Rapid Communication

Cellular FLICE-like Inhibitory Protein Deviates Myofibroblast Fas-Induced Apoptosis Toward Proliferation during Lung Fibrosis

Regina Golan-Gerstl[1]*, Shulamit B. Wallach-Dayan[1]*, Philip Zisman[1], Wellington V. Cardoso[2], Ronald H. Goldstein[3], and Raphael Breuer[1,4]

[1]Lung Cellular and Molecular Biology Laboratory, Institute of Pulmonary Medicine, Hadassah–Hebrew University Medical Center, Jerusalem, Israel; [2]Pulmonary Center, Boston University School of Medicine, Boston; [3]Pulmonary Medicine, Boston VA Healthcare System, Boston; and [4]Department of Pathology, Boston University School of Medicine, Boston, Massachusetts

A prominent feature of fibrotic tissue in general and of lungs in particular is fibroblast proliferation and accumulation. In patients overcoming fibrosis, apoptosis limits this excessive cell growth. We have previously shown resistance to Fas-induced apoptosis of primary lung fibroblasts from mice with bleomycin-induced lung fibrosis, their escape from immune surveillance, and continued accumulation in spite of overexpression of the Fas death receptor. Cellular FLICE-like inhibitory protein (c-FLIP) is a regulator of cell death receptor–induced apoptosis in many cell types. We aimed to determine c-FLIP levels in myofibroblasts from fibrotic lungs and to directly assess c-FLIP's role in apoptosis and proliferation of primary lung myofibroblasts. c-FLIP levels were determined by apoptosis gene array, flow cytometry, Western blot, and immunofluorescence before and after down-regulation with a specific small interfering RNA. Apoptosis was assessed by caspase cleavage in Western blot and by Annexin V affinity labeling after FACS and tissue immunofluorescence. Proliferation was assessed by BrdU uptake, also using FACS and immunofluorescence. We show that myofibroblasts from lungs of humans with idiopathic pulmonary fibrosis and from bleomycin-treated versus normal saline-treated mice up-regulate c-FLIP levels. Using the animal model, we show that fibrotic lung myofibroblasts divert Fas signaling from apoptosis to proliferation and that this requires signaling by TNF receptor–associated factor (TRAF) and NF-κB. c-FLIP down-regulation reverses the effect of Fas activation, causing increased apoptosis, decreased proliferation, and diminished recruitment of TRAF to the DISC complex. This indicates that c-FLIP is essential for myofibroblast accumulation and may serve as a potential target to manipulate tissue fibrosis.

Keywords: fibrosis; c-FLIP; myofibroblast; apoptosis; proliferation

CLINICAL RELEVANCE

As opposed to currently available treatment, which suppresses the immune system in an attempt to block scar formation, the treatment approach we propose boosts immune system activity by blocking the evasion of fibrotic tissue fibroblasts from immune cell–induced apoptosis to alter their uncontrolled accumulation. We show here that an established mechanism for regulating resistance to cell death in cancer cells also affects proliferation in lung myofibroblasts isolated from fibrotic tissue in the experimental model of bleomycin-induced chronic lung inflammation culminating in fibrosis. This involves the caspase 8 inhibitor Flice-like inhibitory protein (FLIP). The role of FLIP in fibrotic tissue is to block fibroblast susceptibility to Fas- and immune cell–induced apoptosis and to impose proliferation during active fibrosis. Thus, the regulation of FLIP expression and function may pave the way for the regulation of fibrotic diseases.

Deregulation of a controlled healing response after injury results in tissue scarring due to uninterrupted accumulation of myofibroblasts (1). Persistent myofibroblast survival and accumulation appear to be important in the evolution of lung fibrosis in animal models and idiopathic pulmonary fibrosis (IPF) in humans (1).

We have recently shown that myofibroblasts accumulating in fibrotic, but not in normal, lungs resist Fas-death receptor-induced apoptosis and escape immune surveillance (2). Fas contains a classic death domain within its cytosolic tail called FADD (Fas-associated death domain) (3, 4). FADD binds to an analogous domain within the zymogen form of caspase 8, also known as FLICE (5). Members of a family of viral proteins called v-FLIP and a related cellular protein called c-FLIP, also known as CFLAR and I-FLICE (6), contain a death effector domain that is similar to the corresponding segment in FADD and caspase 8.

c-FLIP expression usually correlates with resistance to Fas- and immune cell–induced apoptosis that is signaled via death receptors (7) and with escape from immune surveillance *in vivo* (8–11). Down-regulation of c-FLIP confers sensitivity to Fas-mediated apoptosis (7, 12, 13).

Under some conditions, Fas can stimulate proliferation of several types of cells (14, 15), including fibroblasts (16). c-FLIP provides the molecular switch allowing Fas to promote cell proliferation, as opposed to apoptosis, as reviewed by Thome and Tschopp (17) and Peter (18). When overexpressed, c-FLIP induces kinase and NF-κB activation, apparently by binding the upstream kinase Raf-1 (19).

c-FLIP has been shown to be overexpressed in lung myofibroblasts of humans with IPF (12); however, this finding was not confirmed in a more recent study (20). Thus, c-FLIP's role in myofibroblast apoptosis and proliferation in the lungs of humans with IPF remains controversial. It is therefore essential to perform experiments directly and specifically interfering with myofibroblast

(*Received in original form July 6, 2010 and in final form April 26, 2012*)

* These authors contributed equally to this work.

This work was supported by the Israel Science Foundation (grant no. 855/10), the Israeli Ministry of Health, the Israel Lung Foundation, and the David Shainberg Fund.

Correspondence and requests for reprints should be addressed to Shulamit Wallach-Dayan, Ph.D., Lung Cellular and Molecular Laboratory, Institute of Pulmonary Medicine, Hadassah University Hospital, POB 12000, Jerusalem, Israel. E-mail: wallach-dayan@hadassah.org.il

Am J Respir Cell Mol Biol Vol 47, Iss. 3, pp 271–279, Sep 2012

Originally Published in Press as DOI: 10.1165/rcmb.2010-0284RC on May 10, 2012
Internet address: www.atsjournals.org

c-FLIP expression to unequivocally identify its biological properties in apoptosis and proliferation of primary lung myofibroblasts.

We characterized c-FLIP expression on myofibroblasts during the evolution of fibrosis in the lungs of mice and of humans with IPF and determined c-FLIP's role as a potential inhibitor of Fas-induced myofibroblast apoptosis and mediator of proliferation using the experimental model of bleomycin-induced lung fibrosis in mice.

We show that c-FLIP is consistently overexpressed in lung myofibroblasts of bleomycin-treated C57BL/6 mice and humans with IPF. We further show that, upon Fas stimulation, fibrotic lung myofibroblasts resist Fas-induced apoptosis and become sensitive to Fas-induced proliferation. Concomitantly, after Fas stimulation, these fibroblasts form DISC-FADD complexes and show a significant increase in the recruitment of the TNF receptor–associated factor (TRAF) signaling molecule to the DISC-FADD complex. Despite overexpression of other antiapoptotic genes, the selective down-regulation of c-FLIP by small interfering RNA (siRNA) was sufficient to sensitize fibrotic lung myofibroblasts to Fas-induced apoptosis and to annul Fas-induced proliferation.

Therefore, we conclude that c-FLIP is a key regulator of death receptor resistance in fibrotic lung myofibroblasts, that its up-regulation might be a mechanism for myofibroblast proliferation, and that the TRAF signaling molecule is recruited by c-FLIP to uphold this process, leading to myofibroblast accumulation in injured lungs and resulting in fibrosis.

MATERIALS AND METHODS

Animals

Sprague Dawley C57BL/6 mice (11–12 wk old) (Harlan, Indianapolis, IN) were used in these experiments. All animal experiments and animal care procedures were reviewed and approved by our institutional committee for the protection of animals.

Intratracheal Instillation for Induction of Fibrosis

Bleomycin intratracheal instillation was performed in C57BL/6 mice as we have previously detailed (2, 21–25).

Lung Myofibroblast Isolation

Myofibroblasts were isolated 14 days after intratracheal instillation of bleomycin or saline at the time of maximal fibrosis (23) as we have reported previously (2, 21).

Tissue and Myofibroblast Isolation from Human Lung Biopsies

Lung samples were obtained from lung biopsy specimens of seven patients with IPF and from five patients undergoing lung cancer resection. Lung tissues were embedded in paraffin or minced and cultured for myofibroblast isolation (2, 26).

Fas Activation

Myofibroblast Fas activation was performed as we have previously detailed (21).

Gene Array

GEArray/SuperArray (SuperArray Biosciences Corp., Bethesda, MD) analysis was performed using cDNA isolated from lung myofibroblasts as we have previously detailed (21).

Real-Time PCR

RNA samples were reverse transcribed and subjected to PCR analysis (MiniCycler; MJ Research, Waltham, MA) as we have previously detailed (27). The following primers were used: forward, $FLIP_L$ (the long isoform of FLIP) 5′TGCTGAAGTCATCCAT CAGG3′; reverse, $FLIP_L$ 5′ATTCCTAGGGGC TTGCTCT3′. The housekeeping gene was glyceraldehyde 3-phosphate dehydrogenase.

Analysis of Apoptosis

Annexin V affinity labeling was performed, and apoptosis was assessed using low cytometry or confocal analysis as detailed previously (2, 21). Caspase-8 activity was measured as we have detailed previously (28).

Down-Regulation of c-FLIP by siRNA Transfection

Mouse siRNA-FLIP (Qiagen, Hilden, Germany) and control nonspecific siRNA (Qiagen), were delivered into fibrotic lung myofibroblasts in culture as we have detailed (29). Down-regulation of c-FLIP was verified by flow cytometry.

Immunoprecipitation

Myofibroblast lysates incubated with 2 μg anti-FADD pAb (R&D Systems, Minneapolis, MN) were immunoprecipitated with 100 μl protein-A–gel slurry (Pierce, Rockford, IL). FLIP-FADD complex was detected by enhanced chemiluminescence using anti-FLIP antibody as detailed previously (29).

Proliferation Analysis by BrdU

BrdU uptake was analyzed by flow cytometry (FACStar; Becton Dickinson, Franklin Lakes, NJ) or confocal microscopy (Carl Zeiss AG, Oberkochen, Germany) as previously detailed (29).

Immunofluorescence of c-FLIP and α-Smooth Muscle Actin or TRAF or Iκ-B Kinase Expression in Lung Tissue Sections or Monolayers

Lung myofibroblasts were cultured on 0.2-mm-thick coverslips or pelleted and incubated with 1:100 FITC-conjugated, anti–α-smooth muscle actin (α-SMA) (Dako, Carpenteria, CA) and Cy5-conjugated, anti-FLIP (ClonTech, Mountain View, CA) mAbs or FITC-conjugated, anti-TRAF mAb or with I-κB kinase (IKK) mAb (Cell Signaling, Danvers, MA). Myofibroblast monolayers were analyzed by confocal microscopy (Axio Scope 2; Carl Zeiss AG) and cell pellets by flow cytometry as we have previously detailed (2, 21, 29). Lung sections were similarly stained for α-SMA, FLIP, TRAF, or IKK and subjected to confocal analysis.

Immunohistochemical Staining of Tissue Sections

Sections were stained as we have previously described in detail (21). Briefly, lung sections were incubated overnight at 4°C with polyclonal rabbit anti-FLIP (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 in 1% BSA PBS Tween 0.05%. The Envision Detection System (DAKO, Glostrup, Denmark) containing secondary anti-rabbit horseradish peroxidase–conjugated antibody and 3,3′-diaminobenzidine as a substrate was used for staining detection.

Trypan Blue Exclusion

Myofibroblasts were exposed to trypan blue dye (0.04% in 1× PBS), placed on a hemocytometer, and counted under light microscopy.

Western Blot

Myofibroblast protein was blotted, incubated with anti-FLIP (Stressgen, San Diego, CA) or anti-FADD, reincubated for 1 hour in the appropriate horseradish peroxidase–conjugated antiserum (1:2,500 dilution) (Jackson ImmunoResearch Labs, West Grove, PA), and analyzed by enhanced chemiluminescence as previously reported (29).

IKK Inhibition

Myofibroblasts were activated by Fas mAb or IgG with IKK-2 inhibitor (200 μM, 24 h) (Calbiochem, Gibbstown, NJ). Cell mass were determined by methylene-blue assay.

Electrophoretic Mobility Shift Assay

We used the nuclear extraction kit from Active Motive (Carlsbad, CA). Extracts (10 μl) were subjected to bandshift analysis as described elsewhere (30). We used the NF-κB probe 5′-ACCAAGAGGGATTT CACCTAAATC-3′. In each reaction, 2×10^4 cpm of labeled probe was used. Bandshifts were resolved on 5% PAGE.

RESULTS

c-FLIP Is Overexpressed in Fibrotic Murine Lung Myofibroblasts

We have previously shown that fibroblasts from fibrotic lungs overexpress the Fas death receptor (21) yet resist Fas-induced apoptosis (2). To find a molecular mechanism for this phenomenon, we randomly assessed the expression of 96 genes known to interfere with apoptosis using gene array techniques described previously (21). Although several mRNA molecules were overexpressed in cultured lung myofibroblasts isolated from fibrotic, bleomycin-treated mice (14 d after intratracheal instillation) compared with saline-treated normal control mice (Figure 1A), the Fas-inhibitor FLIP mRNA was markedly up-regulated (Figure 1A, *arrow*). Overexpression of FLIP-RNA was validated by real-time RT-PCR (Figure 1B). We also detected an increased c-FLIP expression in bleomycin- versus saline-treated lung myofibroblasts at the protein level using anti-FLIP mAb in Western blot from an optical density (OD) of 0.26 to 2.07 (Figure 1C) and in flow cytometry from 25 to 64% (Figure 1D).

We then determined c-FLIP expression in myofibroblasts *in vivo* in mice with bleomycin-treated versus normal lungs. Incremental c-FLIP expression in α-SMA–positive cells of fibrotic versus normal lungs was detected in lung tissue sections stained with anti-FLIP and/or anti–α-SMA mAbs and analyzed by immunofluorescence (Figure 1E) or immunohistochemistry (Figure 1F). Flow cytometry analysis of myofibroblasts freshly isolated from fibrotic lungs and from control saline-treated lungs confirmed this phenomenon. FACS results revealed that a median of approximately 49% of the fibrotic lung myofibroblasts express c-FLIP, compared with only 28% of those from normal lungs (Figure 1E, *insert*).

c-FLIP Is Overexpressed in Lung Myofibroblasts from Humans with IPF

We assessed whether increased c-FLIP expression detected in the mouse model is a feature of fibrosis in the lungs of humans with IPF. We noted that myofibroblasts isolated from open lung biopsy specimens of seven patients with IPF overexpress c-FLIP at the transcriptional level as assessed by real-time PCR, in comparison with myofibroblasts isolated from biopsy specimens of five patients with no clinical or pathological evidence of lung fibrosis who underwent surgery for lung cancer (Figure 2A). Three samples of lung sections and isolated myofibroblasts from patients with IPF and from non-IPF patients were analyzed. Similar results were obtained at the protein level using Western blot (Figure 2B) after staining for FLIP and detection in fibroblast foci (FF) in lung sections on immunohistochemistry (Figure $2C_1$) and after double staining of FLIP and α-SMA with detection on immunofluorescence (Figure $2C_2$).

c-FLIP Down-regulation in Fibrotic Lung Myofibroblasts Reverses Resistance to Fas-Induced Apoptosis

We examined the potential for a change in myofibroblast cell viability after FLIP down-regulation by siRNA transfection and Fas activation. One day after being transfected with c-FLIP siRNA, myofibroblasts were activated for 24 hours by Jo2 anti-Fas mAb or control IgG (20 μg/ml). After Fas activation, we detected, by light microscope and Trypan blue exclusion,

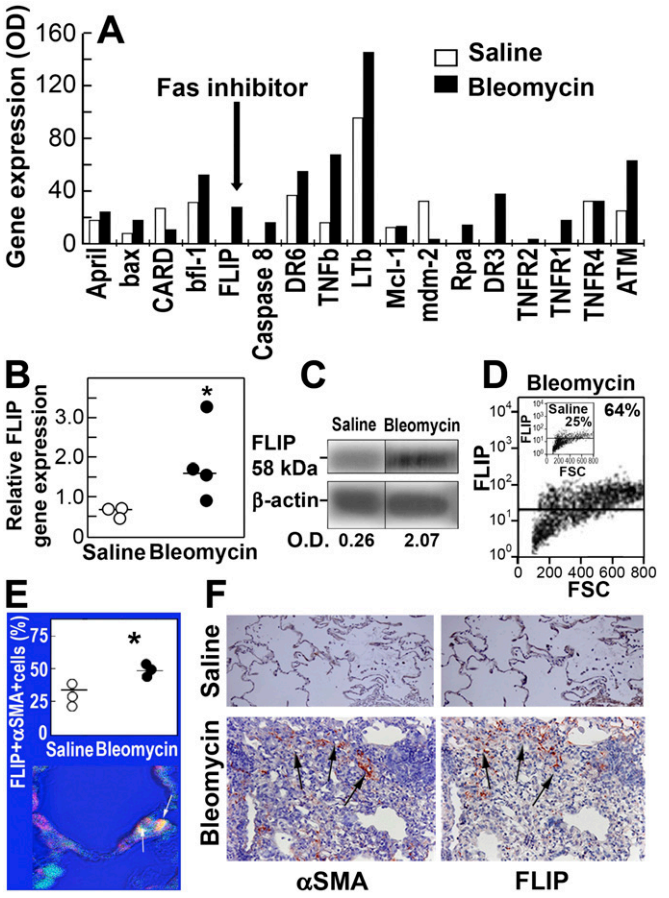

Figure 1. Cellular FLICE-like inhibitory protein (c-FLIP) overexpression in lung myofibroblasts of bleomycin-treated mice. (*A*) Apoptosis gene array analysis of cDNA probes from a pool of three total RNA samples corresponding to cultured myofibroblasts after intratracheal saline or bleomycin instillation into wild-type C57BL/6 mice. Signal optical intensities were compared with the RPLA 13A-housekeeping gene and quantified by densitometry after background subtraction. c-FLIP is marked by an *arrow*. Representative results from two experiments. (*B*) Real-time PCR. $*P < 0.05$. (*C*) Western blot and (*D*) flow cytometry analyses of FLIP expression in RNA and protein levels in myofibroblasts from bleomycin- versus saline-treated mouse lungs. Representative results from three or four experiments in (*B*), four experiments in (*C*), and three experiments in (*D*). (*E*) Immunofluorescence of c-FLIP and α-smooth muscle actin (α-SMA) dual staining in paraffin-embedded consecutive lung sections of bleomycin-treated mice lungs. *Original magnification*: ×40. (*E*, *insert*) Flow cytometry analysis of freshly isolated α-SMA and c-FLIP double-stained cells among total lung cells. Comparisons of double-stained cells (%) were made between the bleomycin (*solid circles*) and saline (*open circles*) groups. $*P < 0.05$. (*F*) Immunohistochemistry of c-FLIP and α-SMA staining in paraffin-embedded consecutive lung sections of bleomycin- versus saline-treated lungs ($n = 6$). *Original magnification*: ×40. Illustrative example (*arrows*).

a significant decrease in cell number (from 2.7 ± 0.7 SD $\times 10^4$ to 0.4 ± 0.2 SD $\times 10^4$) in siRNA FLIP-transfected but not in control siRNA-transfected lung myofibroblasts (Figure 3A; note inserted numbers). The controls showed increments in the cell number from 2.5 ± 0.6 SD $\times 10^4$ to 3.8 ± 0.6 SD $\times 10^4$ (Figure 3A; note inserted numbers, siRNA ctl). This indicates that, after down-regulation of c-FLIP levels, primary fibrotic lung myofibroblasts lose their ability to survive after activation of the Fas death receptor. Concomitantly, the cell death ratio between Fas and IgG treatments in fibrotic lung myofibroblasts, determined by

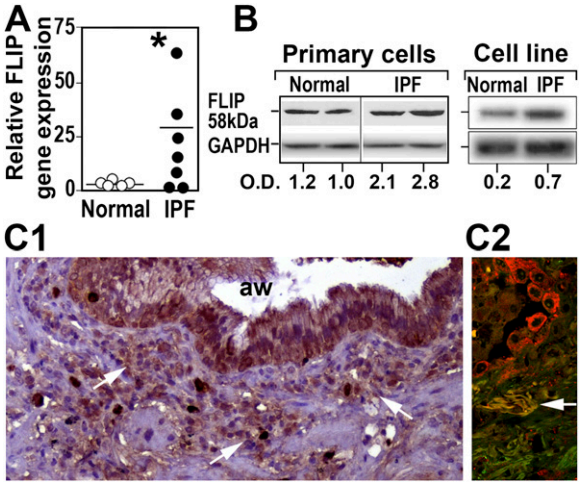

Figure 2. c-FLIP overexpression in myofibroblasts from lungs of humans with idiopathic pulmonary fibrosis (IPF). (*A*) Real-time PCR of c-FLIP$_L$ (the cellular long form of FLIP) RNA levels in myofibroblasts from IPF (*solid circles*) versus normal (*open circles*) lungs. *$P < 0.02$. (*B*) Western blot using rabbit anti-human c-FLIP primary Ab and donkey anti-rabbit secondary antibody detecting c-FLIP in lysates of human fibrotic (IPF) versus normal lung myofibroblasts ($n = 3$). (*C1*) Immunohistochemistry of c-FLIP in paraffin-embedded lung tissue sections from the lungs of humans with IPF (*original magnification*: ×40). Note fibroblastic foci (ff) (*arrow*) ($n = 8$). (*C2*) Immunofluorescence of c-FLIP and α-SMA staining in paraffin-embedded lung tissue sections from the lungs of humans with IPF (*original magnification*: ×40). Illustrative example of double staining (*arrow*) ($n = 3$).

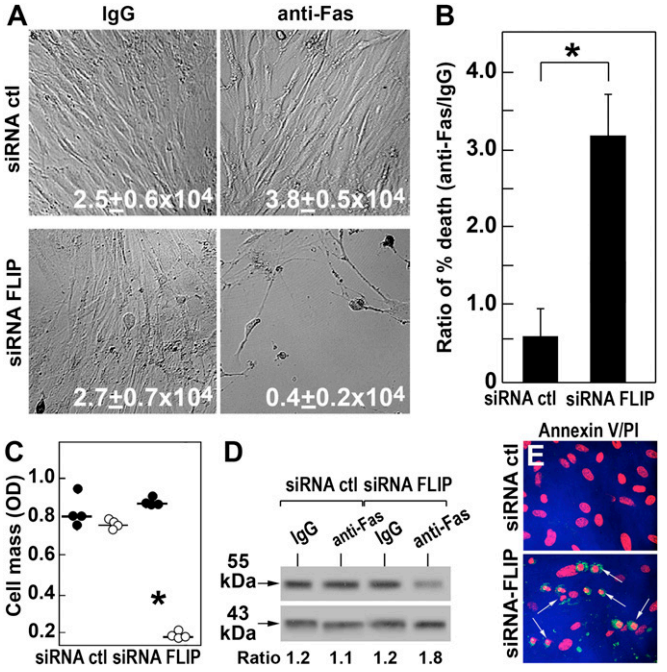

Figure 3. Resistance to Fas-induced cell death in fibrotic lung myofibroblasts is c-FLIP dependent. Fas mAb versus control IgG activation (20 μg, 24 h, room temperature [RT]) of mouse lung myofibroblasts (0.3×10^6) harvested after bleomycin intratracheal instillation, maintained in RPMI medium and 0% serum, and subjected to transient transfection with small interfering RNA (siRNA) c-FLIP or control (24 h, RT). (*A*) Light microscope imaging of myofibroblasts and counts (mean ± SD) in culture after IgG or Fas activation with the addition of siRNA FLIP or control. (*B*) Methylene blue staining quantitated by fold ratios of optical densities (OD) of cell death ($n = 3$ measurements in each treatment). *$P < 0.01$. (*C*) Cell mass was assessed using methylene blue staining after treatment with IgG (*solid circles*) versus anti-Fas (*open circles*) and siRNAs. Four measurements were made in each treatment. *$P < 0.01$. (*D*) Western blot of caspase 8 activation based on the ratio between p43-cleavage product and p55 uncleaved caspase 8. (*E*) Detection of Annexin V (*green*) and propidium iodide (*red*) staining with confocal microscopy. *Arrows* indicate the Annexin V staining and apoptotic condensate nucleus. Representative results of two experiments in each assay.

the percentage of trypan blue–stained cells among the total cell count, was increased after treatment with siRNA FLIP (Figure 3B). In addition, Fas or control IgG activation after c-FLIP down-regulation showed, in comparison to control siRNA-treated cells, a remarkable decrease in cell mass from a median of 0.85 OD to a median of 0.15 OD (Figure 3C).

We then determined changes in the extent of apoptosis induced after Fas activation in siRNA-treated fibroblasts using a variety of assays, including the specific assay of caspase 8 activation by Western blot (Figure 3D) and immunofluorescence of Annexin V together with propidium iodide staining of the nucleus (Figure 3E). All assays were repeated in two independent experiments that yielded similar results. After Fas activation, primary fibrotic lung myofibroblasts with down-regulated c-FLIP showed a 1.8-fold increase in caspase 8 activity, as determined by the ratio of uncleaved (55 kD) and cleaved (43 kD) subunits in anti-Fas–treated versus IgG-treated cells (Figure 3D), confocal microscopy assessment of increased staining with Annexin V, and increased nucleus condensation shown by propidium iodide–positive staining (Figure 3E).

This indicates that, after down-regulation of c-FLIP levels, primary lung myofibroblasts from fibrotic lungs are rendered susceptible to Fas-induced cell death and apoptosis.

Myofibroblasts from Fibrotic Bleomycin-Treated Mouse Lungs Switch Fas-Induced Apoptosis toward Proliferation and Activate TNF Receptor-Associated Factor and the NF-κB Signaling Pathway

The apparent contradiction between Fas overexpression and resistance to Fas-induced apoptosis that we detected in primary fibrotic lung myofibroblasts (2, 21) is consistent with a previously described nonapoptotic function of Fas and with the diversion to Fas-induced cell proliferation (14, 15, 31, 32). We thus initially compared proliferation of primary lung myofibroblasts from bleomycin-treated (fibrotic) and normal saline-treated mice after 24 hours of activation with 20 μg/ml Jo2 anti-Fas mAb or control IgG. After Fas molecule activation, we detected almost no change in cell number (Figure 4A; Saline), with an arrest (3.3% versus 5.4%) in cell proliferation (Figure 4B; Saline) only in myofibroblasts from normal lungs of saline-treated mice. In contrast, myofibroblasts from fibrotic lungs manifested almost a 2.5-fold increase in cell number (Figure 4A; Bleomycin) and an increased rate of cell proliferation, from 19.8 to 31% (Figure 4B; Bleomycin).

Because we found no sign of proliferation in myofibroblasts from normal lungs after Fas induction, we set out to assess the signaling pathway involved in the Fas-induced proliferation of fibrotic-lung myofibroblasts. To deviate Fas activation toward signaling that can induce proliferation, c-FLIP forms complexes with the Fas death domain (FADD). We assessed c-FLIP–FADD interaction in lysates of Fas-activated myofibroblasts in IP with anti-FADD mAb and after Western blot using anti-FLIP mAb. The results clearly demonstrate c-FLIP (58 kD) in FADD IP, indicating FLIP–FADD coimmunoprecipitation in Fas-activated fibrotic-lung myofibroblasts (Figure 4C; IP: FADD$^+$ versus FADD$^-$). We detected no signs of c-FLIP recruitment to the FADD complex after IgG treatment of myofibroblasts (not shown).

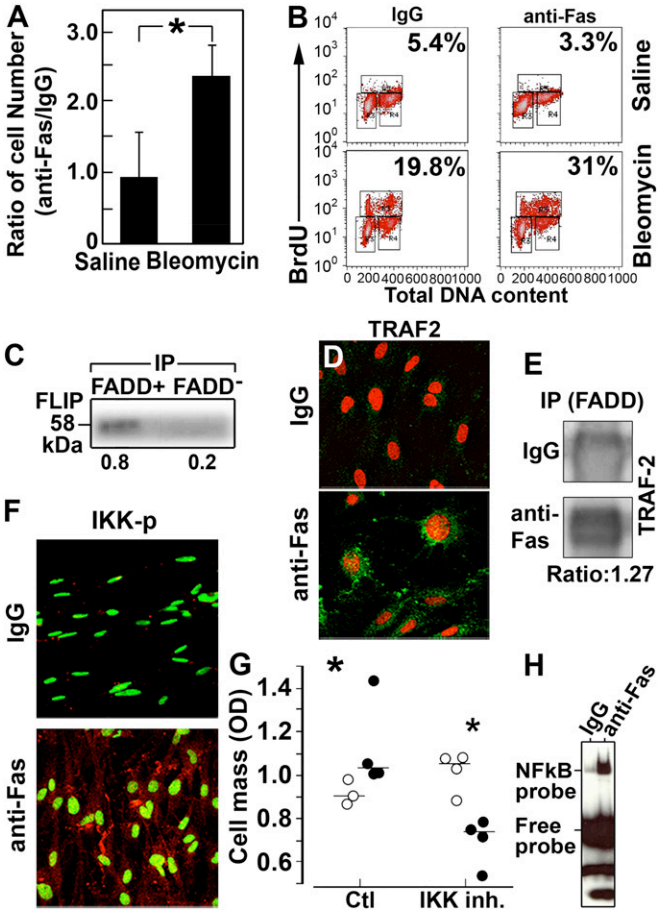

Figure 4. Fas induces proliferation in myofibroblasts from bleomycin-treated but not from saline-treated mice, and activates the NF-κB signaling pathway. (*A*) Trypan blue exclusion ($n = 3$). *$P > 0.05$. (*B*) Flow cytometry analysis (representative of two experiments) of BrdU uptake quantified at the S-phase versus PI-total DNA, at Day 14 of intratracheal instillation, in normal (saline-treated) and fibrotic (bleomycin-treated) lung myofibroblasts after activation with Jo2 anti-Fas versus IgG mAbs (20 μg, 24 h, RT) in 0% FCS. (*C*) Fas-associated death domain (FADD) immunoprecipitate followed by Western blot using anti-FLIP mAb. The 58-kD expected size of FLIP in anti-FADD immunoprecipitate and its absence in the control immunoprecipitation ($FADD^-$) is shown. (*D*) Confocal microscope images of indirect immunofluorescence after staining for TNF receptor–associated factor (TRAF)2 protein using FITC-conjugated antibody (*green*). Cell nuclei were stained with propidium iodide (*red*). (*E*) FADD immunoprecipitate followed by Western blot using anti-TRAF mAb. Quantitative analysis of protein levels was performed by optical densitometry of bands and measured as arbitrary units. (*F*) Confocal microscopy of IKK phosphorylation. (*G*) Methylene blue assay of cell mass before and after treatment with I-κB kinase (IKK) inhibitor after treatment with IgG (*solid circles*) versus anti-Fas (*open circles*). *$P < 0.05$. (*H*) Electrophoretic mobility shift assay of NF-κB activation after induction of Fas (versus IgG) in myofibroblasts from bleomycin-treated lungs. Purified cells were incubated with 10 μg/ml Jo2 anti-Fas. Nuclear extracts of activated myofibroblasts were analyzed by using NF-κB binding probe compared with the control-free probe.

Based on this finding, we further assessed, in fibrotic lung myofibroblasts, Fas-induced expression of downstream signaling molecules that are known to be recruited to the FADD membranal complex, such as TNF receptor–associated factor II (TRAF-2) (Figure 4D). To this end, fibrotic lung myofibroblasts were activated by anti-Fas or control IgG mAbs, stained with immunofluorescent antibody against TRAF-2, and analyzed by confocal microscopy. After Fas activation, positive staining of TRAF localized in the cytoplasmatic side of the cell membrane was detected (Figure 4D; TRAF in anti-Fas–activated versus IgG mAb–activated activated cells), indicating proximity to the FADD complex. Concomitantly, we detected an increased product of coimmunoprecipitation of TRAF in FADD IP in Fas-activated fibroblasts versus IgG (Figure 4E). This is an essential step to allow the execution of Fas signaling of proliferation. We saw almost no traces of TRAF in $FADD^-$ IP (not shown).

In some cases, cell death receptors stimulate phosphorylation of IKK, which in turn inhibits I-κB domains, thereby releasing NF-κB and subsequent promotion of cell proliferation. We assessed IKK activation in Fas- versus IgG-treated fibrotic lung myofibroblasts by immunofluorescence after cell staining with FITC-conjugated antiphosphorylated IKK (IKK-p)-mAb. Cell activation with Jo2 anti-Fas mAb induced IKK phosphorylation in fibrotic lung myofibroblasts (Figure 4F, *red staining*). To further assess the involvement of IKK in myofibroblast cell growth (i.e., changes in cell mass), we determined the effect of Fas signaling in fibrotic lung myofibroblasts with or without the addition of 20 μM SC-414 IKK inhibitor (Calbiochem, San Diego, CA). Fas activation increased myofibroblast cell mass (Figure 4G; Control). However, IKK inhibitor limited this increase (Figure 4G; IKK inhibitor).

We then directly determined the activation of NF-κB in fibrotic lung myofibroblasts after Fas activation by electrophoretic mobility shift assay. We found increased NF-κB in nuclear extracts from Fas- versus control IgG-stimulated fibrotic lung myofibroblasts (Figure 4H versus ctl).

Proliferation and TRAF Recruitment to the DISC Complex in Fas-Activated Myofibroblasts Is c-FLIP Dependent

Having found that fibrotic lung myofibroblasts form FLIP–FADD complexes and deviate Fas toward proliferation, we assessed the critical role of c-FLIP in fibrotic lung myofibroblast proliferation, after activation of the Fas death receptor and specific down-regulation of c-FLIP levels by siRNA.

Fibrotic lung myofibroblast cell proliferation was increased after Fas versus IgG activation only in control siRNA-treated fibroblasts (Figure 5A and *inserts*; siRNA ctl). This was assessed by BrdU uptake in S-phase, followed by immunofluorescent staining of BrdU and then by confocal microscopy analysis (Figure 5A, *green*) and by FACS analysis with increments from 5 to 9% (Figure 5A, *inserts*). No changes in BrdU uptake and cell proliferation were noted in Fas-activated myofibroblasts with down-regulated c-FLIP (Figure 5A and *inserts*; siRNA FLIP).

We then determined c-FLIP dependence of Fas-induced TRAF recruitment to the FADD complex in FADD immunoprecipitates followed by Western blot with anti-TRAF mAb (Figure 5B). Results show that, in spite of Fas induction, siRNA FLIP-transfected myofibroblasts failed to recruit TRAF to the FADD complex (Figure 5B; siRNA FLIP), indicating that TRAF-FADD interaction is at least partly c-FLIP dependent.

We further assessed whether, after Fas activation, c-FLIP affects recruitment of the TRAF signaling molecule to the cell membrane in fibrotic lung fibroblasts where the FADD complex is situated. Fibrotic lung myofibroblasts transfected with c-FLIP or control siRNA were activated by Fas or IgG, stained with immunofluorescent antibodies against TRAF-2, and analyzed by confocal microscopy. After Fas activation, positive staining of TRAF was detected in siRNA control- and siRNA FLIP-transfected cells (Figure 5C; siRNA ctl and siRNA FLIP, anti-Fas versus IgG). However, TRAF was localized in the cytoplasmatic side of the cell membrane only in cells transfected with siRNA control (Figure 5C; siRNA ctl, anti-Fas versus

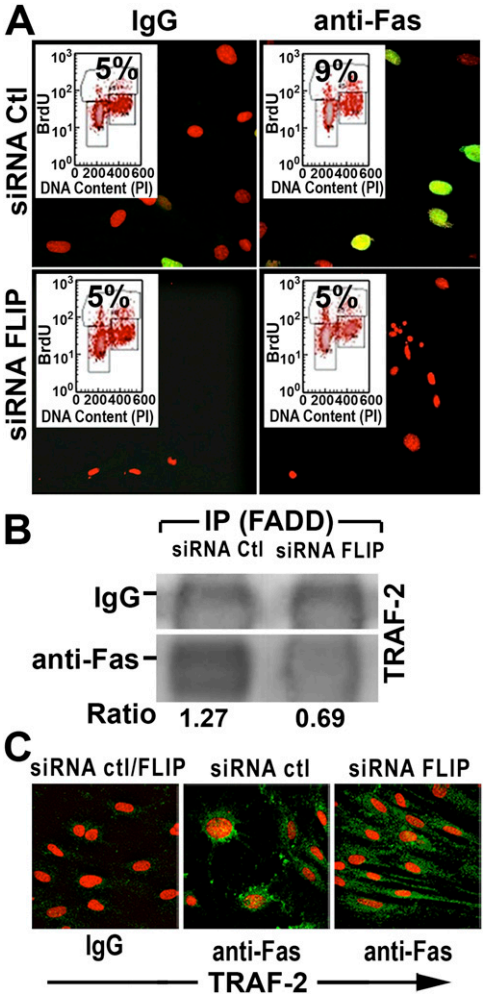

Figure 5. Fas-induced proliferation and TRAF recruitment to the DISC complex in fibrotic lung myofibroblasts are c-FLIP dependent. Fas versus IgG activation combined with siRNA c-FLIP or siRNA control treatments of myofibroblasts from bleomycin-treated lungs. Treated myofibroblasts were analyzed for proliferation and signaling of proliferation. (*A*) BrdU uptake assessed by anti–BrdU-FITC conjugated mAb staining subjected to confocal microscope. *Green staining* corresponds to BrdU-positive cells. *Red* indicates propidium iodide (PI) staining. Quantification of BrdU at the S-phase versus PI-total DNA staining was performed by FACS flow cytometry (*inserts*). (*B*) FADD immunoprecipitate followed by Western blot using anti-TRAF mAb and (*C*) confocal microscope images of indirect immunofluorescence after staining for TRAF2 protein using FITC-conjugated antibody (*green*). Cell nuclei were stained with PI (*red*). Representative results from two experiments. Fold ratios relate to Fas versus IgG treatment.

IgG), indicating its proximity to the FADD complex. This is an essential step to allow the execution of Fas signaling of proliferation. In contrast, we observed a diffuse cytosolic staining in myofibroblasts activated with Fas after down-regulation of c-FLIP levels (Figure 5C; siRNA FLIP, anti-Fas versus IgG). This indicates that Fas activates the expression of the TRAF molecule; however, c-FLIP is responsible for its localization to the inner part of the cell membrane.

DISCUSSION

In the present study, we investigated the role of c-FLIP in Fas signaling in primary myofibroblasts isolated from fibrotic lungs. We demonstrate that, during fibrosis, c-FLIP expression is up-regulated *in vivo* and *in vitro* in lung myofibroblasts from fibrotic lungs of bleomycin-treated mice and from humans with IPF. We demonstrate that, after induction of the Fas death receptor, primary lung myofibroblasts from fibrotic bleomycin-treated lungs of C57BL/6 mice not only resist Fas-induced apoptosis but are also driven to initiate Fas signaling of proliferation, with activation of the NF-κB signaling pathway. We also show that specific reduction of c-FLIP expression using siRNA decreases fibrotic lung myofibroblast Fas-induced proliferation and sensitizes myofibroblasts to Fas-induced apoptosis. These findings are supported by results showing that TRAF recruitment to the inner part of cell membrane and to the FADD complex is deregulated after treatment with specific siRNA-FLIP. We and others (33) have identified TRAF as a binding partner that could be part of the link between Fas-induced cell proliferation and c-FLIP in myofibroblasts via up-regulation of the NF-κB signaling pathway.

The classic role of the Fas pathway is initiation of apoptosis; however, myofibroblast resistance to Fas-induced apoptosis in fibrotic lungs of humans with IPF but not in normal lungs has been reported (12, 34–37). Moreover, Fas-induced proliferation, rather than apoptosis, has been demonstrated in several cell types, including fibroblasts (16, 38, 39).

c-FLIP is a known inhibitor of apoptosis induced by death receptors, such as Fas, DR4, and DR5 (40). Fas activation by FasL or by anti-Fas activating antibodies leads to the recruitment of FADD adaptor protein to form the DISC, as seen in our study. Due to its structural homology with caspase 8, c-FLIP interferes with caspase 8 activation at the DISC ensemble level. Although the mechanism of apoptosis attenuation by c-FLIP has not been completely elucidated, it has been suggested that c-FLIP may be a competitive inhibitor, possibly excluding recruitment of caspase 8 to the DISC. Indeed, we found that cleavage of caspase 8 after activation of Fas on myofibroblasts correlates negatively with c-FLIP activation because down-regulation of c-FLIP increased caspase 8 cleavage. c-FLIP may play a role in myofibroblast proliferation or death, depending on the level of its expression. In addition, differences between c-$FLIP_L$ and $FLIP_S$ (the short isoform of the FLIP molecule) have been described by several investigators (41, 42). It has been suggested that $FLIP_S$ completely inhibits cleavage of caspase 8, whereas $FLIP_L$ does not (42), although virtually all studies with this finding were conducted with lymphoid cells (19, 42). In our studies, we regulated the expression of both c-FLIP isoforms using siRNA designed to the common sequence.

c-FLIP has been shown to induce activation of kinases involved in signaling pathways (19), raising the possibility that c-FLIP involvement in Fas signaling events influences myofibroblast proliferation. It has been suggested that c-FLIP may provide the molecular switch allowing Fas to promote cell proliferation, as opposed to apoptosis (17, 18). We demonstrate that c-FLIP down-regulation inhibited Fas-induced proliferation of fibrotic lung myofibroblasts, rendering them susceptible to Fas-induced apoptosis.

c-FLIP–dependent proliferation, initiated by Fas, is thought to be transmitted through FADD by activation of the NF-κB and ERK transcriptional pathways. c-FLIP is capable of binding to Raf-1, which leads to the activation of ERK and to TRAF1 and TRAF2, which activate NF-κB. Hence, c-FLIP can drive Fas signals toward pathways leading to cell proliferation. We show, for the first time, that TRAF2 in myofibroblasts is associated with the DISC after Fas activation.

TRAF2 belongs to the TNF receptor–associated factors, a family of proteins that plays a pivotal role in diverse biological processes. TRAF2 has a critical role in the activation of NF-κB. TRAF2 recruits the IKK complex to the TNFR, allowing, after a cascade of events, the translocation of NF-κB to the nucleolus, where it activates a variety of genes. TRAF2 was shown to be essential for c-FLIP induction of NF-κB (43). We demonstrate

that TRAF in myofibroblasts is associated with the DISC after Fas activation and that this association is c-FLIP dependent. Our findings show that Fas-dependent c-FLIP signaling of proliferation in myofibroblasts is via TRAF and involves activation of the NF-κB signaling molecule. The role of the ERK pathway in Fas/FLIP signaling must also be investigated because c-FLIP activates this pathway in T cells (44).

c-FLIP expression has been implicated in the progression of various diseases, including autoimmune (45), neoplastic (46), and cardiovascular (13) disorders. Previous studies of the role of c-FLIP in fibroblast survival have been mostly focused on the prevention of apoptosis. Fibroblasts from c-FLIP knockout mouse embryos (13) and dermal- or rheumatoid synovial fibroblasts with down-regulated c-FLIP (47) exhibited increased susceptibility to Fas-mediated apoptosis.

We demonstrate the novel finding that, in primary fibroblasts from fibrotic mouse lungs, c-FLIP is not only involved in the inhibition of Fas-induced apoptosis but also in Fas-induced proliferation. This function of c-FLIP has been demonstrated previously in lymphocytes (48) and hepatocytes (49).

Fas signaling of apoptosis in many cells, including fibroblasts, can be reversed by a mechanism involving c-FLIP up-regulation via profibrotic Th2 cytokines such as TGF-β (37, 50–52), IL-4 (53), IL-10 (54), IL-6 (55), and IL-1β (56); by growth factors such as FGF (57); and by reactive oxygen species (58) and cell attachments (59), which in turn promotes myofibroblast survival (reviewed in Ref. 60) and subsequent fibrosis (61–63). On the other hand, Th1 cytokines such as TNF-α, IFN-γ, and IL-2, which are known to act antifibrotically (61, 64, 65), were reported to annul resistance to Fas-induced apoptosis by a mechanism involving c-FLIP down-regulation (66, 67).

Thus, factors promoting myofibroblast survival with subsequent accumulation and the fibrotic process are linked with up-regulation of c-FLIP, whereas those inhibiting fibrosis are linked with its down-regulation. c-FLIP was also detected by us and others (12, 20) in lung epithelial cells associated with the fibrotic foci. Given its role in promoting cell survival (68–70), there is a possibility that c-FLIP contributes to the survival of epithelial cells undergoing epithelial–mesenchymal transition (71, 72), further supporting a role for c-FLIP in myofibroblast accumulation in pulmonary fibrosis. c-FLIP could also support and mediate epithelial–mesenchymal transition, as previously reported in cancer cells (73) and subsequent accumulation of fibroblasts and fibrosis (71, 72).

Our results demonstrate that c-FLIP is a key regulator of Fas-induced apoptosis and proliferation of primary lung myofibroblasts. Altering the level of c-FLIP expression may mediate the attenuation of lung fibrosis and may pave the way for designing a new approach for therapeutic intervention.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Anita Kol and Reem Bader for performing experimental work and Shifra Fraifeld for editorial assistance in preparing this manuscript.

References

1. Phan SH. The myofibroblast in pulmonary fibrosis. *Chest* 2002;122 (Suppl)286S–289S.
2. Wallach-Dayan SB, Golan-Gerstl R, Breuer R. Evasion of myofibroblasts from immune surveillance: a mechanism for tissue fibrosis. *Proc Natl Acad Sci USA* 2007;104:20460–20465.
3. Filippatos G, Ang E, Gidea C, Dincer E, Wang R, Uhal BD. Fas induces apoptosis in human coronary artery endothelial cells in vitro. *BMC Cell Biol* 2004;5:6.
4. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 1995;81:505–512.
5. Hofmann K, Bucher P, Tschopp J. The CARD domain: a new apoptotic signalling motif. *Trends Biochem Sci* 1997;22:155–156.
6. Yang X, Khosravi-Far R, Chang HY, Baltimore D. Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell* 1997;89: 1067–1076.
7. Wajant H. Targeting the FLICE Inhibitory Protein (FLIP) in cancer therapy. *Mol Interv* 2003;3:124–127.
8. Djerbi M, Screpanti V, Catrina AI, Bogen B, Biberfeld P, Grandien A. The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. *J Exp Med* 1999;190: 1025–1032.
9. French LE, Tschopp J. Inhibition of death receptor signaling by FLICE-inhibitory protein as a mechanism for immune escape of tumors. *J Exp Med* 1999;190:891–894.
10. Medema JP, de Jong J, van Hall T, Melief CJ, Offringa R. Immune escape of tumors in vivo by expression of cellular FLICE-inhibitory protein. *J Exp Med* 1999;190:1033–1038.
11. Tepper CG, Seldin MF. Modulation of caspase-8 and FLICE-inhibitory protein expression as a potential mechanism of Epstein-Barr virus tumorigenesis in Burkitt's lymphoma. *Blood* 1999;94:1727–1737.
12. Tanaka T, Yoshimi M, Maeyama T, Hagimoto N, Kuwano K, Hara N. Resistance to Fas-mediated apoptosis in human lung fibroblast. *Eur Respir J* 2002;20:359–368.
13. Yeh WC, Itie A, Elia AJ, Ng M, Shu HB, Wakeham A, Mirtsos C, Suzuki N, Bonnard M, Goeddel DV, *et al*. Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. *Immunity* 2000;12:633–642.
14. Mapara MY, Bargou R, Zugck C, Dohner H, Ustaoglu F, Jonker RR, Krammer PH, Dorken B. APO-1 mediated apoptosis or proliferation in human chronic B lymphocytic leukemia: correlation with bcl-2 oncogene expression. *Eur J Immunol* 1993;23:702–708.
15. Mitsiades CS, Poulaki V, Fanourakis G, Sozopoulos E, McMillin D, Wen Z, Voutsinas G, Tseleni-Balafouta S, Mitsiades N. Fas signaling in thyroid carcinomas is diverted from apoptosis to proliferation. *Clin Cancer Res* 2006;12:3705–3712.
16. Aggarwal BB, Singh S, LaPushin R, Totpal K. Fas antigen signals proliferation of normal human diploid fibroblast and its mechanism is different from tumor necrosis factor receptor. *FEBS Lett* 1995;364: 5–8.
17. Thome M, Tschopp J. Regulation of lymphocyte proliferation and death by FLIP. *Nat Rev Immunol* 2001;1:50–58.
18. Peter ME. The flip side of FLIP. *Biochem J* 2004;382:e1–e3.
19. Kataoka T, Budd RC, Holler N, Thome M, Martinon F, Irmler M, Burns K, Hahne M, Kennedy N, Kovacsovics M, *et al*. The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. *Curr Biol* 2000;10:640–648.
20. Cha SI, Groshong SD, Frankel SK, Edelman BL, Cosgrove GP, Terry-Powers JL, Remigio LK, Curran-Everett D, Brown KK, Cool CD, *et al*. Compartmentalized expression of c-FLIP in lung tissues of patients with idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2010;42:140–148.
21. Golan-Gerstl R, Wallach-Dayan SB, Amir G, Breuer R. Epithelial cell apoptosis by fas ligand-positive myofibroblasts in lung fibrosis. *Am J Respir Cell Mol Biol* 2007;36:270–275.
22. Izbicki G, Or R, Christensen TG, Segel MJ, Fine A, Goldstein RH, Breuer R. Bleomycin-induced lung fibrosis in IL-4-overexpressing and knockout mice. *Am J Physiol Lung Cell Mol Physiol* 2002;283: L1110–L1116.
23. Izbicki G, Segel MJ, Christensen TG, Conner MW, Breuer R. Time course of bleomycin-induced lung fibrosis. *Int J Exp Pathol* 2002;83: 111–119.
24. Segel MJ, Aqeilan R, Zilka K, Lorberboum-Galski H, Wallach-Dayan SB, Conner MW, Christensen TG, Breuer R. Effect of IL-2-Bax, a novel interleukin-2-receptor-targeted chimeric protein, on bleomycin lung injury. *Int J Exp Pathol* 2005;86:279–288.
25. Segel MJ, Izbicki G, Cohen PY, Or R, Christensen TG, Wallach-Dayan SB, Breuer R. Role of interferon-gamma in the evolution of murine bleomycin lung fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2003; 285:L1255–L1262.
26. Bogatkevich GS, Ludwicka-Bradley A, Highland KB, Hant F, Nietert PJ, Singleton CB, Feghali-Bostwick CA, Silver RM. Impairment of the antifibrotic effect of hepatocyte growth factor in lung fibroblasts

from African Americans: possible role in systemic sclerosis. *Arthritis Rheum* 2007;56:2432–2442.
27. Kohan M, Bader R, Puxeddu I, Levi-Schaffer F, Breuer R, Berkman N. Enhanced osteopontin expression in a murine model of allergen-induced airway remodelling. *Clin Exp Allergy* 2007;37:1444–1454.
28. Wallach-Dayan SB, Izbicki G, Cohen PY, Gerstl-Golan R, Fine A, Breuer R. Bleomycin initiates apoptosis of lung epithelial cells by ROS but not by Fas/FasL pathway. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L790–L796.
29. Cohen PY, Breuer R, Wallach-Dayan SB. Thy1 up-regulates FasL expression in lung myofibroblasts via Src family kinases. *Am J Respir Cell Mol Biol* 2009;40:1–8.
30. Sun Z, Arendt CW, Ellmeier W, Schaeffer EM, Sunshine MJ, Gandhi L, Annes J, Petrzilka D, Kupfer A, Schwartzberg PL, *et al*. PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. *Nature* 2000;404:402–407.
31. Borset M, Hjorth-Hansen H, Johnsen AC, Seidel C, Waage A, Espevik T, Sundan A. Apoptosis, proliferation and NF-kappaB activation induced by agonistic Fas antibodies in the human myeloma cell line OH-2: amplification of Fas-mediated apoptosis by tumor necrosis factor. *Eur J Haematol* 1999;63:345–353.
32. Owen-Schaub LB, Radinsky R, Kruzel E, Berry K, Yonehara S. Anti-Fas on nonhematopoietic tumors: levels of Fas/APO-1 and bcl-2 are not predictive of biological responsiveness. *Cancer Res* 1994;54:1580–1586.
33. Kataoka T, Tschopp J. N-terminal fragment of c-FLIP(L) processed by caspase 8 specifically interacts with TRAF2 and induces activation of the NF-kappaB signaling pathway. *Mol Cell Biol* 2004;24:2627–2636.
34. Buhling F, Wille A, Rocken C, Wiesner O, Baier A, Meinecke I, Welte T, Pap T. Altered expression of membrane-bound and soluble CD95/Fas contributes to the resistance of fibrotic lung fibroblasts to FasL induced apoptosis. *Respir Res* 2005;6:37.
35. Frankel SK, Cosgrove GP, Cha SI, Cool CD, Wynes MW, Edelman BL, Brown KK, Riches DW. TNF-alpha sensitizes normal and fibrotic human lung fibroblasts to Fas-induced apoptosis. *Am J Respir Cell Mol Biol* 2006;34:293–304.
36. Moodley YP, Caterina P, Scaffidi AK, Misso NL, Papadimitriou JM, McAnulty RJ, Laurent GJ, Thompson PJ, Knight DA. Comparison of the morphological and biochemical changes in normal human lung fibroblasts and fibroblasts derived from lungs of patients with idiopathic pulmonary fibrosis during FasL-induced apoptosis. *J Pathol* 2004;202:486–495.
37. Moodley Y, Rigby P, Bundell C, Bunt S, Hayashi H, Misso N, McAnulty R, Laurent G, Scaffidi A, Thompson P, *et al*. Macrophage recognition and phagocytosis of apoptotic fibroblasts is critically dependent on fibroblast-derived thrombospondin 1 and CD36. *Am J Pathol* 2003;162:771–779.
38. Fireman E, Vardinon N, Burke M, Spizer S, Levin S, Endler A, Stav D, Topilsky M, Mann A, Schwarz Y, *et al*. Predictive value of response to treatment of T-lymphocyte subpopulations in idiopathic pulmonary fibrosis. *Eur Respir J* 1998;11:706–711.
39. Jelaska A, Korn JH. Anti-Fas induces apoptosis and proliferation in human dermal fibroblasts: differences between foreskin and adult fibroblasts. *J Cell Physiol* 1998;175:19–29.
40. Hu S, Vincenz C, Ni J, Gentz R, Dixit VM. I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD-95-induced apoptosis. *J Biol Chem* 1997;272:17255–17257.
41. Lacronique V, Mignon A, Fabre M, Viollet B, Rouquet N, Molina T, Porteu A, Henrion A, Bouscary D, Varlet P, *et al*. Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice. *Nat Med* 1996;2:80–86.
42. Krueger A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *J Biol Chem* 2001;276:20633–20640.
43. Guasparri I, Wu H, Cesarman E. The KSHV oncoprotein vFLIP contains a TRAF-interacting motif and requires TRAF2 and TRAF3 for signalling. *EMBO Rep* 2006;7:114–119.
44. Yeh JH, Hsu SC, Han SH, Lai MZ. Mitogen-activated protein kinase kinase antagonized fas-associated death domain protein-mediated apoptosis by induced FLICE-inhibitory protein expression. *J Exp Med* 1998;188:1795–1802.
45. Semra YK, Seidi OA, Sharief MK. Overexpression of the apoptosis inhibitor FLIP in T cells correlates with disease activity in multiple sclerosis. *J Neuroimmunol* 2001;113:268–274.
46. Irisarri M, Plumas J, Bonnefoix T, Jacob MC, Roucard C, Pasquier MA, Sotto JJ, Lajmanovich A. Resistance to CD95-mediated apoptosis through constitutive c-FLIP expression in a non-Hodgkin's lymphoma B cell line. *Leukemia* 2000;14:2149–2158.
47. Palao G, Santiago B, Galindo M, Paya M, Ramirez JC, Pablos JL. Down-regulation of FLIP sensitizes rheumatoid synovial fibroblasts to Fas-mediated apoptosis. *Arthritis Rheum* 2004;50:2803–2810.
48. Zhang N, Hopkins K, He YW. The long isoform of cellular FLIP is essential for T lymphocyte proliferation through an NF-kappaB-independent pathway. *J Immunol* 2008;180:5506–5511.
49. Gilot D, Serandour AL, Ilyin GP, Lagadic-Gossmann D, Loyer P, Corlu A, Coutant A, Baffet G, Peter ME, Fardel O, *et al*. A role for caspase-8 and c-FLIPL in proliferation and cell-cycle progression of primary hepatocytes. *Carcinogenesis* 2005;26:2086–2094.
50. Hinz B. Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 2007;127:526–537.
51. Perlman R, Schiemann WP, Brooks MW, Lodish HF, Weinberg RA. TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. *Nat Cell Biol* 2001;3:708–714.
52. Schlapbach R, Spanaus KS, Malipiero U, Lens S, Tasinato A, Tschopp J, Fontana A. TGF-beta induces the expression of the FLICE-inhibitory protein and inhibits Fas-mediated apoptosis of microglia. *Eur J Immunol* 2000;30:3680–3688.
53. Conticello C, Pedini F, Zeuner A, Patti M, Zerilli M, Stassi G, Messina A, Peschle C, De Maria R. IL-4 protects tumor cells from anti-CD95 and chemotherapeutic agents via up-regulation of antiapoptotic proteins. *J Immunol* 2004;172:5467–5477.
54. Bharhani MS, Borojevic R, Basak S, Ho E, Zhou P, Croitoru K. IL-10 protects mouse intestinal epithelial cells from Fas-induced apoptosis via modulating Fas expression and altering caspase-8 and FLIP expression. *Am J Physiol Gastrointest Liver Physiol* 2006;291:G820–G829.
55. Kovalovich K, Li W, DeAngelis R, Greenbaum LE, Ciliberto G, Taub R. Interleukin-6 protects against Fas-mediated death by establishing a critical level of anti-apoptotic hepatic proteins FLIP, Bcl-2, and Bcl-xL. *J Biol Chem* 2001;276:26605–26613.
56. Maedler K, Schumann DM, Sauter N, Ellingsgaard H, Bosco D, Baertschiger R, Iwakura Y, Oberholzer J, Wollheim CB, Gauthier BR, *et al*. Low concentration of interleukin-1beta induces FLICE-inhibitory protein-mediated beta-cell proliferation in human pancreatic islets. *Diabetes* 2006;55:2713–2722.
57. Kobayashi T, Okamoto K, Kobata T, Hasunuma T, Kato T, Hamada H, Nishioka K. Differential regulation of Fas-mediated apoptosis of rheumatoid synoviocytes by tumor necrosis factor alpha and basic fibroblast growth factor is associated with the expression of apoptosis-related molecules. *Arthritis Rheum* 2000;43:1106–1114.
58. Nitobe J, Yamaguchi S, Okuyama M, Nozaki N, Sata M, Miyamoto T, Takeishi Y, Kubota I, Tomoike H. Reactive oxygen species regulate FLICE inhibitory protein (FLIP) and susceptibility to Fas-mediated apoptosis in cardiac myocytes. *Cardiovasc Res* 2003;57:119–128.
59. Aoudjit F, Vuori K. Matrix attachment regulates Fas-induced apoptosis in endothelial cells: a role for c-flip and implications for anoikis. *J Cell Biol* 2001;152:633–643.
60. Thannickal VJ, Horowitz JC. Evolving concepts of apoptosis in idiopathic pulmonary fibrosis. *Proc Am Thorac Soc* 2006;3:350–356.
61. Pan LH, Ohtani H, Yamauchi K, Nagura H. Co-expression of TNF alpha and IL-1 beta in human acute pulmonary fibrotic diseases: an immunohistochemical analysis. *Pathol Int* 1996;46:91–99.
62. Zhang HY, Gharaee-Kermani M, Phan SH. Regulation of lung fibroblast alpha-smooth muscle actin expression, contractile phenotype, and apoptosis by IL-1beta. *J Immunol* 1997;158:1392–1399.
63. Bergeron A, Soler P, Kambouchner M, Loiseau P, Milleron B, Valeyre D, Hance AJ, Tazi A. Cytokine profiles in idiopathic pulmonary fibrosis suggest an important role for TGF-beta and IL-10. *Eur Respir J* 2003;22:69–76.
64. Nash JR, McLaughlin PJ, Butcher D, Corrin B. Expression of tumour necrosis factor-alpha in cryptogenic fibrosing alveolitis. *Histopathology* 1993;22:343–347.
65. Antoniou KM, Ferdoutsis E, Bouros D. Interferons and their application in the diseases of the lung. *Chest* 2003;123:209–216.

66. Du C, Guan Q, Yin Z, Zhong R, Jevnikar AM. IL-2-mediated apoptosis of kidney tubular epithelial cells is regulated by the caspase-8 inhibitor c-FLIP. *Kidney Int* 2005;67:1397–1409.
67. Siegmund D, Wicovsky A, Schmitz I, Schulze-Osthoff K, Kreuz S, Leverkus M, Dittrich-Breiholz O, Kracht M, Wajant H. Death receptor-induced signaling pathways are differentially regulated by gamma interferon upstream of caspase 8 processing. *Mol Cell Biol* 2005;25:6363–6379.
68. Yu JW, Shi Y. FLIP and the death effector domain family. *Oncogene* 2008;27:6216–6227.
69. Perlman H, Pagliari LJ, Georganas C, Mano T, Walsh K, Pope RM. FLICE-inhibitory protein expression during macrophage differentiation confers resistance to fas-mediated apoptosis. *J Exp Med* 1999;190:1679–1688.
70. Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, *et al*. Inhibition of death receptor signals by cellular FLIP. *Nature* 1997;388:190–195.
71. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 2002;110:341–350.
72. Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, Borok Z. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol* 2005; 166:1321–1332.
73. Kim Y, Park H, Jeoung D. CAGE, a cancer/testis antigen, induces c-FLIP(L) and Snail to enhance cell motility and increase resistance to an anti-cancer drug. *Biotechnol Lett* 2009;31:945–952.