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### Antioxidant c-FLIP Inhibits Fas Ligand-Induced NF- $\kappa$ B Activation in a Phosphatidylinositol 3-Kinase/Akt-Dependent Manner

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Fas ligand (FasL) belongs to the TNF family of death ligands, and its binding to the FasR leads to activation of several downstream signaling pathways and proteins, including NF- $\kappa$ B and PI3K/Akt. However, it is not known whether cross-talk exists between NF- $\kappa$ B and PI3K/Akt in the context of FasL signaling. We demonstrate using both human renal epithelial 293T cells and Jurkat T-lymphocyte cells that although FasL activates both Akt and NF- $\kappa$ B, Akt inhibits FasL-dependent NF- $\kappa$ B activity in a reactive oxygen species-dependent manner. Cellular FLICE-inhibitory protein (c-FLIP), an antioxidant and an important component of the death-inducing signaling complex, also represses NF- $\kappa$ B upstream of the regulatory I $\kappa$ B kinase- $\gamma$  protein subunit in the NF- $\kappa$ B signaling pathway, and positive cross-talk exists between Akt and c-FLIP in the context of inhibition of FasL-induced NF- $\kappa$ B activity. The presence of two death effector domains of c-FLIP and S-nitrosylation of its caspase-like domain were found to be important for mediating c-FLIP-dependent downregulation of NF- $\kappa$ B activity. Taken together, our study reveals a novel link between NF- $\kappa$ B and PI3K/Akt and establishes c-FLIP as an important regulator of FasL-mediated cell death. *The Journal of Immunology*, 2011, 187: 3256–3266.

poptotic cell death plays an important role in a number of physiological and pathophysiological conditions (1). Apoptosis is mediated primarily by the TNF family of proteins, and Fas (APO-1 or CD95) is an important member of this death receptor family, which triggers cell death primarily by binding Fas ligand (FasL) (2). FasL binds Fas as a trimer at the cell surface, initiating the apoptosis cascade by the formation of a death-inducing signaling complex (DISC) (3–5). Caspase-8 is recruited to the DISC, where its gets self-activated by proteolytic cleavage, subsequently activating downstream effector caspases (6). In addition to the formation of the DISC and subsequent activation of proapoptotic proteins, FasL binding also causes the production of reactive oxygen species (ROS) such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide ( $\cdot$ O<sub>2</sub><sup>-</sup>) leading to cell death via oxidative stress signaling (7, 8).

An important mediator of caspase-8 activation at the DISC is a structurally related protein known as cellular FLICE inhibitory protein (c-FLIP) (9). Initially identified as an inhibitor of caspase-8, it is now known that c-FLIP can also exert proapoptotic effects (10, 11). In addition to the full-length form of c-FLIP (55 kDa) or p55-FLIP (c-FLIP<sub>L</sub>) that contains two death effector domains (DEDs) and a caspase-like domain, two main proteolytically processed forms of c-FLIP<sub>L</sub> have been characterized: p43-FLIP (43 kDa) and the shorter p22-FLIP (22 kDa), which contains only the two DEDs (12, 13). In addition to the proteolytic fragments just mentioned, splice variants of cFLIP<sub>L</sub>, known as cFLIP<sub>R</sub> and cFLIP<sub>S</sub>, also exist, which have been shown to play an important role in FasL-induced apoptosis (14, 15).

A growing number of reports demonstrate a direct correlation between carcinogenesis and the ectopic expression levels of c-FLIP. However, studies quantifying total c-FLIP in various cells lines show that the physiological level of c-FLIP expression is extremely low and approximates to only 1% of endogenous procaspase-8 levels (12, 16). The functional role of c-FLIP depends upon its expression levels: extremely low levels of c-FLIP typically induce apoptosis, whereas higher levels are cytoprotective (17–23).

Apart from proteins at the DISC, activation of NF- $\kappa$ B by FasR stimulation has been reported to occur in several cellular systems (16, 24–26). Upon FasL-induced DISC formation, the I $\kappa$ B kinase (IKK) complex is activated by receptor interacting protein, leading to the phosphorylation of I $\kappa$ B and its subsequent degradation via the ubiquitin-proteosome pathway (27). Degradation of I $\kappa$ B releases the NF- $\kappa$ B complex (composed of p50 and p65 subunits) into the nucleus, leading to subsequent activation of a variety of target genes (28, 29). Mice knockout studies that involve silencing of NF- $\kappa$ B and IKK- $\beta$  genes showed massive apoptosis of hepatocytes and embryonic lethality (30–32), indicating that NF- $\kappa$ B plays a critical antiapoptotic role in death receptor-induced signaling.

An important mediator of NF- $\kappa$ B activity that also regulates cell death is the serine/threonine protein kinase Akt/PKB, which is activated via phosphoinositides produced by PI3K (33, 34). Depending upon a variety of factors, Akt may have either proapoptotic or antiapoptotic effects in response to FasL stimulation (35). Akt can stimulate expression of antiapoptotic proteins such

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Abbreviations used in this article: c-FLIP, cellular FLICE inhibitory protein; DCF, dichlorofluorescein diacetate; DED, death effector domain; DHE, dihydroethidium; DISC, death-inducing signaling complex; FasL, Fas ligand; IKK, IkB kinase; NAC, *N*-acetylcysteine; ROS, reactive oxygen species.

as c-FLIP and inhibitors of apoptosis and suppress proapoptotic proteins such as caspase-9 and cytochrome c, thus exhibiting a dual role (33, 36–38).

Although both Akt and NF- $\kappa$ B have been extensively studied for their role in death-receptor signaling, and both are sensitive to FasL, it is not known whether cross-talk exists between the two pathways in the context of FasL-induced cell death. In this study, we report that FasL can activate both Akt and NF- $\kappa$ B; however, Akt exerts an inhibitory effect on NF- $\kappa$ B in a ROS-dependent and c-FLIP-dependent manner. c-FLIP is also capable of independently inhibiting FasL-mediated NF- $\kappa$ B activity through downregulation of ROS, which occurs upstream of the IKK complex. S-nitrosylation and processing of c-FLIP are also important factors in determining c-FLIP-dependent NF- $\kappa$ B downregulation.

This study may broaden the scope of the signaling potential of c-FLIP in the regulation of apoptosis at extremely low levels and establish a hitherto unknown link between the PI3K/Akt and NF- $\kappa$ B pathway in FasL-mediated cell death. Further, it expands the role of c-FLIP to include a novel role in mediating Akt–NF- $\kappa$ B cross-talk, which has significant implications in inflammation, cytotoxicity, and cellular homeostasis.

### **Materials and Methods**

### Chemicals and reagents

Recombinant Fas ligand (*Super*FasL) and mAb to c-FLIP (Dave-2) were purchased from Alexis Biochemicals (San Diego, CA). Wortmannin, *N*acetylcysteine (NAC), and 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) were obtained from Calbiochem (La Jolla, CA). The oxidative probes dichlorofluorescein diacetate (DCF) and dihydroethidium (DHE) were from Molecular Probes (Eugene, OR). Abs for IkB, phospho-IkB, GAPDH, myc, and peroxidase-labeled secondary Abs were obtained from Cell Signaling Technology (Danvers, MA). The transfecting reagent Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). For transfection of Jurkat cells, Metafectene Pro was procured from Biontex (San Diego,



**FIGURE 1.** FasL-dependent NF-κB activity is mediated by hydrogen peroxide. *A*, Cells were cotransfected with 100 ng/well of NF-κB-Luc and 10 ng/ well of pRL-tk normalizing luciferase plasmid for 24 h. Transfected cells were treated with increasing concentrations of FasL (0–200 ng/ml) for 12 h and assayed for NF-κB luciferase activity. Plots show relative NF-κB activity over nontreated control. Concurrently, cells that were treated with increasing concentrations of FasL were assayed for apoptosis using the Hoechst assay and graphed. \**p* < 0.05 (for each FasL data point compared with control without FasL). *B*, Cells cotransfected with the NF-κB-Luc (100 ng/well) and pRL-tk (10 ng/well) vectors for 24 h. Transfected cells were pretreated with 10 mM NAC for 1 h followed by treatment with increasing doses (0–200 ng/ml) of FasL for 12 h. NF-κB activity was measured by luciferase assay. *C* and *D*, Cells were either left untreated or were pretreated with 10 mM NAC for 1 h followed by treatment with various concentrations (0–200 ng/ml) of FasL. Cells were then analyzed for either (*B*) H<sub>2</sub>O<sub>2</sub> or (*C*) ·O<sub>2</sub><sup>-</sup> production by measuring DCF and DHE fluorescence intensity, respectively. Plots show relative fluorescence intensity over nontreated control at the peak response time of 1 h. *E*, Cells were pretreated with 1,000 U/ml catalase for 1 h followed by FasL treatment (0– 200 ng/ml) for 12 h and were analyzed for H<sub>2</sub>O<sub>2</sub> production by measuring DCF fluorescence intensity. Plots show relative fluorescence intensity over nontreated control at the peak response time of 1 h. *F*, Cells cotransfected with the NF-κB-Luc (100 ng/well) and pRL-tk (10 ng/well) vectors were pretreated with 1,000 U/ml catalase for 1 h prior to treatment with FasL (0–200 ng/ml) for 12 h. FasL-dependent NF-κB activity was measured by luciferase assay. \**p* and \*\**p* < 0.05 where indicated for each data point treated with inhibitor (NAC or Catalase) and FasL as compared to FasL alone.



FIGURE 2. FasL activates PI3K/Akt in a dose-dependent manner. Akt negatively regulates NF-kB. A, 293T cells were treated with increasing doses of FasL (25-200 ng/ml) or left in serum-free medium alone without FasL for 1 h and assayed for total Akt and phosphorylated Akt-473 levels by immunoblot analysis. Blots were reprobed with GAPDH Ab to confirm equal loading of the samples. The immunoblot signals for phosphorylated Akt were quantified by densitometry. B, Cells cotransfected with the NFκB-Luc and pRL-tk vectors were either left untreated or pretreated with 20 µM LY294002 or 10 µM wortmannin for 1 h. After pretreatment, cells were treated with FasL (0-200 ng/ml) for 12 h and assayed for NF-KB activity. Concurrently, cells were lysed and assayed for phosphorylated Akt to confirm modulation of Akt using Western blotting. Blots were reprobed with GAPDH Ab to confirm equal loading of samples. C, In addition to the luciferase vectors described in A, cells were cotransfected with either 1 µg control pcDNA3 plasmid or constitutively active Akt plasmid (Akt-WT). Posttransfection, cells were treated with 200 ng/ml FasL for 12 h in the presence or absence of 20 µM LY294002 (1 h pretreatment) and assayed for NF-KB activity by luciferase assay. Further, FasL-treated lysates were probed for phosphorylated Akt to confirm effect of Akt-WT plasmid. Plots show relative NF- $\kappa$ B levels over nontreated control. Values are mean  $\pm$  SD (n = 4). \*p < 0.05 (versus nontreated control), \*\*p < 0.05, #p < 0.05

CA), and the transfections were carried out using the manufacturerrecommended protocol. The dual-luciferase assay kit was purchased from Promega (Madison, WI).

### Plasmids

The pcDNA3-FLIP (c-FLIP) plasmid was generously provided by Dr. Christian Stehlik (Northwestern University, Chicago, IL). The open reading frame of c-FLIP was amplified by high-fidelity PCR (Stratagene, Cedar Creek, TX) from the corresponding expressed sequence tags and cloned into pcDNA3 expression vectors containing the N-terminal myc epitope tag. FLIP domain mutants containing either the first DED ( $\Delta$ 3), both death domains ( $\Delta 2/p22$ -FLIP), and part of the caspase-like domain ( $\Delta 1/p43$ -FLIP) were generous gifts from Dr. Robert C. Thome (Burnham Institute, La Jolla, CA). Myc-tagged non-nitrosylable mutant of FLIP (FLIP-C2A) was generated using the Quik-Change mutagenesis kit (Stratagene). Authenticity of all constructs was verified by DNA sequencing. The IKK-y mutant plasmids for both the constitutively active form (IKK-y-WT) and the dominant negative form (IKK-y-DN) were a generous gift from Dr. Fei Chen (National Institute for Occupational Safety and Health, Morgantown, WV). The constitutively active Akt plasmid (Akt-WT) was generated by inserting the open reading frame of Akt into the pcDNA3 expression vector. The NF-KB reporter plasmid (NF-KB-Luc) was a kind gift from Dr. Peter Johnson (National Cancer Institute, Frederick, MD). The amount of DNA was normalized in all transfection experiments with pcDNA3. Expression of proteins was verified by Western blotting.

### Cell culture

Both human renal epithelial 293T cells and Jurkat T-lymphocyte cells were obtained from the American Type Culture Collection (Manassas, VA). 293T cells were cultured in DMEM, supplemented with 10% FBS, 200 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 5% CO<sub>2</sub> in plates precoated with 0.02% gelatin prepared in PBS. Twenty-four hours prior to transfection, cells were seeded into the required format (12- or 48-well) on plates precoated with 10  $\mu$ g/ml collagen IV (rat tail) in 10% DMEM medium without antibiotics. Jurkat suspensions were maintained in RPMI 1640 medium supplemented with 10% FBS, 400 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and maintained in 5% CO<sub>2</sub>. On the day of experiment, cells were spun down, split, and resuspended in appropriate medium depending on the specific assay.

### ROS detection

Intracellular peroxide and  $\cdot O_2^{-}$  production was determined by fluorometric analysis using specific probes DCF and DHE, respectively. After appropriate treatments, cells (1 × 10<sup>5</sup>/ml) were incubated with the fluorescent probes (5  $\mu$ M) for 30 min at 37°C, after which the cells were washed, resuspended in PBS, and analyzed for DCF (485/535 nm) and DHE (535/610 nm) fluorescence intensity using a multiwell plate reader (FLUOstar Optima, BMG Labtech, Durham, NC).

### Western blotting

After specific treatments, 293T cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM PMSF, and a protease inhibitor mixture (Roche Molecular Biochemicals, Basel, Switzerland) for 20 min on ice. Alternatively, for Jurkat cells, the cells were first collected and spun down at 2,000 rpm for 5 min and then incubated in lysis buffer. After insoluble debris was precipitated for both 293T cells and Jurkat cells by centrifugation at 14,000  $\times$  g for 15 min at 4° C, the supernatants were collected and assayed for protein content using bicinchoninic acid method (Pierce Biotechnology, Rockford, IL). Equal amount of protein per sample (30 µg) was resolved on a 10% SDS-PAGE and transferred onto a 0.45-µm nitrocellulose membrane (Pierce). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.05% Tween 20) and incubated with the appropriate primary Abs and HRP-conjugated isotypespecific secondary Abs. The immune complexes were detected by chemiluminescence (Supersignal West Pico; Pierce) and quantified by imaging densitometry using UN-SCAN-IT automated digitizing software (Silk Scientific, Orem, UT). Mean densitometry data from independent

<sup>(</sup>versus FasL-treated data sets). Ntx, 293T cells in serum-free medium alone without FasL.

experiments were normalized to the control where indicated. The data were presented as mean  $\pm$  SD and analyzed by Student *t* test.

#### NF-KB reporter gene assays

Cells were seeded in 48-well plates and cultured to 80–90% confluence. Cells were cotransfected with 100 ng/well of NF- $\kappa$ B reporter (NF- $\kappa$ B-Luc) plasmid, 10 ng/well of the renilla luciferase vector (pRL-tk), and differing amounts of c-FLIP plasmid (either full length or domain mutants). Empty pcDNA-3 control vector was used either as a control or added to c-FLIP such that the total DNA in each well was equal. In other experiments, plasmid constructs for Akt and IKK (described earlier) were cotransfected (1  $\mu$ g/well) as described, and equal concentration of empty pcDNA3 vector was used as control. Cell were transfected using the Lipofectamine 2000 transfection reagent (Invitrogen) for 293T cells or Metafectene Pro for Jurkat cells, respectively, according to the manufacturer's protocol. After a 24-h recovery period, transfected cells were treated with CD95/FasL with or without pretreatment with inhibitors for 12 h. Cell extracts were prepared and analyzed for luciferase activity using the Promega dual luciferase assay kit.

#### Luciferase activity assay

Luciferase activity was measured by enzyme-dependent light production using a luciferase assay kit (Promega). After each experiment, cells were washed and incubated at room temperature for 10 min in 250  $\mu$ l lysis buffer (Promega). For analysis, 10  $\mu$ l of each sample was loaded in an automated luminometer (Bio-Rad, Hercules, CA). At the time of measurement, 100  $\mu$ l luciferase assay reagent was automatically injected in each sample, and total firefly luminescence was measured over a 10-s time interval. This was immediately followed by the injection of 100  $\mu$ l stop-and-glow reagent to measure the control renilla luminescence. The output was quantified as total firefly light units relative to the control renilla luminescence for each sample.

#### Apoptosis measurements

Subconfluent (80%) densities of cells were treated with FasL and incubated with 10  $\mu$ g/ml Hoechst 33342 nuclear stain for 30 min at 37°C. The percentage of cells having intensely condensed chromatin and/or fragmented nuclei was scored by fluorescence microscopy (Axiovert 100; Carl Zeiss) using Pixera software. Data from at least 10 separate fields were recorded and plotted.

### Statistical analysis

The data represent mean  $\pm$  SD from three or more independent experiments. Statistical analysis was performed by Student *t* test at a significance level of p < 0.05 for all experiments for the indicated data sets.

### Results

# FasL-dependent NF- $\kappa B$ activity is mediated by hydrogen peroxide

The activation of NF-κB upon FasL stimulation was first ascertained in human renal epithelial cells (293T cells) using an NF-κB luciferase reporter system, which has been shown to be a reliable indicator of NF-κB promoter activity (39). FasL induced NF-κB activation in a dose-dependent manner; a dose-dependent increase in overall apoptosis was also observed (Fig. 1*A*). Because the role of ROS in mediating the proapoptotic effect of death ligands such as FasL TNF- $\alpha$  is well established (40, 41), we assessed for ROS involvement in mediating NF-κB activity observed with FasL stimulation. Therefore, cells were pretreated with the general antioxidant NAC prior to assaying for NF-κB. NAC inhibited FasL-dependent NF-κB activation, suggesting that ROS may play



**FIGURE 3.** c-FLIP is a negative regulator of ROS generation and FasL-induced NF-κB activity. In addition to transfection with 100 ng/well NF-κB-Luc and 10 ng/well pRL-tk normalization luciferase construct, cells were transfected with either empty pcDNA3 vector or increasing concentrations (0–4 ng/ml) of c-FLIP. Total DNA in each cell was kept equal by addition of empty control vector. Further, cells were lysed and assayed for total c-FLIP protein using immunoblot analysis. Blots were reprobed for GAPDH to ensure equal loading. *A*, Transfected cells were treated with (0–200 ng/ml) FasL for 12 h and analyzed for NF-κB activity by luciferase assay. Plots show relative luciferase activity over pcDNA3 control. *B* and *C*, Cells transfected with either control pcDNA3 or c-FLIP plasmids (0–4 ng/ml) were assessed for total IκB levels in the absence (*B*) or presence (*C*) of FasL (200 ng/ml) by immunoblotting. Blots were reprobed with GAPDH Ab to confirm equal loading of the samples. The immunoblot signals were quantified by densitometry. *D*, Cells transfected with either control pcDNA3 or c-FLIP plasmids (0–4 ng/ml) were treated with 200 ng/ml FasL (12 h) with or without 1,000 U/ml catalase (1 h pretreatment) and analyzed for H<sub>2</sub>O<sub>2</sub> by measuring DCF fluorescence. Plots show relative fluorescence intensity over nontreated control at the peak response time of 1 h. Values are mean ± SD (*n* = 4). \**p* < 0.05 (versus nontreated control), \**p* < 0.05 (versus pcDNA3 or c-FLIP plasmids. Twenty-four hours posttransfection, cells were treated with 200 ng/ml FasL (12 h), with or without 10 ng/well of NF-κB luciferase activity and the activity plotted. Ntx, 293T cells in serum-free medium alone without FasL. \**p* < 0.05 (versus pcDNA controls).

a positive role in mediating FasL-induced NF- $\kappa$ B activation (Fig. 1*B*). Both  $\cdot O_2^-$  and H<sub>2</sub>O<sub>2</sub> are important second messengers generated upon FasL treatment in mouse macrophages (39). To identify the dominant ROS involved in FasL-induced NF- $\kappa$ B activation, we used specific fluorescent probes for H<sub>2</sub>O<sub>2</sub> (DCF) and  $\cdot O_2^-$  (DHE). FasL induced H<sub>2</sub>O<sub>2</sub> in a dose-dependent manner, and NAC significantly inhibited this effect (Fig. 1*C*); however,  $\cdot O_2^-$  levels remained largely unchanged with increasing doses of FasL, even in the presence of NAC (Fig. 1*D*). Pretreatment of cells with catalase, a specific H<sub>2</sub>O<sub>2</sub> scavenger, was sufficient to cause inhibition of both FasL-induced NF- $\kappa$ B activity and overall H<sub>2</sub>O<sub>2</sub> levels (Fig. 1*E*, 1*F*). Thus, H<sub>2</sub>O<sub>2</sub> seemed to be the specific ROS that seemed to play a predominant role in FasL signaling and subsequent NF- $\kappa$ B activity.

### FasL activates PI3K/Akt in a dose-dependent manner

Akt negatively regulates NF- $\kappa B$ . In conformity with previous studies that demonstrated PI3K/Akt regulation by FasL (7, 39, 42), we observed that increasing doses of FasL induced phosphorylation of PI3K/Akt in 293T cells without affecting total Akt levels (Fig. 2A). Because FasL had a positive effect on both PI3K/Akt and NF-KB, we assessed for potential cross-talk between the two molecules in the context of FasL signaling. 293T cells were pretreated with PI3K inhibitors LY294002 and wortmannin and assayed for NF-KB activity using the luciferase reporter assay. Surprisingly, inhibition of PI3K/Akt led to a marked increase in NF- $\kappa$ B activity in the presence of FasL (Fig. 2B). A similar effect was observed in Jurkat cells as well (Supplemental Fig. 2). To ascertain whether Akt was directly involved in NF-KB inhibition or acted downstream of other molecules in the PI3K/Akt pathway, cells were cotransfected with a plasmid encoding constitutively active Akt (Akt-WT) along with the NF-kB reporter plasmid in 293T cells, and luciferase activity was measured in the absence or presence of FasL. We observed a decrease in NF-KB luciferase activity with overexpression of Akt plasmid, and Akt-mediated inhibition was observed even in the face of LY294002, suggesting a direct role for Akt in downregulation of FasL-dependent NF- $\kappa$ B signaling (Fig. 2C). Thus, although FasL activates both PI3K/Akt and NF-KB, Akt inhibits NF-KB upon FasL stimulation.

# *c*-*FLIP* is a negative regulator of ROS generation and FasL-induced NF- $\kappa$ B activity

Because several studies have implicated c-FLIP in the regulation of NF- $\kappa$ B (13, 43), we investigated its role in mediating the effect of FasL on NF-KB. Transfection of 293T cells with increasing doses of plasmid encoding c-FLIP at low concentrations (0-4 ng) alone caused a small albeit significant increase in basal NF-KB activity; however, c-FLIP strongly inhibited NF-KB levels in the presence of FasL in a dose-dependent manner (Fig. 3A, Supplemental Fig. 2). To assess whether the observed changes in NF-KB reporter levels were reflected in modulation of protein expression of NFκB-regulatory proteins, we measured protein expression levels of total IkB subunit, an inhibitory protein that remains bound to inactive NF-KB and sequesters it in the cytoplasm (44), in the presence or absence of FasL. As with NF-KB, c-FLIP alone downregulated IkB protein levels in a dose-dependent manner (Fig. 3B); however, in the presence of FasL, c-FLIP upregulated I $\kappa$ B (Fig. 3C). This suggested a dichotomy in c-FLIP signaling depending upon the absence or presence of FasL stimulation. Also, in addition to NF-kB, c-FLIP downregulated H2O2 levels in the presence of FasL (Fig. 3D). Given that inhibition of  $H_2O_2$ leads to direct downregulation of NF-KB (Fig. 1B, 1F), the inhibitory effects of c-FLIP on NF-KB may also be driven by its antioxidant effect in the presence of FasL. To assess whether



FIGURE 4. Akt-dependent NF-KB inhibition occurs through c-FLIP. A, Cells transfected with either 4 ng/ml of either pcDNA3 or c-FLIP were treated with 200 ng/ml FasL (12 h) in the presence or absence of 20 µM LY294002 or 10 mM NAC (1-h pretreatment). Treated cells were analyzed for phospho-Akt levels by immunoblotting. Blots were reprobed with GAPDH Ab to confirm equal loading of the samples. The immunoblot signals were quantified by densitometry. \*p < 0.05 (for all data points compared with FasL treatment alone without c-FLIP [data point 1]), \*\*p < 0.05 (for c-FLIP versus pcDNA-transfected data sets for both FasL treatment and FasL treated with LY294002). B, Cells transfected with 4 ng/ml c-FLIP, in addition to 1 mg of either Akt-WT or pcDNA control plasmid, were exposed to 200 ng/ml FasL in the absence or presence of 20 µM LY294002 (1 h pretreatment) and probed for c-FLIP levels using Western blotting. Blots were reprobed with GAPDH Ab to confirm equal loading of the samples. The immunoblot signals were quantified by densitometry. C, Cells were either transfected with 1 µg Akt-WT plasmid or pcDNA3 control plasmid for 24 h. pcDNA3-transfected cells were then pretreated with either 20 µM LY294002 or with serum-free medium for 1 h, followed by FasL treatment for 12 h. Akt-WT transfected cells were directly treated with FasL for 12 h. The samples were then analyzed for  $H_2O_2$ production by measuring DCF fluorescence intensity. Plots show relative fluorescence intensity over nontreated control at the peak response time of 1 h. Values are mean  $\pm$  SD (*n* = 4). \**p* < 0.05, \*\**p* < 0.05 (versus FasLtreated controls). Ntx, 293T cells in serum-free medium alone without FasL.

modulation of  $H_2O_2$  by c-FLIP led to an effect on NF- $\kappa$ B activity, cells cotransfected with c-FLIP and NF- $\kappa$ B reporter plasmid were assayed for luciferase activity in the presence of both catalase and NAC. As shown previously, preincubation with NAC and catalase caused a decrease in NF- $\kappa$ B activity in the presence of FasL. Notably, transfection with c-FLIP caused a further decrease in NF- $\kappa$ B activity compared with ROS inhibitors alone (Fig. 3*E*).

### Akt-dependent NF-KB inhibition occurs through c-FLIP

Thus far, the data suggest that both c-FLIP and Akt repress FasLinduced NF-kB activity. Furthermore, this inhibitory action of both proteins is mediated at least in part by ROS, suggesting that cross-talk may exist between Akt and c-FLIP in the context of FasL signaling. To assess whether such an interaction exists, 293T cells were transfected with either empty pcDNA3 vector or 4 ng c-FLIP and assayed for activated Akt. Transfection with c-FLIP led to increased levels of phosphorylated Akt in the presence of FasL, and this increase was sustained even in cells pretreated with LY294002 and NAC (Fig. 4A). Pretreatment with NAC also upregulated basal level of FasL-induced Akt in the absence of c-FLIP (Fig. 4A, fifth lane), which suggests that the positive effect of c-FLIP on Akt could at least partially be due to its antioxidant role. Conversely, modulation of Akt activity also led to a concurrent change in levels of c-FLIP (Fig. 4B). Inhibition of Akt by pretreatment with LY294002 decreased c-FLIP protein expression, whereas transfection with the constitutively active Akt-WT plasmid increased c-FLIP protein levels.

Because Akt has a positive effect on c-FLIP, this suggests that Akt may also indirectly possess antioxidant activity in the context of FasL signaling, which was ascertained by measuring the effect of modulation of Akt on  $H_2O_2$  levels. Indeed, pretreatment with LY294002 led to an increase in  $H_2O_2$  levels, whereas transfection with Akt-WT plasmid had an inhibitory effect (Fig. 4*C*).

### Regulation of NF- $\kappa B$ is mediated by positive cross-talk between c-FLIP and Akt

As we have independently demonstrated above that Akt and c-FLIP exerted inhibitory effect on NF-kB in the presence of FasL, we wanted to now assess for cross-talk between these two molecules in the context of NF-KB inhibition and the putative role of ROS in such an interaction. We first used PI3K/Akt modulators in the absence or presence of low levels of c-FLIP and assayed for FasLinduced NF-KB activity. Cells transfected with c-FLIP significantly inhibited LY294002- and wortmannin-mediated increase in FasL-dependent NF-KB activity (Fig. 5A). This was reflected even in phosphorylated IkB, with c-FLIP inhibiting phospho-IkB levels in a dose-dependent manner in the presence of LY294002 and NAC (Fig. 5B). Therefore, regulation of FasL-induced NF- $\kappa$ B by Akt seems to be mediated by c-FLIP. This was further confirmed by assaying for NF-KB luciferase activity in cells transfected with constitutively active Akt-WT plasmid. c-FLIP inhibited NF-KB in cells transfected with Akt-WT plasmid, and this effect was sustained even in the presence of LY294002 and NAC (Fig. 5C). Finally, assaying for H<sub>2</sub>O<sub>2</sub> levels with c-FLIP-transfected cells in the presence of PI3K/Akt modulators showed an inhibition of DCF fluorescence with c-FLIP, which confirmed a role for ROS in c-FLIP-mediated downregulation of FasL-induced NF-KB levels (Fig. 5D). Taken together, the data suggest that both Akt and c-FLIP play a coregulatory role in inhibiting FasL-induced NF-KB activity. Furthermore, the data show that the antioxidant role of



**FIGURE 5.** Regulation of NF-κB is mediated by positive cross-talk between c-FLIP and Akt. *A*, Cells were cotransfected with 100 ng/well of NF-κB-Luc plasmid, 10 ng/well of pRL-tk plasmid, and 4 ng/ml of either pcDNA3 or c-FLIP plasmids. Twenty-four hours posttransfection, cells were treated with 200 ng/ml FasL (12 h), with or without 1 h pretreatment with 20  $\mu$ M LY294002 and 10  $\mu$ M wortmannin. Treated cells were lysed and assayed for NF-κB luciferase activity. *B*, Levels of phosphorylated IκB were assessed for cells transfected with increasing doses of c-FLIP (0–4 ng/ml) by Western blotting. Transfected cells were treated with 200 ng/ml FasL (12 h) alone or in the presence of 20  $\mu$ M LY294002 or 10 mM NAC (1-h pretreatment). Blots were reprobed with GAPDH Ab to confirm equal loading of the samples. The immunoblot signals were quantified by densitometry. *C*, Cells were cotransfected with 1  $\mu$ g Akt-Wt and 4 ng/ml c-FLIP and assessed for NF-κB activity in the presence of 200 ng/ml FasL (12 h) with or without 20  $\mu$ M LY294002 or 10 mM NAC (1-h pretreatment). *D*, Cells were either transfected with 1  $\mu$ g Akt-WT plasmid or pcDNA3 control plasmid for 24 h, in addition to 4 ng/ml c-FLIP plasmid. pcDNA3-transfected cells were pretreated with 20  $\mu$ M LY294002 for 1 h, followed by FasL treatment (0–200 ng/ml) for 12 h. Akt-WT transfected cells were directly treated with FasL (0–200 ng/ml) for 12 h. The samples were then analyzed for H<sub>2</sub>O<sub>2</sub> production by measuring DCF fluorescence intensity. Plots show relative fluorescence intensity over nontreated control at the peak response time of 1 h. Values are mean ± SD (*n* = 4). \**p* < 0.05, \*\**p* < 0.05 (versus control data sets as shown in the respective figures). Ntx, 293T cells in serum-free medium alone without FasL.

c-FLIP is important in driving its cross-talk with Akt and also suggest an important overall role for ROS in FasL-induced NF- $\kappa$ B activity.

### Regulation of NF-KB by c-FLIP is upstream of IKK

Because both c-FLIP and Akt can regulate NF-κB, we wanted to assess which regulatory components of the NF-κB pathway could mediate this inhibitory effect. The IKK complex lies upstream of NF-κB and is composed of three subunits:  $\alpha$ ,  $\beta$ , and  $\gamma$ . Previous studies have shown that the IKK- $\gamma$  subunit plays a regulatory role in NF-κB activity and can be directly modulated by c-FLIP (13). Therefore, we cotransfected cells with either a constitutively active (IKK-WT) or dominant negative (IKK-DN) form of the IKK- $\gamma$  subunit along with 4 ng/ml c-FLIP, and assessed the effect on NF-κB luciferase activity. In the absence of c-FLIP, IKK-WT caused a basal increase in NF-κB activity, whereas IKK-DN decreased it both in the presence or absence of FasL (Fig. 6A, 6B). Transfection with c-FLIP downregulated both IKK-WT– and IKK-DN–mediated NF-κB activity (Fig. 6B, 6C). This suggested



**FIGURE 6.** Regulation of NF-κB by c-FLIP is upstream of IKK. *A*, Cells transfected with either pcDNA control plasmid or 4 ng/ml c-FLIP and exposed to 200 ng/ml FasL in the absence or presence of 10 mM NAC were probed for phosphorylated IKK- $\alpha/\beta$  using Western blotting. Blots were reprobed with GAPDH Ab to confirm equal loading of the samples. *B* and *C*, Cells were cotransfected with 100 ng/well of NF-κB-Luc plasmid, 10 ng/well of pRL-tk plasmid, 4 ng/ml of either pcDNA3 or c-FLIP plasmids, and 1 µg pcDNA3, IKK-WT, or IKK-DN plasmids. Twenty-four hours posttransfection, cells were treated with 200 ng/ml FasL along with (*B*) 10 mM NAC or (*C*) 20 µM LY294002. Treated cells were analyzed for NF-κB levels by luciferase assay. Plots show relative NF-κB levels over nontreated control. Values are mean ± SD (*n* = 4). \**p* < 0.05 (versus nontreated control), \*\**p* < 0.05 (versus pcDNA-transfected control data sets). Ntx, 293T cells in serum-free medium alone without FasL.

that c-FLIP acted upstream of the IKK complex. Further, overexpression of IKK- $\gamma$  by transfection of the IKK-WT plasmid was not sufficient to overcome c-FLIP-mediated downregulation of FasL-induced NF- $\kappa$ B activity. This effect was at least partially mediated by ROS and PI3K/Akt, as c-FLIP could further inhibit FasL-dependent NF- $\kappa$ B levels in the presence of NAC (Fig. 6*B*) and LY294002 (Fig. 6*C*). Overall, the data suggested that the inhibitory effect of c-FLIP on NF- $\kappa$ B occurs upstream in the IKK pathway, possibly by modulation of the IKK complex.

### *Role of c-FLIP processing and S-nitrosylation in FasL-induced NF-κB activation*

Previous studies have shown that FLIPs is mainly responsible for the activation of NF- $\kappa$ B by the regulation of the IKK complex (44). Upon activation, full-length c-FLIP is processed into shorter forms such as p43-FLIP and p22-FLIP (45). Because the processing of c-FLIP may also be important in the downregulation of FasL-induced NF-KB activity, we wanted to identify and assess individual domains in c-FLIP that may be responsible for NF-KB inhibition. We found that c-FLIP was mainly undetectable in non-transfected cells (left lanes in both blot panels in Fig. 7A) but was processed predominantly into p43-FLIP upon FasL stimulation in cells transfected with 4 ng/ml c-FLIP (Fig. 7A, second lane in right panel). Therefore, we generated relevant domain deletion mutants of c-FLIP and cotransfected cells with either full-length c-FLIP or its deletion constructs along with NF-kB luciferase construct (Fig. 7B). In the presence of FasL, full-length c-FLIP inhibited NF- $\kappa$ B as expected. However, the shorter forms of c-FLIP (p22-FLIP and p43-FLIP) could also lead to inhibition of NF-KB activity, with p22-FLIP driving inhibition of FasL-dependent NF-KB activity the most compared with the other c-FLIP constructs (Fig. 7C). In contrast, NF-kB inhibitory activity was lost when transfected with c-FLIP construct comprising only death effector domain 1 (DED1), suggesting that both DED1 and DED2 were required for NF-KB inhibition. Previous studies by our group had suggested an important role for S-nitrosylation in mediating c-FLIP expression and stability in FasL signaling (46, 47). Therefore, we assessed the importance of c-FLIP S-nitrosylation in mediating NF-KB activity. Cells were transfected with a non-nitrosylable c-FLIP mutant (46) and assessed for FasL-dependent NF-KB activity. The non-nitrosylable mutant further downregulated FasL-dependent NF-KB activity, suggesting that S-nitrosylation of c-FLIP may lead to a decrease in its effectiveness in inhibition of FasL-dependent NF-KB activity (Fig. 7D). The overall effects of c-FLIP signaling in our model have been summarized in Fig. 8.

### Discussion

Homeostasis is a tightly regulated phenomenon and requires the continuous interplay of both cell death and cell survival pathways. NF-κB and PI3K/Akt are important regulators of cellular homeostasis. In the context of FasL-mediated apoptosis, NF-κB primarily exerts a prosurvival signal, probably as a defense mechanism to protect cells from superfluous death ligand-induced apoptosis (48). We found that FasL-induced NF-κB activity was ROS dependent in 293T cells, with H<sub>2</sub>O<sub>2</sub> being the predominant species responsible for NF-κB activation (Fig. 1*E*, 1*F*). This is consistent with previous studies showing that transcriptional inhibition of FasL using antioxidants also leads to the inhibition of NF-κB and has a net inhibitory effect on FasL (49–52).

Both PI3K/Akt and NF-κB play a role in protecting cells from undergoing apoptosis (53–56). However, this is the first study, to our knowledge, that demonstrates an inhibitory effect of PI3K/Akt on FasL-induced NF-κB activity. This result is certainly surprising, given that FasL positively stimulates both PI3K/Akt and NF-



**FIGURE 7.** Role of c-FLIP processing and S-nitrosylation in FasL-induced NF-κB activation. *A*, Cells transfected with either pcDNA3 control plasmid or 4 ng/ml c-FLIP were exposed to 200 ng/ml FasL and probed for c-FLIP levels using Western blotting. Blots were reprobed with GAPDH Ab to confirm equal loading of the samples. *B*, Domain mutants were generated using standard techniques as mentioned in *Materials and Methods* and were cloned into the pcDNA3 vector. The physiologically relevant processed forms include p43-FLIP (D1), p22-FLIP (D2), and domain mutant expressing only the DED1 (D3). In addition, full-length c-FLIP with the two cysteines in its caspase-like domain substituted by alanines (D4) was generated such that the mutated form cannot undergo S-nitrosylation. Western blots probed with c-myc Ab show the expression of the c-FLIP domain mutants. *C*, Cells cotransfected with 100 ng/well NF-κB-Luc, 10 ng/well pRL-tk, and domain mutants of c-FLIP (4 ng/ml) were treated with 200 ng/ml FasL (12 h) in the absence or presence of 20 μM LY294002 and 10 mM NAC (1-h pretreatment) and then assayed for NF-κB activity by luciferase assay. *D*, Cells were cotransfected with 100 ng/well NF-κB-Luc, 10 ng/well pRL-tk, and non-nitrosylable c-FLIP mutant (D4). Transfected cells were either left untreated or pretreated with 20 μM LY294002 and 10 mM NAC for 1 h followed by 200 ng/ml FasL treatment for 12 h. Treated cells were analyzed for NF-κB activity by luciferase assay. Values are mean ± SD (*n* = 4). \**p* < 0.05, \*\**p* < 0.05 (for respective data versus pcDNA-transfected control data sets as indicated). Ntx, 293T cells in serum-free medium alone without FasL.

 $\kappa$ B activity (Figs. 1*A*, 2*A*). However, given that PI3K/Akt has also been shown to play a proapoptotic role in response to FasL (42), inhibition of NF-κB fits well with the model of PI3K/Akt-induced apoptosis, as NF-κB is a strong antiapoptotic signaling molecule, and its inhibition may be required for potent induction of cell death (57, 58).

Furthermore, this study is among a select few that demonstrate that c-FLIP can inhibit FasL-induced NF-KB activity (Fig. 3). c-FLIP is a known antioxidant that is regulated by the NF-KB signaling pathway (59), and c-FLIP in turn can lead to a direct and potent increase in NF-KB activity (10, 60-62). The role of c-FLIP as an oncogenic factor in cancer progression and chemoresistance has been well documented (60, 63). However, studies assessing its role in tumors predominantly involve high levels of c-FLIP expression (more than 100 ng/ml). The "physiological" expression levels of c-FLIP vary depending on cell type and are typically extremely low, which has hindered the delineation of c-FLIP function under normal cellular conditions (16). In fact, basal c-FLIP levels were undetectable even in 293T cells in our study (Fig. 7A), which has also been corroborated by others (61). However, there have been a few important studies assessing the role of c-FLIP under physiological conditions, the overall results of which are reaffirmed in our study. For example, Kreuz et al. (16) suggested that physiological levels of c-FLIP exerted an inhibitory effect on FasL-induced NF-kB-driven IL-8 activity in a caspase-dependent manner. Also, Chang et al. (23) demonstrated that c-FLIP may play a dual role depending upon its expression levels: at low levels that mimic physiological conditions, c-FLIP is proapoptotic, whereas at higher protein levels of c-FLIP such as that observed in tumors, c-FLIP promotes survival. Recent studies investigating the role of c-FLIP and its processed forms in FasL signaling by Neumann et al. (64), Lavrik (65), and Fricker et al. (66) from the German Cancer Research Center have suggested a similar proapoptotic effect for c-FLIP at physiological concentrations using a systems biology approach. Overall, our results effectively complement the data presented by the aforementioned studies while demonstrating a novel role for PI3K/Akt in regulating c-FLIP–mediated inhibition of NF- $\kappa$ B upon FasL treatment.

We demonstrate that at low levels, c-FLIP alone activates NF-KB as expected. However, in the presence of FasL, NF-KB activity was inhibited by c-FLIP in a dose-dependent manner (Fig. 3A, 3C). Furthermore, low levels of c-FLIP also inhibited H<sub>2</sub>O<sub>2</sub> levels in the presence of FasL (Fig. 3D), which suggested that the inhibition of NF-KB by c-FLIP might be due at least partially to its antioxidant properties, which have been shown to be important in FasL signaling (67). However, c-FLIP can further inhibit NF-ĸB even in the presence of NAC, suggesting that a ROS-independent mechanism of NF-KB regulation by c-FLIP may also exist. We now know from corroborating evidence provided by the studies performed in Germany that c-FLIP-driven inhibition of NF-KB may be due to direct interaction of the p43 form of c-FLIP with the IKK-y subunit. The increased processing of c-FLIP upon FasL stimulation observed in our study supports this idea (Fig. 7A). Also, c-FLIP is capable of inhibiting NF-KB activity even when IKK is upregulated using constitutively active IKK-y construct (Fig. 6). Lastly, much higher inhibition of NF-KB is observed when transfected with the p43-FLIP construct compared with c-FLIP<sub>L</sub>. However, the important aspect of this result is the fact that c-FLIP seems to play a regulatory role depending upon its expression levels, with higher levels leading to an exactly opposite effect as proposed by the recent studies (64–66). Such concentration-dependent duality in function for c-FLIP is very significant in that it suggests an extremely critical function for c-FLIP in regulation of the cell death. Some of the important findings in 293T cells have also been confirmed using Jurkat T-lymphocytes, which offers further credibility to this study and makes these findings translatable to physiological conditions (Supplemental Fig. 2).

We also observe that both PI3K/Akt and c-FLIP positively regulate each other in the presence of FasL (Fig. 4A, 4B) and can inhibit NF-KB, which has not been reported previously. A feedback loop is observed between c-FLIP and Akt, leading to an overall decrease in NF-KB levels, which is corroborated by data observed with coexpression of c-FLIP and Akt-WT (Fig. 5C). The modulation of c-FLIP by PI3K/Akt was observed only in c-FLIPtransfected cells (Fig. 6A), which suggests either that any increase in endogenous c-FLIP expression with Akt stimulation is undetectable, or that there might be a high turnover of c-FLIP protein, which has also been observed by others (16). Our results also show that c-FLIP promotes PI3K/Akt-mediated downregulation of NF- $\kappa$ B, which may be due to the antioxidant properties of c-FLIP as suggested by recapitulation of this effect upon cotreatment with NAC. Also, we modulated levels of the regulatory IKK- $\gamma$  subunit (Fig. 6) and not other subunits of the IKK complex because both c-FLIP and viral FLIP have been previously shown to directly act on IKK- $\gamma$ , thereby exerting effects on overall NF- $\kappa$ B levels (68, 69).

As mentioned earlier, processing of c-FLIP was important for inhibition of NF- $\kappa$ B. This is supported by the fact that c-FLIP is found to be processed in the presence of FasL and not in its absence at least in 293T cells (compare the second and fourth lanes in Fig. 6A), and opposite effects are seen with c-FLIP modulation of NF-KB depending upon either the presence or absence of FasL (Fig. 2). Assessment of NF-kB activity using deletion mutants of c-FLIP that lack one or more important domains indicates that p22-FLIP was particularly effective in inhibiting NF-κB, leading to a much higher decrease in NF-KB activity compared with fulllength c-FLIP (Fig. 6C). Although previous studies show that p22-FLIP promotes NF-KB activity, the seeming inconsistency can be explained by the fact that only low levels of c-FLIP (up to 4 ng/ ml) were used in our study compared with the much higher levels used in previous work. This provides further validation to the dichotomy observed with NF-kB regulation in the context of c-FLIP. Notably, the non-S-nitrosylable mutant of c-FLIP caused further inhibition of NF-KB activity, with levels even lower than that those observed with p22-FLIP. This suggests that not only is the processing of c-FLIP important for its inhibitory effect on NFκB, but also posttranslational modifications such as S-nitrosylation may impede this downregulation under physiological conditions and may have an important role to play in c-FLIP-associated pathophysiological effects.

In addition to the effects of Akt on c-FLIP, previous studies have also shown that the stress-activated protein kinase/JNK pathway may also regulate c-FLIP and can potentially play a regulatory role in the inhibition of NF- $\kappa$ B. However, our data indicate that modulation of JNK activity (using the JNK inhibitor SP600125) did not lead to any significant changes in Akt levels and did not affect downstream proteins at the time points included in this study (see Supplemental Fig. 1). However, treatment with SP600125 was able to counter the increases in NF- $\kappa$ B activity when Akt was repressed (see Supplemental Fig. 1*D*), suggesting that Akt and JNK may have some form of cross-talk, which needs to be investigated further. In addition, no significant changes in FasR levels were observed for the time points assayed, suggesting that inhibitory effects of c-FLIP on NF- $\kappa$ B may be independent of FasR levels (Supplemental Fig. 1).

FasL is an important stimulator of apoptosis, and we observe a decrease in NF-KB levels with an increase in c-FLIP levels in our system. Because NF-KB is an important prosurvival factor, cells transfected with low levels of c-FLIP show a higher level of FasLdependent apoptosis compared with that of non-transfected cells (Fig. 8A). Thus, our study purports the importance of c-FLIPmediated downregulation of NF-kB as a novel and significant mechanism in induction of FasL-induced apoptosis. Also, in our system, we observe an increase in apoptosis but a decrease in c-FLIP-mediated H<sub>2</sub>O<sub>2</sub> production. Although this may be counterintuitive, given that a positive correlation exists between ROS levels and degree of apoptosis, several studies also show that ROS may also have antiapoptotic effects (70). In contrast, this may suggest that inhibition of NF-kB by physiological levels of c-FLIP may have a more significant impact on overall apoptosis compared with the putative protective mechanism as a result of the antioxidant effects of c-FLIP. Therefore, our model may serve as an alternate and updated representation of the role of c-FLIP signaling in the context of FasL-induced inflammatory response under physiological conditions (Fig. 8B). The data presented in this article recapitulate some of the important conclusions drawn



FIGURE 8. Model for c-FLIP-induced apoptosis at low levels of c-FLIP expression. A, Cells transfected with increasing levels of c-FLIP (0-4 ng/ ml) were either treated with 200 ng/ml FasL and assayed for apoptosis or cotransfected with 100 ng NF-KB-Luc and 10 ng pRL-tk vectors and assayed for luciferase levels. In the presence of FasL, increasing c-FLIP levels caused a dose-dependent increase in apoptosis but a corresponding decrease in NF-KB activity. B, FasL binding causes the formation of the DISC, composed of Fas, FADD, procaspase-8, and receptor interacting protein (not shown). Formation of the DISC recruits c-FLIP, where it is activated and cleaved into its shorter forms such as p43-FLIP (45). In this study, we show that FasL can activate Akt through activation of c-FLIP protein, and vice versa. Increased c-FLIP levels at the physiological range leads to decreased H<sub>2</sub>O<sub>2</sub> (primary ROS involved), which occurs upstream of the IKK complex, and leads to a decrease in NF-KB activity. Further, Akt may also cause NF-KB downregulation in a c-FLIP-independent manner. Downregulation of NF-kB by either mechanism leads to decrease in apoptosis.

by recent studies investigating the physiological role of c-FLIP and highlight both the duality of c-FLIP signaling based on levels of both c-FLIP itself and of FasL and the resulting effect on overall apoptosis (Fig. 8A). In addition, our data also hint at the importance of other factors such as NO (which contributes to S-nitrosylation of c-FLIP), which may have an influence on the function of c-FLIP and will be pursued in the future.

We believe that this study may help pave the way for further understanding of FasL-mediated inflammatory response and shed new light on the role of PI3K/Akt and c-FLIP in apoptosis, which are traditionally understood to be prosurvival factors. Particularly, such studies are important to extend the understanding of c-FLIP under normal biological conditions, including growth and immune response, and may lay the foundation for detailed mechanistic studies on c-FLIP and its role in apoptosis.

### Disclosures

The authors have no financial conflicts of interest.

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Supplemental Fig. 1. The effects of FasL on Akt- and c-FLIP-mediated inhibition of NF-KB are independent of JNK and Fas Receptor levels. A, B. 293T cells were transfected with either empty vector control pcDNA vector, or with c-FLIP plasmid using LipofectAMINE 2000 using standard protocols. Following transfection, cells were exposed to 200ng/ml FasL in the absence or presence of JNK inhibitor SP600125 (10 $\mu$ M) as indicated in (A), or with Akt inhibitor LY294002 (20 $\mu$ M) as shown in (B) for 12 h. Cells were lysed and assayed for levels of proteins indicated using immunoblot analysis. Blots were re-probed for GAPDH to ensure equal loading. Addition of FasL, and co-treatment with either JNK or Akt inhibitors did not have any significant effect on Fas receptor levels. Phospho-JNK (A) and phospho-Akt (B) levels were inhibited by SP600125 and LY294002 respectively, indicating that the inhibitors were active. Further, addition of SP600125 did not affect phospho-Akt levels in the absence or presence of FasL as shown in (A). Similarly, Akt inhibitor LY294002 also did not affect levels of activated JNK either (B), suggesting a lack of cross-talk between Akt and JNK in the context of Akt-mediated inhibition of NF-KB in the presence of FasL. C. 293T cells were co-transfected with 100ng/well NF-kB-Luc, 10ng/well pRL-tk normalization luciferase construct, and either empty pcDNA3 vector or increasing concentrations (2ng/ml, 4ng/ml) of c-FLIP. Total DNA in each well was kept equal by addition of empty control vector. Transfected cells were treated either with FasL alone (200ng/ml) or in the presence of Akt inhibitor LY294002 (20µM) or JNK inhibitor SP600125 (10μM) for 12 h. Cells were then harvested and assayed for NF-κB luciferase activity. Plots show relative NF- $\kappa$ B activity over non-treated control. Values are mean ± SD (n = 4). \*P < 0.05 versus corresponding samples treated with LY294002 alone. Pretreatment with LY294002 released NF-κB from Akt-mediated repression in the presence of FasL. NF-κB levels remained comparable to SFM-treated controls (compare data sets for *Ntx* and *FasL+SP*) for cells treated with SP600125. However, treatment of cells with both LY294002 and SP600125 together did have a significant effect on NF-κB levels. SP294002 inhibited the increase in NF-κB levels observed using LY294002 alone (see data set for *FasL+LY*), suggesting a probable role for JNK in Akt-mediated effects on NF-κB. However, a lack of effect of SP294002 on Akt levels (see immunoblot in (*A*)) was also observed, suggesting that the effect of SP294002 on the levels of NF-κB, if any, may be independent of Akt levels.

**Supplemental Fig. 2. Effects of FasL on Akt and c-FLIP and their cross-talk observed in 293T cells is recapitulated in Jurkat T-cell lymphocytes.** *A*. 1 x 10<sup>6</sup> Jurkat T-cells were plated into a 6-well plate in complete medium, and transfected with 50ng/ml eGFP plasmid using Metafectene Pro reagent. Cells were imaged 24 h post transfection for green fluorescence. The image indicates low albeit measurable level of transfection of the cells. *B*. In addition to transfection with 200ng/well NF- $\kappa$ B-Luc and 20ng/well pRLtk normalization luciferase construct, cells were transfected with either empty pcDNA3 vector or 50ng/ml c-FLIP. 24 h post transfection, cells were treated with 200ng/ml FasL in the absence or presence of 20 μM LY294002 for 12h, and assayed for NF- $\kappa$ B luciferase activity, and data plotted as shown. Values are mean ± SD (n = 3). \*P < 0.05 versus non-treated control; \*P < 0.05 and \*\*P < 0.05 versus respective pcDNA controls as shown. The data shows that inhibition of Akt (*FasL+LY*) led to increased activation of NF- $\kappa$ B, but SP600125 did not have any significant effects. Transfection with c-FLIP could further inhibit FasL mediated activation of NF- $\kappa$ B in the presence and absence of inhibitors, recapitulating the results obtained with 293T cells (see Fig. 5C). *C*. Jurkats treated with 50ng/ml c-FLIP in the absence or presence of FasL with or without LY294002 were assayed for several proteins using immunoblotting analysis. An increase in both phospho-I- $\kappa$ B and NF- $\kappa$ B are observed in the presence of Akt inhibitor LY294002, confirming the inhibitory role for Akt on NF- $\kappa$ B in the presence of FasL. *D*. Jurkats treated with FasL in the presence or absence of inhibitors showed no significant changes in FasR levels, suggesting that regulation of NF- $\kappa$ B by Akt may be independent of protein levels of death receptor.

### Supplemental Figure 1:



### Supplemental Figure 2:

