NF-κB–Regulated Expression of Cellular FLIP Protects Rheumatoid Arthritis Synovial Fibroblasts From Tumor Necrosis Factor α–Mediated Apoptosis

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Objective. Little apoptosis has been observed in rheumatoid arthritis (RA) synovial tissues. Tumor necrosis factor α (TNFα) is expressed in the joints of patients with RA, yet RA synovial fibroblasts are relatively resistant to apoptosis induced by TNFα. Recently, we demonstrated that FLIP is highly expressed in the RA joint. These studies were performed to determine if TNFα-induced NF-κB controls the expression of FLIP long (FLIPL) and FLIP short (FLIPS) in RA synovial fibroblasts and to determine the role of FLIP in the control of TNFα-induced apoptosis.

Methods. RA synovial fibroblasts were isolated from RA synovial tissues and used between passages 3 and 9. RA synovial or control fibroblasts were sham infected or infected with a control adenovirus vector or one expressing the super-repressor IκBα (srIκBα). The cells were stimulated with TNFα or a control vehicle, and expression of FLIPL and FLIPS mRNA and protein. The TNFα-induced, but not the basal, expression of FLIP was regulated by NF-κB. When NF-κB activation was suppressed by the expression of srIκBα, TNFα-mediated apoptosis was induced. TNFα-induced apoptotic cell death was mediated by caspase 8 activation and was prevented by the ectopic expression of FLIPL or the caspase 8 inhibitor CrmA.

Results. TNFα induced the expression of both isoforms of FLIP messenger RNA (mRNA) in RA synovial fibroblasts; however, FLIPL was the dominant isoform detected by Western blot analysis. In control fibroblasts, TNFα induced the expression of FLIPL and FLIPS mRNA and protein. The TNFα-induced, but not the basal, expression of FLIP was regulated by NF-κB. When NF-κB activation was suppressed by the expression of srIκBα, TNFα-mediated apoptosis was induced. TNFα-induced apoptotic cell death was mediated by caspase 8 activation and was prevented by the ectopic expression of FLIPL or the caspase 8 inhibitor CrmA.

Conclusion. The TNFα-induced, but not the basal, expression of FLIP is regulated by NF-κB in RA synovial fibroblasts. The resistance of RA synovial fibroblasts to TNFα-induced apoptosis is mediated by the NF-κB–regulated expression of FLIP. These observations support the role of NF-κB and FLIP as attractive therapeutic targets in RA.

Rheumatoid arthritis (RA) is a chronic inflammatory synovitis characterized by hyperplasia of the synovial lining (composed of macrophages and fibroblasts) and infiltration of chronic inflammatory cells, including lymphocytes, dendritic cells, and macrophages, into the sublining region (1,2). Although evidence of DNA damage has been identified in RA joints, several studies using electron microscopy have not detected evidence of substantial apoptosis in the joints of patients with RA (for review, see ref. 3). The absence of morphologic evidence of apoptosis in the presence of DNA damage, which is a stimulus for the induction of apoptosis, further indicates that the environment of the RA joint is highly resistant to apoptosis. Nonetheless, the means of apoptosis are present in the RA joint, since tumor necrosis factor α (TNFα), which is capable of
inducing apoptosis by ligation of TNF receptor I (TNFR1), is highly expressed locally (4). Additionally, Fas ligand (FasL), which is expressed on macrophages in the RA joint, is capable of inducing apoptosis following ligation of the death receptor Fas, which is expressed on macrophages, lymphocytes, and synovial fibroblasts (5–7).

In the synovial lining of the RA joint, Fas-expressing synovial fibroblasts are in intimate contact with FasL-expressing macrophages, yet apoptosis is not induced. Fas–FasL interactions result in the recruitment of the adapter molecule FADD, which recruits pro-caspase 8, forming the death-inducing signaling complex (DISC) (8). Caspase 8 activation, which occurs in the DISC, is capable of inducing apoptosis mediated either directly through the activation of caspase 3 or by the activation of the Bid protein, which results in mitochondrial injury, the release of cytochrome c, and activation of caspases 9 and 3. Under certain conditions, the ligation of TNFR1 may also induce caspase 8–mediated apoptosis by a different mechanism that involves TRADD and FADD (9). FLIP is capable of interacting with caspase 8 and FADD, thus inhibiting the activation of caspase 8 (10). FLIP is expressed as 2 isoforms, FLIP long (FLIP_L) and FLIP short (FLIP_S). FLIP_L possesses 2 death-effector domains (DEDs) and an inactive caspase activation–like domain. FLIP_S possesses only the DEDs (10). Both FLIP isoforms are expressed in macrophages (11) and T and B lymphocytes following activation (12,13). While FLIP_L may promote or inhibit apoptosis, depending upon the experimental conditions, the major function of FLIP_S is the suppression of death receptor–mediated caspase activation (10,11,14,15).

We previously demonstrated that FLIP was highly expressed in the RA synovial lining in both macrophages and synovial fibroblasts (11). Others have shown that FLIP messenger RNA (mRNA) was highly expressed at sites of cartilage and bone erosion, further supporting the role of FLIP in the pathogenesis of RA (16). Additionally, the expression of FLIP mRNA was increased in RA synovial fibroblasts after the addition of TNFα (16). In this study, we further examined the role of TNFα-induced NF-κB activation in the regulation of the expression of FLIP_L and FLIP_S and the control of apoptosis in RA synovial fibroblasts. In RA synovial and control fibroblasts, NF-κB activation was responsible for TNFα-induced, but not basal, expression of both FLIP_L and FLIP_S mRNA. In contrast to control fibroblasts, at the protein level FLIP_L was the major isoform induced in RA synovial fibroblasts by TNFα. Additionally, following the inhibition of NF-κB activation, TNFα-induced apoptosis was associated with the reduction of FLIP and was mediated by caspase 8. Apoptosis was prevented by caspase 8 inhibitors and by the ectopic expression of FLIP_L. These observations demonstrate the importance of NF-κB–induced FLIP as the switch between life and death in RA synovial fibroblasts in the presence of TNFα.

**MATERIALS AND METHODS**

**Reagents.** Fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Dulbecco’s modified Eagle’s medium (DMEM) and glutamine were obtained from Cellgro (Rockville, MD). The RNase Minikit was from Qiagen (Valencia, CA) and the reverse transcription system was purchased from Promega (Madison, WI). The TaqMan Universal polymerase chain reaction (PCR) master mix was purchased from Applied Biosystems (Foster City, CA). PCR primers and TaqMan probe were synthesized by Dr. Ka-Leung Ngai (Northwestern University, Chicago, IL). The anti-FLIP antibody (Dave 2) was obtained from Alexis (San Diego, CA), the antibodies against caspases 8 and 3 were purchased from BioSource (Worcester, MA), and the antitu- bulin antibody was obtained from Sigma-Aldrich (St. Louis, MO). The ECL chemiluminescence system for developing Western blots was purchased from Amersham Biosciences (Buckinghamshire, UK). Recombinant human TNFα was purchased from R&D Systems (Minneapolis, MN), and the Z-VAD-fluoromethylketone (Z-VAD-FMK) was obtained from Enzyme System Products (Livermore, CA). Caspase 8 and caspase 3 fluorometric assay reagents were purchased from Roche Laboratories (Basel, Switzerland), and the annexin V–fluorescein isothiocyanate (FITC) was purchased from Onco- gene Research Products (Cambridge, MA).

**Cell cultures.** RA synovial tissues were obtained from patients undergoing total joint replacement who met the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA (17). RA synovial fibroblasts were isolated and used between passages 3 and 9 for most experiments, as previously described (18,19). Some experiments were conducted using adherent cells, isolated in the same manner, but used after the first passage. Normal dermal fibroblasts (CRJ-1475) were obtained from American Type Culture Collection (Manassas, VA) or from newborn foreskins (kindly provided by Dr. M. K. Rundell, Northwestern University). All cells were maintained in DMEM supplemented with 10% FBS with glutamine and antibiotics (18,19).

**Adenovirus infections.** For infections, cells were plated in growth media and cultured until they achieved 70–80% confluence. They were then transferred to low-serum media (0.5% FBS) for 24 hours, and then infected with AdGFP, Adβ-gal, AdIxBα, AdTet-FLIP_L (AdTet-FLIP_L kindly provided by Dr. Ken Walsh [20]), or AdCrmA (21). After another 24 hours in low-serum media, the cells were washed, and DMEM containing 10% FBS was added.

**Real-time PCR.** Total RNA was isolated from fibroblasts using the RNase Minikit, according to the manufacturer’s protocol. Reverse transcription with oligo(dT) primers was
performed with the reverse transcription system. Real-time PCR reactions were performed with the ABI Prism 770 apparatus (Perkin-Elmer Applied Biosystems, Emeryville, CA). Thermocycling was done in a final volume of 25 μl containing 60 ng of complementary DNA, 800 nM of each of the forward and reverse primers, and 250 nM of the probes. The sequences for the FLIP forward and reverse primers were as follows: forward (for both FLIP<sub>L</sub> and FLIP<sub>S</sub>) 5'-CAAGCAGTTGTTCAAGGA, reverse (FLIP<sub>L</sub>) 5'-GCCAACGTTCCATTAAAGA, reverse (FLIP<sub>S</sub>) 5'-ATGGGCATAGGGTTATTC, probe 5'-TGTICTCCAAAGCA-GCAATCCA. The probe was labeled with FAM. The human GAPDH primers and VIC-labeled probe were used as a control, following the manufacturer’s recommendations (Applied Biosystems). TaqMan universal PCR master mix was used in the PCR reaction mixture. PCR was performed with the following program: 95°C for 10 minutes, and 40 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 60°C for 30 seconds. Quantitative values were derived from the threshold cycle number (C<sub>t</sub>) at which the increase in the signal associated with exponential growth of PCR products was first detected using Perkin-Elmer biosystems analysis software. For each PCR product, the relative amount in each sample was calculated from standard curves generated from pooled RNA of control samples. The expression value was normalized to a housekeeping gene, GAPDH. A relative gene expression was determined by assigning the control a relative value of 1.0, with all other values relative to the control.

**Western blot analysis.** Cells were washed, and total cell lysates were prepared as previously described (19). Cell lysates (30 or 50 μg, as indicated in the Results) were separated on 12% denaturing polyacrylamide gels and transferred to polyvinylidene difluoride membrane, blocked, and then washed with 5% nonfat dried milk in phosphate buffered saline containing 0.1% Tween 20 (PBS–TWEEN 20) (20). The primary antibodies in PBS–TWEEN 20 were added at room temperature for 1 hour. The membranes were washed with PBS–TWEEN 20 and incubated with horseradish peroxidase–conjugated anti-mouse IgG or anti-rabbit or anti-rat IgG for an additional hour. The signals were developed using an ECL chemiluminescence system (Amersham Biosciences).

**Apoptosis assessment.** The percentage of apoptotic cells was determined by annexin V–FITC, which was quantitated by flow cytometry (11). For some experiments, apoptosis was also determined by subdiploid DNA (<2N) analyzed by flow cytometry, as previously described (11). Caspase activation was also used to characterize apoptosis. To measure the activity of caspases 8 and 3, colorimetric assays were used. Cells were harvested and lyzed on ice for 20 minutes. The supernatant (50 μl) was diluted with reaction buffer containing 10 mM dithiothreitol plus 5 μl of 20 mM IETD–7-amino-4-trifluoromethylcoumarin (AFC) or DEVD-AFC and incubated at 37°C for 1 to 2 hours in the dark. Absorbance was measured at 405 nm using a microtiter plate reader. The values were normalized by the protein concentration in each lysate.

**Cell viability test.** The cells were cultured in 96-well tissue culture plates. Cell viability was determined by the formazan dye XTT. Cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye occurred in metabolically active cells. Briefly, cells were incubated with XTT (1 mg/ml) and phenazine methosulfate (1.53 mg/ml) for 4 hours at 37°C. The soluble formazan dye was detected at 450 nm, with a reference wavelength of 630 nm.

**Statistical analysis.** Results are expressed as the mean ± SEM. The statistical difference between groups was determined by the t-test and the difference between 2 treatments was determined by Student’s paired t-test. A corrected P value was determined for the inhibition of apoptosis by applying the Bonferroni adjustment for multiple comparisons. P values less than 0.05 were considered statistically significant.

**RESULTS**

**TNFα-induced expression of FLIP in RA synovial fibroblasts.** To determine the effect of TNFα on the expression of FLIP<sub>L</sub> and FLIP<sub>S</sub> in RA synovial fibroblasts, real-time PCR was performed using isoform-specific primers. In preliminary studies, FLIP mRNA was optimally induced at 8 hours and did not increase over the next 16 hours (data not shown). Therefore, further studies were performed with cells harvested after 8 hours of incubation with TNFα. FLIP<sub>L</sub> and FLIP<sub>S</sub> mRNA were each significantly (P < 0.01–0.001) induced by TNFα at 8 hours (Figure 1A). When RA synovial fibroblasts were examined by Western blot analysis, FLIP<sub>L</sub> was readily detected constitutively in every sample, and was strongly induced by TNFα when examined at 12 and 24 hours, using 30 μg (n = 4) (data not shown) or 50 μg (n = 3) (Figure 1B) of cell lysate. In contrast to the mRNA, at the protein level, FLIP<sub>S</sub> was not detected constitutively in any of the 7 RA synovial fibroblasts (Figure 1B, and results not shown). After TNFα treatment, FLIP<sub>S</sub> was induced at 12 hours, but was only weakly detected after 24 hours in 2 of the patients examined (Figure 1B). Therefore, with isolated RA synovial fibroblasts, while TNFα comparably induced the expression of both isoforms of FLIP mRNA, at the protein level FLIP<sub>L</sub> was the dominant isoform detected, and little or no FLIP<sub>S</sub> was detected by Western blot analysis 24 hours after the addition of TNFα.

Control dermal foreskin fibroblasts were also used to determine the effects of TNFα on the expression of FLIP. Similar to RA synovial fibroblasts, FLIP<sub>L</sub> and FLIP<sub>S</sub> mRNA were comparably induced by TNFα (Figure 1C). The mRNA for FLIP<sub>S</sub>, but not FLIP<sub>L</sub>, was more strongly induced by TNFα in the control fibroblasts compared with the RA synovial fibroblasts (P < 0.02). With the control fibroblasts, both FLIP<sub>L</sub> and FLIP<sub>S</sub> proteins were readily detected when the cell lysates were examined by immunoblot analysis at 12 hours and 24 hours (Figure 1D) after the addition of TNFα. Since these effects were different from those
observed in RA synovial fibroblasts, a different fibroblast cell line (CRL-1475) was also examined. Similar to the foreskin fibroblasts, FLIP_L and FLIP_S were readily induced at both the message and the protein levels (data not shown). Therefore, the TNFα-induced expression of FLIP_S mRNA was greater in the control fibroblasts. Additionally, at the protein level, while FLIP_L was readily detected constitutively and at 12 and 24 hours in both the control and the RA synovial fibroblasts, FLIP_S was not strongly expressed constitutively or at 24 hours after the addition of TNFα to the RA synovial fibroblasts.
NF-κB regulation of the TNFα-induced expression of FLIP in RA synovial fibroblasts regulated. Since NF-κB activation may contribute to the regulation of FLIP, the effect of NF-κB inhibition was examined. When synovial fibroblasts were infected with AdIkBa, the induction of both FLIPα (Figure 2A) and FLIPS (data not shown) by TNFα was suppressed compared with cells infected with the control adenoviral vector. As a control, culture supernatants were examined for the expression of interleukin-6 (IL-6). The infection with AdIkBa prevented the TNFα-induced induction of IL-6, which is highly dependent upon NF-κB activation (data not shown). Although the induction of FLIPα and FLIPS mRNA was suppressed by the ectopic expression of super-repressor IkBa (srIkBa), there was no reduction of the basal expression of either isoform (Figure 2A and data not shown). Additionally, the ectopic expression of IkBa also prevented the TNFα-stimulated induction of FLIPα and FLIPS, but did not reduce the basal level of either isoform in either control fibroblast cell line (data not shown).

To determine the effects of the inhibition of
NF-κB on the expression of FLIP protein, NF-κB activation was inhibited by the infection of cells with AdIκBa for 24 hours prior to the addition of TNFα. When the RA synovial fibroblasts were infected with the control Adβ-gal, TNFα induced the expression of FLIP<sub>L</sub> in RA synovial fibroblasts (Figure 2B). However, when the RA synovial fibroblasts were infected with AdIκBa, TNFα failed to induce FLIP<sub>L</sub>. In each experiment, the addition of TNFα appeared to result in a greater reduction of FLIP<sub>L</sub>, determined by Western blot analysis, compared with cells infected with AdIκBa alone (Figure 2B). Additionally, the induction of both FLIP<sub>L</sub> and FLIP<sub>S</sub> in the control fibroblasts was prevented by the sIκBa (results not shown). In the control fibroblasts, expression of FLIP<sub>L</sub> and FLIP<sub>S</sub> protein also appeared to be reduced after the addition of TNFα, compared with cells expressing IκBa alone (results not shown). Therefore, the TNFα-induced expression of FLIP was mediated by the activation of NF-κB in RA synovial and control fibroblasts. Further, when NF-κB activation was suppressed, TNFα treatment resulted in a greater reduction of FLIP, determined by immunoblot analysis.

Induction of apoptotic cell death in RA synovial fibroblasts following inhibition of NF-κB. Experiments were performed to determine if the inhibition of NF-κB sensitizes RA synovial fibroblasts to TNFα-induced apoptosis. Cells were infected with the control Adβ-gal or AdIκBa for 24 hours and then TNFα was added. No inhibitors of mRNA or protein synthesis were included in the cultures. In contrast to expectations, 24 hours after the addition of TNFα, neither loss of viability nor apoptosis was observed (data not shown). However, when the cells were examined 48 hours after adding TNFα, there was a significant reduction of viability when the cells expressed the sIκBa, compared with uninfected or Adβ-gal–infected cells (data not shown). To characterize the mode of cell death, apoptosis was determined by binding of annexin V (Figure 2C). TNFα-induced apoptosis was observed 48 hours after the addition of TNFα in the cells expressing the sIκBa, but not in the Adβ-gal–infected or the uninfected cells (Figure 2C). Additionally, analysis of subdiploid DNA demonstrated TNFα-induced apoptosis 48 hours after the addition of TNFα when NF-κB activation was inhibited (Figure 2D). These observations demonstrate that following the inhibition of NF-κB activation, TNFα-induced apoptotic cell death was readily apparent at 48 hours, although it was not observed at 24 hours.

Since the expression profile of genes may be different in more recently isolated RA synovial fibroblasts (23,24), the expression of FLIP and the induction of apoptosis were also examined using the synovial fibroblasts of 3 RA patients from passage 1. FLIP<sub>L</sub> (results not shown), but not FLIP<sub>S</sub> (results not shown), was constitutively expressed in each sample, determined by Western blot analysis. As noted with synovial fibroblasts from passages 3–9, following the addition of TNFα, FLIP<sub>L</sub> was increased, while FLIP<sub>S</sub> was barely detectable at 24 hours (results not shown). The inhibition of NF-κB activation by ectopically expressed IκBa abolished the TNFα-induced FLIP<sub>L</sub> expression and resulted in apoptosis, determined by DNA fragmentation (results not shown). Even though cells from the first passage may possess adherent cells other than synovial fibroblasts, these observations demonstrate that the expression of FLIP and TNFα-induced apoptosis was similar in cells examined following the first passage or passages 3–9.

Long-lasting NF-κB activation following the addition of TNFα. Since NF-κB activation occurs within minutes of the addition of TNFα, and since the induction of apoptosis by TNFα was not observed until 48 hours, experiments were performed to determine the time course of the effects of TNFα on NF-κB activation. Following the addition of TNFα, IκBa is phosphorylated, ubiquitinated, and degraded. The degradation of IκBa permits the nuclear translocation of transcriptionally active NF-κB p50/p65 complexes, which results in the increased expression of NF-κB–regulated genes including IκBa. The increased IκBa binds the p50/p65 complex, retaining the complex in the cytoplasm, terminating the NF-κB activation response. Following the addition of TNFα, IκBa was initially degraded and its level was restored after 4 hours, as expected (Figure 3). However, a reduction of IκBa was again observed at 24 and 48 hours, suggesting the ongoing activation of NF-κB by TNFα. These observations suggest that the effects of TNFα may be biologically active over 48 hours.
in culture, providing a potential explanation for the delayed onset of TNFα-induced apoptosis following the inhibition of NF-κB activation.

**Suppression of TNFα-induced apoptosis by ectopic expression of FLIP_L.** Since the TNFα-induced expression of FLIP_L was suppressed following the inhibition of NF-κB, experiments were performed to determine if the ectopic expression of FLIP_L prevented TNFα-induced apoptosis. RA synovial fibroblasts were infected with AdTet-FLIP_L to obtain levels of FLIP_L that were comparable with those observed following the addition of TNFα alone (Figure 4A). The ectopic expression of FLIP_L significantly suppressed the induction of TNFα-induced apoptosis when NF-κB activation was inhibited (Figure 4B). These observations suggest that the NF-κB–induced expression of FLIP_L was important in preventing TNFα-induced apoptosis in RA synovial fibroblasts.

**Figure 4.** Prevention of TNFα-induced apoptotic cell death of RA synovial fibroblasts by ectopic expression of FLIP_L. RA synovial fibroblasts were infected with AdTet-FLIP plus AdTet-on; 6 multiplicities of infection [MOI] of each) or control AdTet-on (12 MOI) together with AdIκBa at 200 MOI for 24 hours. Then, infected cells were treated with TNFα for an additional 24 hours (A) or 48 hours (B). Cells were harvested and the expression of FLIP_L was determined by Western blot analysis using a monoclonal antibody to FLIP (A), or cells were used for detection of apoptosis by annexin V staining (B). Values are the mean ± SEM. ** = P < 0.001 versus control adenoviral vector. Results are representative of 3 independent experiments. See Figure 1 for other definitions.

**Figure 5.** Suppression of TNFα-induced activation of caspases 8 and 3 by the ectopic expression of FLIP_L. RA synovial fibroblasts were infected with Adβ-gal alone or AdTet-FLIP; 6 multiplicities of infection [MOI] of each) or a control vector (AdTet-on; 12 MOI) together with AdIκBa at 200 MOI for 24 hours. The infected cells were treated with TNFα for an additional 48 hours. Cells were harvested and the activities of caspase 8 (A) and caspase 3 (B) were determined using specific fluorescent substrates IETD-AFC (for caspase 8) and DEVD-AFC (for caspase 3). The activation of caspase 8 and caspase 3 was also determined by detecting the cleavage of the proform of caspase 8 and caspase 3 using antibodies that specifically target procaspase 8 or procaspase 3 (C). Values in A and B are the mean ± SEM. ** = corrected P < 0.003 versus control adenoviral vector. Results are representative of 3 independent experiments using synovial fibroblasts from 3 different patients with RA. See Figure 1 for other definitions.
Activation of caspases 8 and 3 suppressed by FLIP. To characterize the mechanism of TNFα-induced apoptosis in RA synovial fibroblasts, the activation of caspase 8 and one of its downstream targets, caspase 3, was examined. Even though infection with AdIκBα (plus AdTet-on) alone resulted in increased (P < 0.05) caspase 8 activity (Figure 5A), caspase 3 activity was not increased and no procaspase 8 or 3 cleavage (Figure 5C) or apoptotic cell death (results not shown) was induced. However, when NF-κB activation was inhibited, caspase 8–like activity (Figure 5A) and caspase 3–like activity (Figure 5B) were both significantly induced by TNFα (Figure 5B). The caspase 8–like and caspase 3–like activity was suppressed by the ectopic expression of FLIP_L (Figures 5A and B). The activation of these caspases was confirmed by examining the cleavage of procaspases 8 and 3. Both procaspase 8 and procaspase 3, determined by Western blot analysis, were cleaved in the presence of TNFα when NF-κB was inhibited (Figure 5C). The expression of FLIP_L prevented the cleavage of both procaspases 8 and 3 by TNFα, when NF-κB activation was inhibited (Figure 5C). These observations demonstrate that FLIP_L suppresses the TNFα-induced activation of the initiator caspase 8 and one of its downstream targets, caspase 3.

Prevention of TNFα-induced cell death by caspase inhibition. To confirm that the effects of FLIP_L on caspase activation were responsible for protecting against cell death, experiments were performed to determine the effects of other forms of caspase inhibition on the TNFα-induced cell death of RA synovial fibroblasts. The addition of Z-VAD-FMK, a broad-based caspase inhibitor, suppressed the loss of viability induced by TNFα when NF-κB activation was inhibited by the srIκBα (Figure 6A). To more specifically determine the effects on the inhibition of caspase 8, AdCrmA was used. The expression of CrmA suppressed the TNFα-induced cell death in RA synovial fibroblasts when NF-κB activation was inhibited (Figure 6B). These observations support the role of caspase activation in the induction of TNFα-induced cell death following the inhibition of NF-κB activation.

DISCUSSION

Previous studies have demonstrated that FLIP is highly expressed in the RA joint, both in the synovial lining and the pannus (7,16); however, the regulation of the expression of the FLIP isoforms and their role in protecting against death receptor–mediated apoptosis in
RA synovial fibroblasts has not been well characterized. In RA synovial fibroblasts, FLIP_L and FLIP_S mRNA were constitutively expressed. However, at the protein level only FLIP_L was detected, even with lysates prepared from cells following the first passage. The detection of only FLIP_L in the extracts of unstimulated RA synovial fibroblasts by Western blot analysis is consistent with the results of others (25). The lack of detection of FLIP_S was not due to the antibody used, since the antibody readily detected FLIP_S in the control fibroblasts.

Since TNFα is expressed locally in the RA joint, studies were performed to determine how TNFα regulates the expression of FLIP_L and FLIP_S and apoptosis in RA synovial fibroblasts. Although the induction of FLIP_L and FLIP_S mRNA in RA synovial fibroblasts by TNFα was similar, only the induction of FLIP_L was consistently observed at the protein level 24 hours after treatment with TNFα. In contrast, the induction of FLIP_S, both message and protein, by TNFα was greater in the control fibroblasts. The reduced expression of FLIP_S in the RA synovial fibroblasts may be due in part to decreased transcription induced by TNFα, and by increased turnover of the expressed protein. These observations are consistent with the interpretation that the expression of the FLIP isoforms is cell type and stimulus specific (26), and that the isoforms may be differentially regulated posttranscriptionally. Differences in the expression of FLIP_L and FLIP_S may be important since the isoforms may differentially regulate protection against apoptosis and nonapoptotic signaling (27–29).

The limited induction of FLIP_S protein following the activation of RA synovial fibroblasts by TNFα contrasts with the expression of FLIP_S in lymphocytes following activation. Circulating B lymphocytes express little FLIP; however, following in vitro activation by either CD40 ligand or B cell receptor crosslinking, both FLIP_L and FLIP_S were expressed (12). Additionally, T cell receptor activation of T lymphocytes resulted in a marked increase of FLIP_S (13). We have also shown that freshly isolated human monocytes express little FLIP; however, during in vitro differentiation into macrophages, the expression of both FLIP_L and FLIP_S mRNA and protein, was increased (11). Although FLIP_S was highly expressed in the Hep-G2 and KYM-1 rhabdospharcoma cell lines, in a variety of cell lines representing 8 other histotypes, under basal conditions, FLIP_L protein was the dominant isoform (30). These observations also support the conclusion that the ratio of the FLIP isoforms expressed at the protein level is cell type and stimulus specific.

The data presented document differences in the mechanisms regulating the basal and TNF-α-stimulated expression of FLIP. TNF-α-induced NF-κB was sufficient to induce FLIP mRNA in many (26,31–33), but not all, cell types (34). In RA synovial fibroblasts, following the inhibition of NF-κB by the ectopic expression of sIκBα, the TNF-α-induced expression of FLIP mRNA was prevented. These observations document that NF-κB activation mediates the TNF-α-induced expression of FLIP in RA synovial fibroblasts. In contrast, the inhibition of the constitutive activation of NF-κB had no appreciable effect on the basal expression of FLIP in either control or RA synovial fibroblasts examined between passages 3 and 9. The slight reduction noted in passage 1 cells may have been due to contamination with other adherent cells, such as macrophages, in which the basal expression of FLIP mRNA is partially regulated by the constitutive activation of NF-κB (data not shown).

The mechanisms contributing to the constitutive expression of FLIP in RA synovial fibroblasts are not clear. Notch-1 is activated in vivo in RA synovial fibroblasts (35), and Notch-1 activation has been shown to regulate FLIP expression in T cells (36). Additionally, the phosphatidylinositol 3-kinase/Akt-1 and ERK pathways are activated in RA synovial tissue and synovial fibroblasts (37,38) and might contribute to the basal expression of FLIP (39–41). Therefore, in RA synovial fibroblasts, mechanisms other than those mediated by NF-κB activation may contribute to the constitutive expression of FLIP.

The limited expression of FLIP_S, constitutively or following treatment with TNFα, may have an effect on protection against death receptor-mediated apoptosis. Upon Fas ligation, FLIP_L is processed or cleaved in the DISC (30,42). Both isoforms of FLIP possess identical DEDs, but FLIP_L also contains a caspase-like domain (10). The effects of FLIP_L on Fas-mediated apoptosis appear to be concentration dependent. At higher physiologic concentrations, FLIP_L protects against FasL- or TNFα-induced apoptosis, whereas at lower concentrations, it may contribute to Fas-mediated apoptosis (15). In contrast, FLIP_S only suppresses, and does not promote, death receptor-mediated apoptosis (11,42). Since FLIP_L was the dominant isoform detected in RA synovial fibroblasts by immunoblot analysis, these cells may be more sensitive to death receptor-mediated apoptosis compared with cells also expressing FLIP_S, such as macrophages or activated B or T lymphocytes.

It is possible that the 2 FLIP isoforms may
contribute differently to nonapoptotic signaling following Fas ligation. Fas ligation on T cells was capable of activating NF-κB and ERK, which was mediated by the recruitment of FLIP\textsubscript{L}, but not FLIP\textsubscript{S}, to the Fas DISC, which resulted in the interaction of FLIP\textsubscript{L} with receptor-interactive protein, TNF receptor–associated factors (TRAFs), and Raf-1 (27). FLIP may modulate activation through other pathways. We recently demonstrated that Fas–FasL interactions on macrophages enhanced stimulation through the Toll-like receptor 4 (TLR-4) and IL-1 receptor type I (IL-1RI) pathways (43). The crosstalk between the Fas and the TLR-4/IL-1RI pathways was mediated by FADD. When FADD was sequestered to the Fas DISC, the interaction between FADD and MyD88 was reduced, enhancing signaling through the TLR-4 and IL-1RI pathways, which is dependent upon MyD88. It is possible that FLIP\textsubscript{L}, which is expressed in macrophages, might contribute to the process, not only because of its potent antiapoptotic effects, but also since it does not promote the activation of NF-κB or ERK when recruited to the Fas DISC. Since RA synovial fibroblasts predominantly expressed FLIP\textsubscript{L}, it is possible that Fas ligation by adjacent macrophages may have a direct proinflammatory effect on the synovial fibroblasts by promoting the activation of NF-κB and ERK, as observed in T lymphocytes (27). Further studies will be required to characterize the nonapoptotic signaling role of FLIP\textsubscript{L} in RA synovial fibroblasts.

Previous studies demonstrated that TNFα resulted in apoptotic cell death in RA synovial fibroblasts when the activation of NF-κB was inhibited using a truncated 1κBα or a proteasome inhibitor (44,45); however, the mechanism responsible for the NF-κB–induced protection of the initiation of apoptosis was not characterized. Our results are the first to demonstrate that FLIP\textsubscript{L} plays an essential role in the prevention of TNFα–induced apoptosis in RA synovial fibroblasts. The ectopic expression of FLIP\textsubscript{L} blocked TNFα–induced caspase 8 activation and apoptotic cell death. Additionally, supporting the role of caspase 8 activation, the broad-specificity caspase inhibitor Z-VAD-FMK and the caspase 8 inhibitor CrmA protected against TNFα–induced apoptotic cell death.

A prior study suggested that TNFα–induced X-linked inhibitor of apoptosis (XIAP), an inhibitor of caspase 3 activation, was important in protecting against TNFα–induced apoptosis of RA synovial fibroblasts (45). However, since XIAP is not effective at protecting against caspase 8 activation, it is not likely to be the factor regulating the initiation of cell death. It is possible that other molecules, such as TRAF1 or TRAF2 and cellular inhibitor of apoptosis 1 or 2 (cIAP1 or 2), may also contribute to the protection against TNFα–induced apoptosis in RA synovial fibroblasts. However, cIAP2 and TRAF2 were not increased in response to TNFα (26), and when NF-κB activation was prevented, these molecules were individually not effective at preventing caspase 8 activation and TNFα–induced apoptosis (26,46). Another study suggested that Akt-1 activation may contribute to the regulation of TNFα–induced apoptosis in RA synovial fibroblasts (37). Although the results of this study suggested that the TNFα-mediated effects on the Akt-1 and NF-κB pathways were independent, it is possible that each pathway contributed to the regulation of FLIP. While we cannot exclude the possibility that other molecules might contribute to the apoptosis resistance during the first 24 hours, the importance of FLIP\textsubscript{L} was documented by the fact that the ectopic expression of FLIP\textsubscript{L} prevented caspase 8 activation and protected RA synovial fibroblasts from apoptotic cell death.

The mechanism of apoptosis induced in RA synovial fibroblasts is distinct from that responsible for TNFα–induced apoptosis in normal, in vitro–differentiated human macrophages, when NF-κB activation is prevented (47). In contrast to the RA synovial fibroblasts, 60–70% of macrophages had undergone apoptotic cell death by 24 hours, and caspase 8 activation was not essential (47). In macrophages, since caspase 8 activation was not essential, it is unlikely that a reduction of FLIP contributed to the induction of apoptosis (47). In contrast, in RA synovial fibroblasts, the expression of FLIP\textsubscript{L} was highly effective at preventing TNFα–induced caspase 8 activation and apoptosis in RA synovial fibroblasts. These observations suggest that in RA, locally expressed TNFα (and possibly other cytokines that activate NF-κB) promotes the expression of FLIP\textsubscript{L} in synovial fibroblasts, which contributes to the resistance to apoptosis in the RA joint. These observations suggest that FLIP is an attractive therapeutic target in the RA joint, and that selective suppression of FLIP locally may promote apoptosis and provide long-term clinical benefit.

REFERENCES


