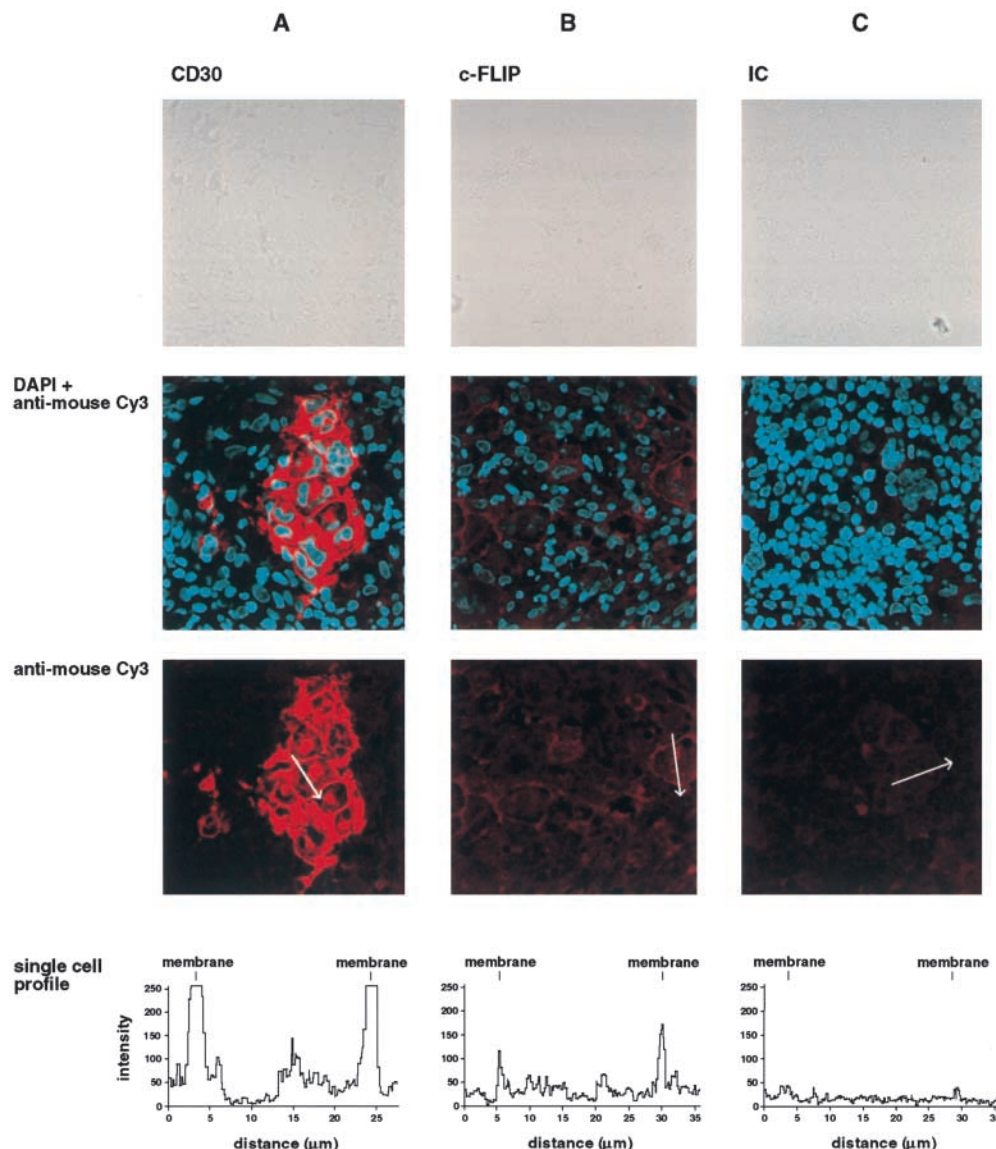

Figure 6. Confocal microscopy of c-FLIP and CD30 in a representative biopsy specimen. Biopsy specimen were stained with CD30 (A), c-FLIP (B), or isotype control (IC; C) mAbs. Detection was performed after staining with an anti–mouse Cy3 conjugate. Nuclei were counterstained with DAPI. For each antibody, the staining intensity for a representative single HRS cell is shown as single cell profile. Localization of the profile is indicated by a white arrow.

gue for an activation of the CD95 pathway in primary HRS cells.

Discussion

The escape of cells from death receptor–induced apoptosis, especially the CD95–CD95L system, has been implicated in tumor progression and is thought to be an important mechanism by which tumor cells evade the control of the immune system (26–28). Consequently, many tumor cells develop strategies to escape control by the death receptor system. The most prominent examples are CD95 mutations or down-modulation of CD95 expression. In contrast to these observations, the CD95 system appears to be up-regulated on HRS cells (Fig. 5; references 40 and 41). Furthermore, structural alterations of the CD95 system in HRS cells are rare (29, 30) and although down-regulation of C-8 (32) or expression of CD95 decoy receptors (44) were reported in HRS cells, a conclusive mechanism for CD95 resistance of HRS cells is lacking. Knowing why HRS cells are resistant to death receptor–induced apoptosis might be critical for the understanding of HRS cell biology for two reasons. First, it is unexplained why HRS cells survive the GC reaction, in which the CD95–CD95L system has been proposed as an important regulator (7, 9), even though they lack Ig expression (17, 25). Second, although CD95-induced apoptosis is supposed to be an important mechanism for CTL-dependent tumor cell elimination in vivo (26, 27, 45), HRS cells are obviously not eliminated by surrounding cells.

Several of our experiments indicated that the resistance of HRS cells to death receptor–induced apoptosis is not due to structural, but to functional inhibition of death receptor pathways by c-FLIP proteins. First, a DISC is formed in HRS cells (Fig. 1 B). FADD, C-8, C-10, and most importantly, c-FLIP$_S$ and c-FLIP$_L$ coimmunoprecipitated with CD95 after CD95 activation. An exception is the cell line L1236, in which we did not observe a clear DISC formation. L1236 cells might be type II cells, or a recently described CD95 mutation in this cell line (29) might inhibit DISC formation, as shown for CD95 mutations identified in patients with ALPS (14). Second, treatment of HRS cell lines with CHX, which leads to the loss of c-FLIP expression, allowed activation of C-8 and induction of apoptosis after death receptor stimulation (Fig. 3, B and C). Third, selective down-regulation of c-FLIP proteins by siRNAs sensitized HRS cell lines to death receptor–induced apoptosis, including CD95- and TRAIL-induced apoptosis (Fig. 4).

The proposed mechanisms of c-FLIP$_S$– or c-FLIP$_L$–mediated inhibition of C-8 activation differ (6). Although c-FLIP$_S$ is supposed to completely inhibit activation of procaspase-8 at the DISC, c-FLIP$_L$ allows generation of the C-8 p43/p41 intermediate cleavage products, but no processing to active C-8 p18/p10. In line with these results we detected procaspase-8 and C-8 p43/p41 in the DISC (Fig. 1 B), but no active C-8 was detectable in whole cell lysates af-

ter CD95 ligation (Fig. 3 B). Activation of C-8 after CHX pretreatment and CD95 stimulation (Fig. 3 B) is most likely explained by the loss of c-FLIP expression (Fig. 3 A). c-FLIPs are short-lived proteins in HRS cells (Fig. 2 A). These results are in contrast to previously published data (31). In our hands, lower concentrations of CHX (10 and 1 μg/ml), which were used by Thomas et al. (31), also induced a rapid loss of c-FLIP in HRS cell lines (unpublished data). The discrepancy between these results might be due to methodical differences. Due to the short half-life of c-FLIP, a strong transcriptional activation is required for maintenance of high expression level. Indeed, c-FLIPs strictly depend on constitutive NF-κB activity in HRS cells (Fig. 2). This finding is supported by data that show NF-κB–dependent c-FLIP regulation in other cell types (42). Recently, FLIP proteins were reported to activate NF-κB itself (46). In our experiments we did not observe an alteration of the NF-κB activity after specific c-FLIP down-regulation (unpublished data), suggesting that c-FLIP does not contribute to the NF-κB activation in HRS cells.

Thus, activated NF-κB in HRS cells not only triggers proliferation and is a key survival factor for these cells (20, 22, 23), but also prevents tumor cell death mediated by the microenvironment. This is suggested by our data regarding c-FLIP expression in HRS cells and the interaction of CD95 in HRS and CD95L on surrounding cells. This interpretation is further supported by recently described animal models, which show that c-FLIP overexpression protects tumor cells from the in vivo immune response (27, 28, 47). Furthermore, NF-κB was reported to render cells resistant to CD95- and TRAIL-induced apoptosis (48, 49). Consequently, the NF-κB–dependent c-FLIP expression in HRS cells offers an explanation for HRS cell immune escape. Similarly, c-FLIP might protect HRS cells against apoptotic stimuli regarding the GC B cell origin because there is evidence that c-FLIP prevents GC B cell apoptosis (9, 13).

Remarkably, down-regulation of c-FLIP alone is sufficient for sensitization of HRS cells to death receptor–induced apoptosis, although the cells express other NF-κB–dependent, antiapoptotic proteins (22). That CD95- and TRAIL-mediated induction of apoptosis after specific c-FLIP down-regulation is not observed in the whole cell population (Fig. 4) is most likely explained by the fact that c-FLIP down-regulation is not complete and not similarly effective in all cells of the population due to different transfection efficiencies.

In contrast to data shown for other tumor entities, in which CD95 often became down-regulated (50–52), CD95 is strongly expressed in HRS cells (Figs. 1 and 5; references 40 and 41). Furthermore, staining of CD95L suggests that CD95 signaling is triggered on primary HRS cells (Fig. 5). However, in other studies different frequencies of CD95L expression in HRS and surrounding cells were reported (41, 53). Therefore, we performed confocal microscopy of c-FLIP in primary HRS cells with the aim to directly analyze a DISC formation (Fig. 6). Although analysis was only

possible in a limited number of cases and c-FLIP might be associated with other death receptors than CD95, the membranous staining pattern of c-FLIP in HRS cells argues for an activation of the CD95 system. In addition, activation of the CD95 system in primary HRS cells might occur in a ligand-independent fashion, as described for GC B cells (13), although this is not observed in HRS cell lines (see above). If c-FLIP proteins block CD95-mediated signaling toward apoptosis, what are the consequences of the strong CD95 expression, together with FADD and c-FLIP, which might facilitate signaling by these receptors? Recent studies revealed several functions of CD95 signaling in the context of blocked apoptosis induction (for review see reference 54). These data are in accordance with the dual function of other TNFR family members concerning apoptosis or survival triggering (1). Thus, in CD95-resistant cells, CD95 induction can trigger proliferation and differentiation (55, 56). Most excitingly regarding the biology of HRS cells, CD95 signaling activates transcription factor NF-κB (57), can induce transcriptional activation of different cytokines (58, 59) including IL-1 and IL-6, and down-regulates Ig expression in B cells, which is independent of apoptosis–inducing signaling events (60). However, the lack of a suitable in vivo model for cHL makes it difficult to further analyze a possible contribution of CD95 signaling to the pathogenesis of cHL. There might be two indications that CD95 signaling could contribute to the pathogenesis of cHL. First, despite long–term culture of HRS cell lines, they maintain an elevated CD95 expression level (Fig. 1). The strong CD95 expression also holds true for primary cases of cHL (Fig. 5). Second, patients with ALPS carry dominant negative CD95 mutations (14), which block apoptosis induction but might allow other CD95-associated signaling events. These patients are predisposed to lymphoma development and, intriguingly, with the highest incidence among these lymphomas they develop cHL (15).

Finally, the combined strong expression of different members of the TNFR family, including CD30, CD40, RANK (61–63), and CD95, is unique among human lymphomas. This suggests a common mechanism for their up–regulation and points to an important role in the pathogenesis of cHL. Given the strong activation of NF-κB and AP-1 in HRS cells (21, 22), it will be important to clarify the contribution of the TNFR family to activation of these transcription factors that are central for the biology of HRS cells.

Our data show that resistance of HRS tumor cells to death receptor stimulation is due to functional inhibition mediated by a strong, NF-κB–dependent up-regulation of c-FLIP proteins. c-FLIP mediates protection of HRS cells from CD95 and TRAIL death receptor stimuli, but also probably other TNFR family members. The manipulation of death receptor systems by pharmacological agents that down-regulate NF-κB activity and subsequently c-FLIP expression, or the specific down-regulation of c-FLIP proteins, might render HRS cells susceptible to death receptor–induced apoptosis and thus offer new therapeutic strategies for cHL.

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