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Activation-induced Degradation of $FLIP_L$ Is Mediated via the Phosphatidylinositol 3-Kinase/Akt Signaling Pathway in Macrophages*

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Cellular FLIP (Flice-like inhibitory protein) is critical for the protection against death receptor-mediated cell apoptosis. In macrophages, FLIP long ($FLIP_L$) and FLIP short ($FLIP_S$) mRNA was induced by tumor necrosis factor (TNF) α, mediated through NF-κB. However, we observed TNFα reduced the protein level of $FLIP_L$, but not $FLIP_S$, at 1 and 2 h. Similar results were observed with lipopolysaccharide. The reduction of $FLIP_L$ by TNFα was not mediated by caspase 8, or through JNK or Itch, but was suppressed by inhibition of the phosphatidylinositol 3-kinase/Akt pathway employing chemical inhibitors, a dominant negative Akt-1, or Akt-1 small interfering RNA. The reduction of $FLIP_L$ resulted in the short term induction of caspase 8-like activity, which augmented NF-κB activation. A co-immunoprecipitation assay demonstrated that Akt-1 physically interacts with $FLIP_L$. Moreover, TNFα enhanced $FLIP_L$ serine phosphorylation, which was increased by activated Akt-1. Serine 273, a putative Akt-1 phosphorylation site in $FLIP_L$, was critical for the activation-induced reduction of $FLIP_L$. Thus, these observations document a novel mechanism where by TNFα facilitates the reduction of $FLIP_L$ protein, which is dependent on the phosphatidylinositol 3-kinase/Akt signaling.

Cellular FLIP (Flice-like inhibitory protein) negatively modulates the caspase 8-dependent apoptotic cascade triggered by the activation of death receptors, such as the tumor necrosis factor (TNF)[2] receptor-1 and Fas (1–3). Although the *flip* gene is expressed as multiple splice variants in many tissues (1, 4), it produces two major isoforms at the protein level, FLIP long ($FLIP_L$) and FLIP short ($FLIP_S$). Both isoforms contain two death effector domains in the N terminus that allow for interaction with the adapter molecule Fas-associated death domain. $FLIP_L$, the more abundantly expressed isoform in most cell types, additionally contains an nonfunctional caspase activation-like domain in its C terminus (1). The increased expression of FLIP renders tumor cells and macrophages from the joints of patients with rheumatoid arthritis resistant to death receptor and chemotherapeutic drug-mediated apoptosis (5–8), whereas the level of FLIP correlates with tumor progression and patient outcomes (9).

To date, the transcriptional and post-transcription regulation of FLIP has not been fully elucidated. NF-κB strongly upregulates the expression of $FLIP_L$ and $FLIP_S$ (10), and TNFα and LPS, which activate NF-κB, are known to induce FLIP (11, 12). Additionally, Akt has been implicated in the induction of FLIP (13–16). Post-transcriptionally, $FLIP_L$ and $FLIP_S$ are regulated by ubiquitin-proteasome pathway (17–20). As reported recently (21), the TNFα-accelerated $FLIP_L$ turnover in mouse hepatocytes and mouse embryonic fibroblasts was mediated through JNK activation that occurs when NF-κB activation is suppressed. The JNK-mediated phosphorylation of E3 ubiquitin ligase Itch results in the ubiquitination and degradation of $FLIP_L$. However, the role of TNFα in the post-transcriptional regulation of FLIP in the absence of NF-κB inhibition or the prolonged activation of JNK is not known.

The dysregulation of macrophage function contributes to the pathogenesis of rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, and chronic obstructive pulmonary disease (22–26). Our prior observations demonstrate that FLIP is critical for the protection of macrophages against death receptor-mediated apoptosis in rheumatoid arthritis (8, 27); however, knowledge of the mechanisms that regulate FLIP in macrophages is incomplete. While characterizing the expression of FLIP in macrophages, in contrast to expectations, we noticed that activation with TNFα or LPS actually reduced the protein levels of $FLIP_L$, but not $FLIP_S$, at 1 and 2 h, even though the mRNA of both isoforms was increased. The activation-induced reduction of $FLIP_L$ resulted in the increased activation of caspase 8, which promoted the activation of NF-κB and the expression of FLIP mRNA. Our observations also demonstrate that the PI3K/Akt-1 (Akt) pathway mediates the activation-induced reduction of $FLIP_L$ by the phosphorylation of $FLIP_L$ at serine 273. These observations identify a novel pathway regulating the decision for life and death of the macrophage following activation, and they identify the regulation of $FLIP_L$ as a potential therapeutic target.

EXPERIMENTAL PROCEDURES

Cell Culture—Human macrophages were differentiated from monocytes isolated from the buffy coats (Lifesource, Glenview,

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[2] The abbreviations used are: TNF, tumor necrosis factor; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering RNA; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; FBS, fetal bovine serum; CMV, cytomegalovirus; DN, dominant negative; PBS, phosphate-buffered saline; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; CHX, cycloheximide; DMSO, dimethyl sulfoxide.

IL) from healthy donors. Mononuclear cells were obtained by histopaque gradient centrifugation and monocytes by counter-current centrifugal elutriation (JE-6B; Beckman Coulter, Palo Alto, CA) in the presence of 10 μg/ml polymyxin B sulfate (Sigma), as previously describe RPMI medium without serum and were differentiated *in vitro* for 7 days in RPMI containing 20% heat-inactivated FBS, 1 μg/ml polymyxin B sulfate, 0.35 mg/ml L-glutamine, 120 units/ml penicillin and streptomycin (20% FBS/RPMI) (27–31). RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS.

Adenovirus Infection of Primary Macrophages—A control adenoviral empty vector (CMV-blank) and adenoviral vectors expressing a $FLIP_L$, a super-repressor IκBα, a constitutively activated myristilated AKT mutant (Myr-AKT), and a dominant negative form of AKT (DN-AKT) were employed. Macrophages were infected with adenoviral vectors in RPMI medium without serum for 2 h. After infection, 20% FBS/RPMI was added, and the cells were incubated overnight. The macrophages were then washed twice with PBS and incubated in 20% FBS/RPMI for an additional 24 h and then employed as described under "Results."

Caspase 8-like Activity—Macrophages were treated as indicated and harvested. Cell lysates were prepared according to the manufacturer's instructions (BioVision Research Products, Palo Alto, CA). Cell lysates containing 180 μg of protein were incubated for 1 h at 37 °C with the caspase 8 (Ac-IETD-AFC) synthetic fluorogenic substrate. The samples were read on a fluorometer at 400-nm excitation and 505-nm emission.

Apoptosis—Macrophages were harvested, fixed in 70% ethanol, and stained with propidium iodide (50 μg/ml). The apoptotic profile was determined by flow cytometry utilizing a Beckman-Coulter EpicsXL flow cytometer and system 2 software, as described (27, 31, 32). The hypodiploid DNA peak (<2 N DNA) immediately adjacent to the G_0/G_1 peak (2 N DNA) represented apoptotic cells and was quantified by histogram analyses. Objects with minimal light scatter representing debris were excluded.

Quantitative Reverse Transcription-PCR—Total RNA was isolated from macrophages using the TRIzol (Invitrogen). Reverse transcription with oligo(dT) primer was performed employing the reverse transcription system kit (Promega) according to the manufacturer's protocol. Real time PCR was carried out employing TaqMan® Universal PCR Master Mix kit (Applied Biosystems). The sequences for the FLIP forward and reverse primers were as follows: forward primer for both $FLIP_L$ and $FLIP_S$ 5′-CAAGCAGTCTGTTCAAGGA, reverse primer of $FLIP_L$ 5′-GCCAAGCTGTTCCTTAAGA, reverse primer of $FLIP_S$ 5′-ATGGGCATAGGGTGTTATC. The probe, 5′-TGTTCTCCAAGCAGCAATCCA, was labeled with carboxyfluorescein-aminohexyl amidite. The control human glyceraldehyde-3-phosphate dehydrogenase primers and VIC-labeled probe were obtained from Applied Biosystems and employed according to the manufacturer's instructions. The PCR was performed with a ABI PRISM™ 7500 sequence detection system (Applied Biosystems) in a final volume of 20 μl containing 60 ng of complementary DNA, 800 nM each of the forward and reverse primers, and 250 nM of the probes. The amplification program was: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Quantitative values were derived from the threshold cycle number (C_t) (11, 32). The experiments were normalized to glyceraldehyde-3-phosphate dehydrogenase. A relative gene expression was determined by assigning the control a relative value of 1.0, with all other values relative to the control.

siRNA Transfection—The forced reduction of caspase 8, Akt-1, and Itch in macrophages was achieved employing the SMARTpools of caspase 8, Akt-1, or Itch siRNA, (Dharmacon, Lafayette, CO). A nonspecific siRNA was employed as the control. Macrophages in 6-well plates were transfected with siRNAs by Lipofectamine methods following vender's protocol, as previously described (32, 33). After 4 h, FBS was added to bring the culture medium to 20% FBS, and the cells were cultured for an additional 48 h.

Immunoblot Analysis—Immunoblot analysis was performed as previously described (27–31). Briefly, whole cell extracts were prepared from macrophages that were treated as indicated under "Results." The proteins (60 μg) were electrophoresed on SDS-PAGE 12% polyacrylamide gels and transferred to Immobilon-P (Millipore) by semidry electroblotter (Bio-Rad). The membranes were then blocked in 5% nonfat milk PBS/0.2% Tween 20 (PBST) and subsequently incubated overnight at 4 °C with the following primary antibodies: anti-human FLIP (Alexis Biochem, Carlsbad, CA), anti-Akt, anti-phospho-Akt Ser^{473}, anti-caspase 8 (Cell Signaling, Danvers, MA), anti-Itch, anti-phosphoserine (BD Biosciences Pharmingen, San Jose, CA), anti-IκBα (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HA-horseradish peroxidase-conjugated (Roche Applied Science), and anti-β-actin (Sigma). The membranes were washed in PBST and incubated with either donkey anti-rat, anti-rabbit, or anti-mouse secondary antibodies conjugated with horseradish peroxidase (1:3300 dilution; Amersham Biosciences). The specific proteins were detected by Enhanced Chemiluminescent detection reagent (Amersham Biosciences).

Immunoprecipitation and Co-immunoprecipitation Method—To characterize the $FLIP_L$ serine phosphorylation status, $FLIP_L$ was pulled down by immunoprecipitation and detected by anti-phosphoserine antibody. Briefly, whole cell extracts containing 600 μg of protein were precleared with 50 μl of protein G-agarose beads with rotation at 4 °C for 1 h. The precleared lysates were collected and incubated with FLIP antibody (5 μg) for 1 h, followed by the addition of 50 μl of protein G-agarose beads, which were incubated with rocking overnight at 4 °C. After being washed four times, the immunoprecipitates were analyzed by immunoblot analysis for the serine phosphorylation of $FLIP_L$ employing an anti-phosphoserine specific antibody (BD Pharmingen, San Jose, CA). To detect the interaction between $FLIP_L$ and Akt, 600 μg of precleared cell lysates from macrophages that were co-infected with adenoviral vectors expressing $FLIP_L$ and HA-Myr-Akt were incubated with anti-FLIP or anti-HA antibodies for 1 h and then further incubated overnight after adding 50 μl of protein G-agarose beads. The rat IgG and rabbit IgG were employed as negative controls for FLIP and HA antibodies. After washing, the samples were subjected to immunoblot analysis and probed with FLIP or anti-HA antibodies.

In Vivo [^{32}P]Orthophosphate Cell Labeling for FLIP Phosphorylation—Macrophages in 6-well plates were infected with an adenoviral vector expressing Myr-Akt or the CMV control vector. After 24 h, the cells were washed twice with PBS and incubated with [^{32}P]orthophosphate (0.42 mCi/ml) in minimum Eagle's medium without phosphate for 2 h. FLIP was immunoprecipitated with anti-FLIP antibody and separated by SDS-PAGE. The gel was then dried, and $FLIP_L$ phosphorylation status was detected by autoradiography.

Site-directed Mutagenesis of $FLIP_L$ and Development of Cell Lines—Serine residue 273 on $FLIP_L$, a putative Akt phosphorylation site, was point mutated to alanine by a QuikChange site-directed mutagenesis kit according to the supplier's protocol (Stratagene, La Jolla, CA). In brief, we used retroviral pLXIN-$FLIP_L$ expression vector as template and used the following oligonucleotides to make point mutation serine 273 to alanine (mutated codon indicated as underlined): 5′-CGA GAC ACC TTC ACT GCC CTG GGC TAT GAA GTC-3′ (forward), 5′-GAC TTC ATA GCC CAG GGC AGT GAA GGT GTC TCG-3′ (reverse). The sequences of wild type and mutant form of $FLIP_L$ cDNA in pLXIN vectors were verified to be correct by DNA sequencing. The pLXIN-$FLIP_L$ wild type or mutant plasmids were transfected into packaging cell line PT67, and recombinant retroviral RNAs were packaged into infectious, replication-incompetent particles. Culture media containing the viral particles were collected and used to infect RAW264.7 cells. Stably expressing wild type and mutant $FLIP_L$ expressing cell lines were selected in medium containing 600 μg/ml geneticin (G418).

Statistical Analysis—The experimental data are presented as the means ± S.E. The statistical differences between groups were determined by *t* test. *p* values less than 0.05 were considered statistically significant.

RESULTS

$FLIP_L$ Is Regulated Differently by TNFα at the mRNA and Protein Levels—Studies were performed to determine the effect of activation through the TNF receptor pathway on the expression of FLIP employing normal *in vitro* differentiated macrophages. Incubation of macrophages with TNFα resulted in the induction of mRNA for both $FLIP_L$ and $FLIP_S$ (Fig. 1*A*), which were significantly ($p < 0.05$ and 0.01, respectively) increased by 2 h (Fig. 1, *A* and *C*) and diminished between 12 and 24 h (Fig. 1*A*). The induction of FLIP at the protein level was very different. By immunoblot analysis TNFα-induced $FLIP_S$ was significantly increased ($p < 0.01$) at 2 h, peaked at 8 h, and returned to basal levels by 24 h (Fig. 1*B*). In contrast, there was a significant ($p < 0.01$) reduction of $FLIP_L$ at 2 h (Fig. 1*D*), although $FLIP_L$ protein was increased at 8 and 12 h (Fig. 1*B*). To determine whether these observations were unique to TNFα, macrophages were incubated with LPS to activate the TLR4 pathway. The effects on $FLIP_L$ and $FLIP_S$ were the same as observed with TNFα (Fig. 1, *A–D*). These observations suggest that a regulatory mechanism at protein level is involved in reducing the intracellular level of $FLIP_L$ following activation with TNFα or LPS and that these effects were reversed by 8 h.

NF-κB activation is one of the pathways known to contribute to the expression of FLIP (10, 11). Therefore, macrophages were infected with an adenoviral vector expressing a super repressor IκBα (Ad-IκBα) to characterize the effects of the inhibition of NF-κB activation on the expression of FLIP. The ectopic expression IκBα dramatically suppressed the TNFα-induced (Fig. 1, *E* and *F*) and LPS-induced (data not shown) $FLIP_L$ and $FLIP_S$ at both the mRNA (Fig. 1*E*) and protein (Fig. 1*F*) levels. Compared with its basal level in the presence of IκBα alone, at 2 h $FLIP_L$ protein was reduced by TNFα when IκBα was expressed, suggesting that the activation induced reduction of $FLIP_L$ also occurred when NF-κB activation was suppressed. Of note, the suppression of NF-κB activation in macrophages by infection with the Ad-IκBα for 24 h did not suppress the constitutive expression of $FLIP_L$ and $FLIP_S$ mRNA (Fig. 1*E*) or the expression of $FLIP_L$ protein (Fig. 1*F*). These observations demonstrate that TNFα- and LPS-induced FLIP in macrophages is predominantly regulated transcriptionally and that the basal levels $FLIP_L$ and $FLIP_S$ may be regulated by pathways other than NF-κB.

PI3K Is Involved in TNFα-mediated Degradation of $FLIP_L$—Besides activating the NF-κB pathway, TNFα and LPS also activate the MAPK, PI3K/Akt-1, and protein kinase C signaling pathways (34, 35). Because additional studies demonstrated that the reduction of $FLIP_L$ was more pronounced at 1 h (40–60%) compared with 2 h (30–40%, $p < 0.02$), the 1-h time point was employed to define the mechanism for the reduction of $FLIP_L$. The reduction of $FLIP_L$ induced by TNFα at 1 h was not observed when macrophages were co-incubated with the PI3K inhibitors LY 294002 (20 μM) or wortmannin (200 nM) (Fig. 2, *A* and *B*). In contrast, the TNFα-induced reduction of $FLIP_L$ was still present when the macrophages were co-incubated with the p38 MAPK inhibitor SB203580 (10 μM) or the protein kinase C inhibitors bisindolylmaleimide III (20 μM) or bisindolylmaleimide VIII (10 μM) (data not shown). The experiments were also performed to determine whether the reduction of $FLIP_L$ may be due to caspase activation, because caspase 8 may form heterodimers with $FLIP_L$ (55kDa), processing $FLIP_L$ into p43, p22, and p12 fragments (36, 37). Treatment with TNFα alone resulted in very little p43 FLIP (data not shown). Additionally, incubation of macrophages with caspase 8 inhibitor IETD prior to the addition of TNFα failed to prevent the reduction of $FLIP_L$ (Fig. 2, *A* and *B*). Further, the forced reduction of caspase 8 by a specific siRNA failed to protect $FLIP_L$ from TNFα-induced reduction (Fig. 2, *C* and *D*). These observations suggest that the TNFα-induced reduction of $FLIP_L$ was mediated through the PI3K pathway and that activated caspase 8 was not responsible.

Akt Mediates the TNFα-induced Reduction of $FLIP_L$—Because Akt-1 (Akt) is downstream of PI3K, the experiments were performed to determine the role of Akt in the TNFα-induced reduction of $FLIP_L$. Employing macrophages, TNFα induced the activation of Akt, determined with an antibody specific for phosphorylation of serine 473 of Akt, and LY 294002 suppressed this activation (Fig. 3*A*). Similar results were obtained for LPS (data not shown). To confirm the role of Akt, macrophages were infected with an adenoviral vector expressing a dominant negative form of Akt (Ad-DN-Akt), previously demonstrated to suppress Akt activity (29, 38), or a CMV control vector (Ad-CMV) prior to the addition of TNFα. Following infection with the Ad-CMV vector, incu-

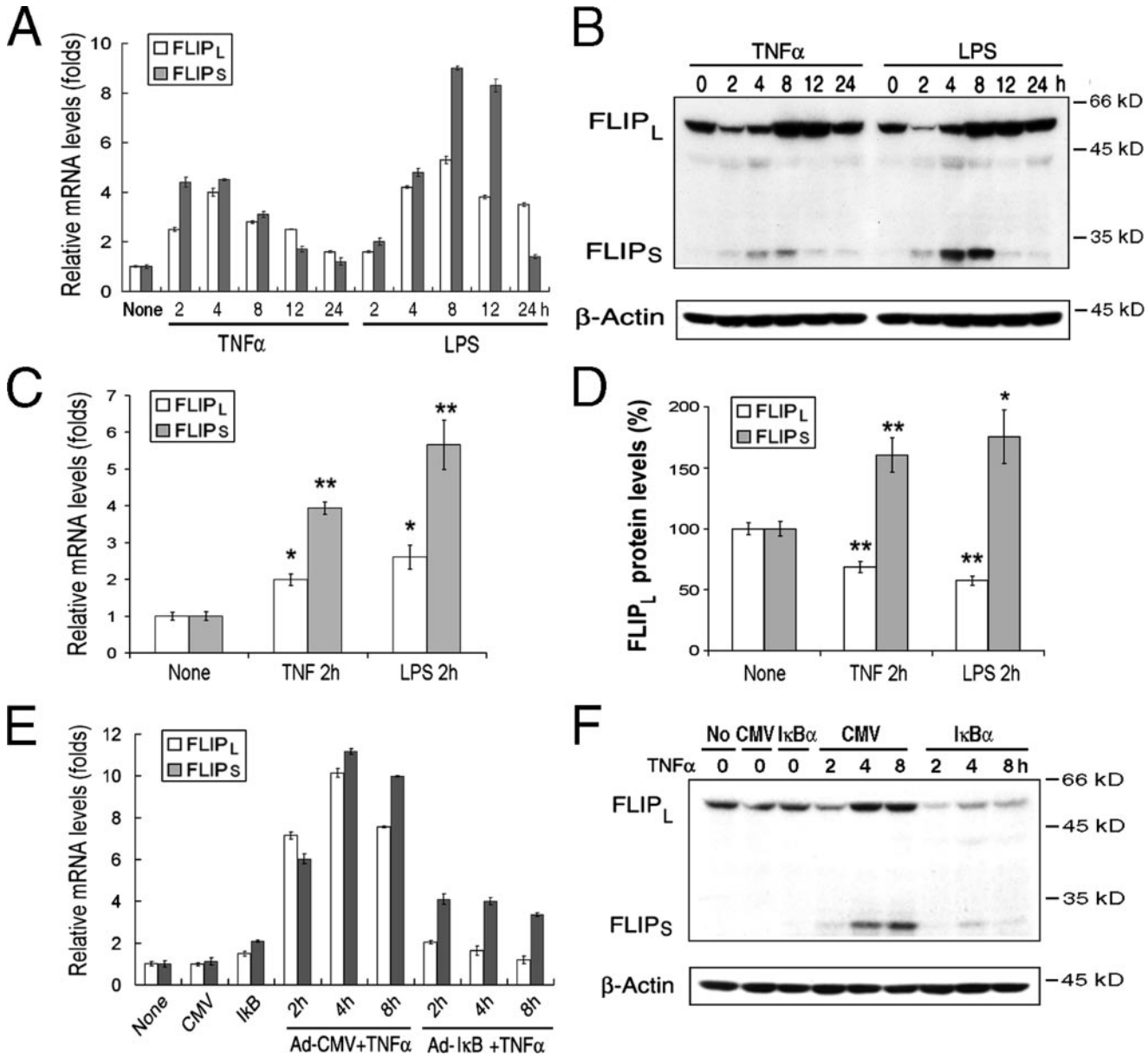

FIGURE 1. **TNFα- and LPS-mediated regulation of FLIP.** *A* and *B*, recombinant human TNFα (R&D, Minneapolis, MN) or LPS (Sigma) induces $FLIP_L$ and $FLIP_S$. Macrophages were incubated with TNFα or LPS (10 ng/ml), and the cells were harvested at the indicated time points and employed to determine the level of $FLIP_L$ and $FLIP_S$ mRNA by quantitative reverse transcription-PCR (*A*) and protein by immunoblot analysis (*B*). The results are representative of four independent experiments. *C* and *D*, $FLIP_L$ is reduced at 2 h. The expression of $FLIP_L$ and $FLIP_S$ mRNA (*C*) and protein (*D*) in response to incubation with TNFα or LPS at 2 h, is presented as the means ± S.E. of four independent experiments. The data in *D* represent the expression of FLIP, determined by densitometry normalized with β-actin. *, $p < 0.05$; **, $p < 0.01$ compared cells treated with control medium (*None*). *E* and *F*, NF-κB activation regulates TNFα induced FLIP. Macrophages were infected with control (Ad-CMV) or super repressor IκBα (Ad-IκBα) expressing vectors (multiplicity of infection of 100) for 24 h. Following the addition of TNFα the cells were harvested at the indicated time points, and the expression of $FLIP_L$ and $FLIP_S$ at mRNA (*E*) and protein by immunoblot analysis (*F*) was determined. The results presented are representative of two independent experiments.

bation with TNFα resulted in a 54% reduction ($p < 0.01$) of $FLIP_L$ at 1 h, whereas no reduction of $FLIP_L$ was noted in cells expressing the DN-Akt (Fig. 3, *B* and *C*). To more specifically examine the role of Akt, siRNA was employed to transfect macrophages. Overall there was a minor increase of $FLIP_L$ in macrophages transfected with the Akt-specific siRNA, compared with the nonspecific siRNA (Fig. 3, *D* and *E*). Consistent with the observations obtained employing the chemical inhibitors and the DN Akt, the forced reduction of Akt (Fig. 3*D*) prevented the reduction of $FLIP_L$ induced by incubation with TNFα (Fig. 3*E*). These observations demonstrate that Akt is necessary for the TNFα-induced suppression of $FLIP_L$.

Akt Promotes TNFα-induced Caspase 8 and NF-κB Activation and Increased FLIP mRNA—Experiments were performed to determine the relevance of the Akt-mediated reduction of $FLIP_L$, by examination of caspase 8 activation. When macrophages were transfected with nonspecific siRNA, TNFα induced caspase 8-like activity at 1 h, which returned to base line by 4 h (Fig. 4*A*). No apoptosis, defined by DNA fragmentation, was observed at 16 h (data not shown). In contrast, the forced reduction of Akt prevented the induction of caspase

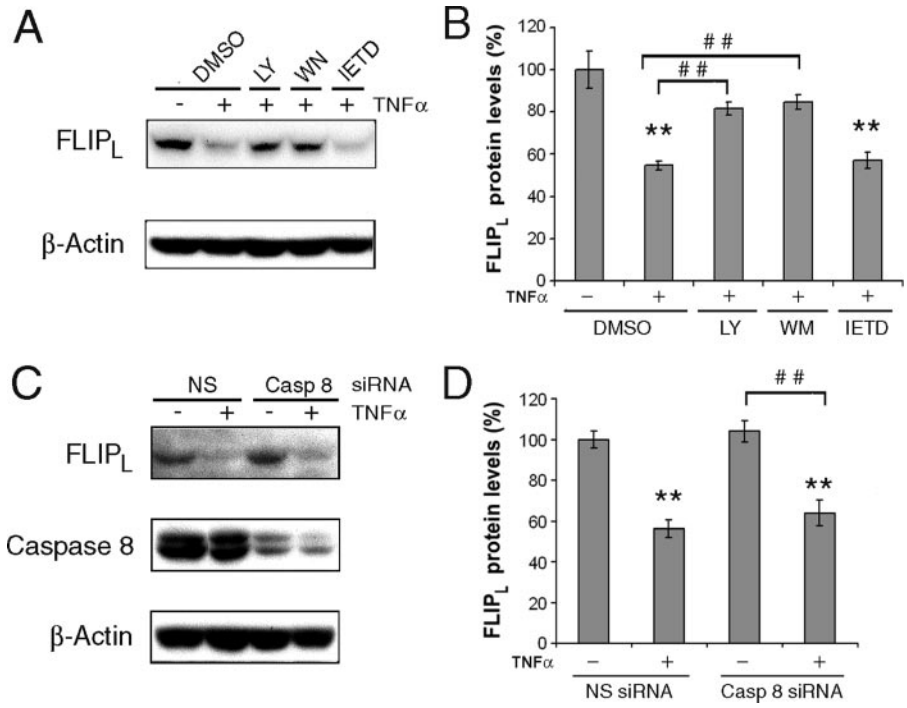

FIGURE 2. **Inhibition of PI3K, but not caspase 8, suppresses the TNFα-induced reduction of FLIP$_L$.** *A* and *B*, macrophages were incubated for 30 min with LY294002 (20 μM), wortmannin (100 nM), IETD (20 μM) (Calbiochem, San Diego, CA), or control DMSO and then incubated with TNFα (10 ng/ml) for 1 h. The cells were harvested, and whole cell lysates were employed for immunoblot analysis employing anti-FLIP and β-actin antibodies. *C* and *D*, macrophages were transfected with 100 nM caspase 8 or nonspecific (*NS*) siRNA for 48 h then and then incubated with TNFα (10 ng/ml) for 1 h. Cell lysates were employed for immunoblot analysis. *B* and *D*, the summaries of three independent experiments are presented as the means ± S.E. of immunoblots. The *bars* represent the expression of FLIP$_L$ relative to β-actin determined by densitometry. **, $p < 0.01$ compared the NS siRNA in the absence of TNFα; ##, $p < 0.01$ compared with the indicated treatments.

8-like activity (Fig. 4*A*). To determine the biological relevance of the caspase 8-like activity, NF-κB activation was measured by examining IκBα by immunoblot analysis. TNFα resulted in the reduction of IκBα at 15 and 30 min, which returned to base line by 60 min (Fig. 4, *B* and *C*). The suppression of caspase 8-like activity by IETD (Fig. 4*B*) or the forced reduction of caspase 8 by siRNA (Fig. 4*C*) resulted in the attenuation of NF-κB activation. Because NF-κB activation is responsible for the TNFα-mediated induction of FLIP, the effect of caspase inhibition on the expression of FLIP mRNA was examined. Both IETD and caspase 8 siRNA resulted in the significant ($p < 0.05$-0.01) reduction of both FLIP$_L$ and FLIP$_S$ mRNA at 1 and 2 h (Fig. 4, *D* and *E*). Therefore, the TNFα-induced reduction of FLIP$_L$ promotes NF-κB activation, which in turn enhances the expression of FLIP$_L$ and FLIP$_S$ mRNA.

The Forced Reduction of Akt Suppresses TNFα-induced Caspase 8 Activation and Apoptosis When NF-κB Is Suppressed—Next the role of Akt in regulation FLIP$_L$ and caspase 8-like activity was examined when NF-κB activation was suppressed. Macrophages were transfected with nonspecific or Akt siRNA and then infected with a control or a super repressor IκBα expressing adenoviral vectors. When NF-κB activation was suppressed, the forced reduction of Akt diminished the reduction of FLIP$_L$ induced by TNFα, compared with transfection with nonspecific siRNA (Fig. 5*A*). The forced reduction of Akt also lessened the TNFα-induced caspase 8-like activity and DNA fragmentation (Fig. 5, *B* and *C*) observed when NF-κB activation was suppressed. The forced reduction of Akt resulted in a minor but significant ($p < 0.05$) reduction of the basal caspase 8-like activity when IκBα was expressed ectopically, and the fold induction of caspase 8-like activity was similar in the presence or absence of Akt siRNA (Fig. 5*B*). Therefore, the Akt-mediated reduction of FLIP$_L$, promotes TNFα-induced caspase 8 activation and subsequent apoptosis, independent of NF-κB.

The Proteasome Participates in the TNFα-triggered Reduction of FLIP$_L$—Because the ubiquitin-proteasome pathway contributes to the degradation of FLIP$_L$ (17, 18, 21, 39–41), studies were performed to determine the effect of proteasomal inhibition on the TNFα-induced reduction of FLIP$_L$. Macrophages were preincubated with LY294002 (20 μM) and/or MG132 (10 μM), a cell-permeable proteasome inhibitor, for 30 min prior to the addition of TNFα. Incubation with TNFα alone resulted in the reduction of FLIP$_L$ ($p < 0.01$), whereas preincubation MG132 or LY294002 or the combination of both resulted in significant ($p < 0.05$ to 0.01) protection against the TNFα-induced reduction of FLIP$_L$ (Fig. 6). There was no difference noted in the protection provided by MG132 or LY294002 employed alone or in combination. These results support the role of the proteasome in the TNFα-mediated reduction of FLIP$_L$ and suggest that PI3K/Akt-1 signaling promotes the proteasomal degradation of FLIP$_L$.

Itch Does Not Contribute to the Reduction of FLIP$_L$ in Macrophages—A recent report (21) demonstrated that TNFα accelerates FLIP$_L$ turnover through JNK-mediated phosphorylation and activation of the E3 ubiquitin ligase Itch. To determine whether JNK was involved in the TNFα-induced degradation of FLIP$_L$, macrophages were preincubated with the JNK inhibitor SP600125 (20 μM), LY294002 (20 μM), or the combination of both for 30 min prior to incubation with TNFα. Preincubation with the JNK inhibitor, at a concentration that effectively suppressed JNK (42, 43), had no effect on the TNFα-induced reduction of FLIP$_L$ and the combination of the JNK and PI3K inhibitors was not different from PI3K inhibition alone (Fig. 7, *A* and *B*). To examine the specific role of Itch, macrophages were transfected with nonspecific or Itch-specific siRNA. The forced reduction of Itch (Fig. 6*C*) did not protect against the TNFα-induced reduction of FLIP$_L$ (Fig. 7, *C* and *D*). These observations do not provide evidence that the JNK-Itch pathway was involved in the TNFα-induced reduction of FLIP$_L$ observed at 1 h in macrophages.

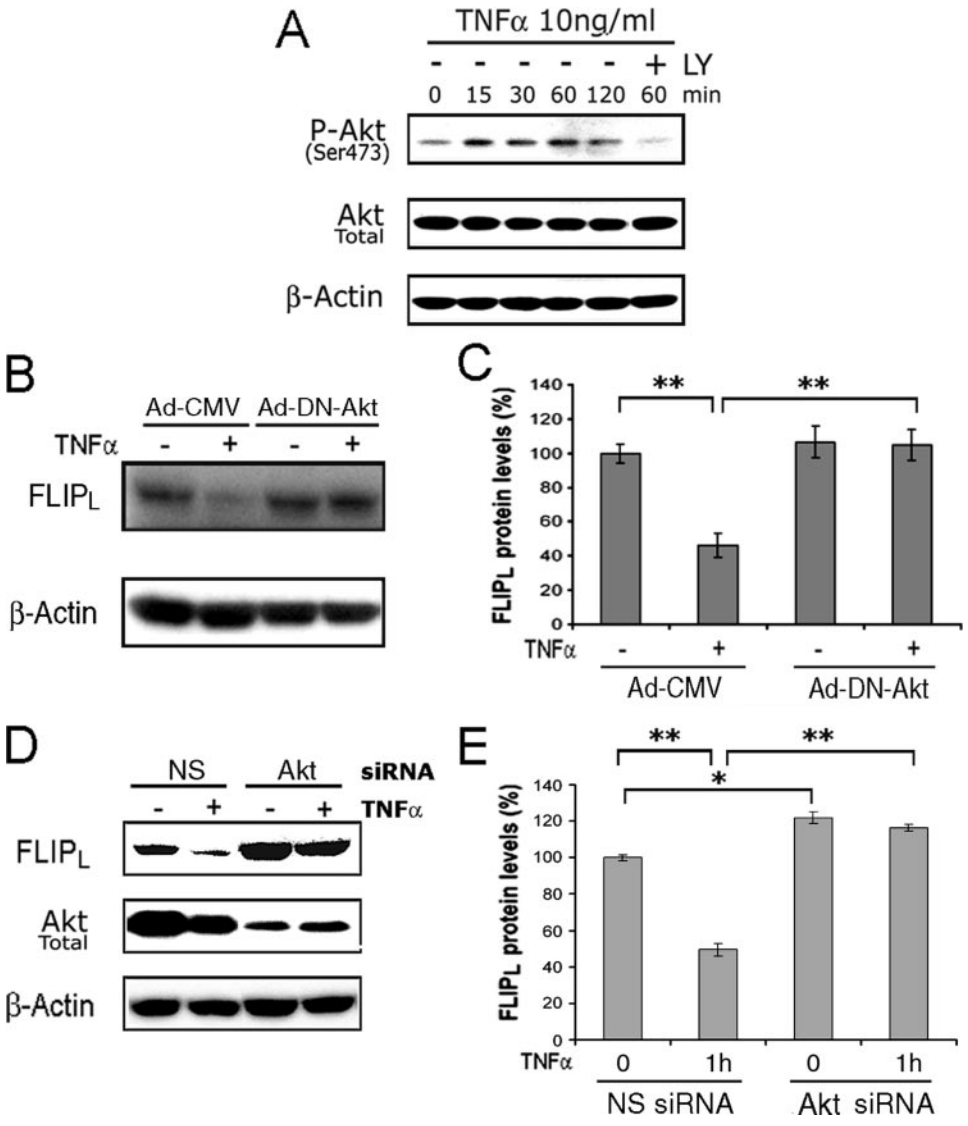

FIGURE 3. **Akt-1 mediates the TNFα-induced reduction of $FLIP_L$.** *A*, TNFα activates PI3K/Akt signaling detected by increasing Akt phosphorylation at serine 473. Macrophages were treated with 10 ng/ml of TNFα for the indicated times. Akt serine 473 phosphorylation was detected by Western blot analysis. Total Akt and β-actin were presented as controls. *B* and *C*, macrophages were infected with an adenoviral vector expressing a dominant negative Akt (DN-AKT) or a CMV control vector for 24 h prior to the addition of TNFα (10 ng/ml) for 1 h. The results of a representative immunoblot (*B*) and the summary (means ± S.E.) of three independent experiments (*C*) are presented. *D* and *E*, the forced reduction of Akt protects against the TNFα-induced reduction of $FLIP_L$. The macrophages were transfected with 100 nM Akt1 or nonspecific (*NS*) siRNA for 48 h then the cells were incubated with TNFα (10 ng/ml) for 1 h. The results of a representative immunoblot (*D*) and the summary (means ± S.E.) of three independent experiments (*E*) are presented. **, $p < 0.01$ between the indicated groups.

PI3K Pathway Has Minimal Effects on $FLIP_L$ Turnover in the Absence of Activation—The studies employing Akt siRNA suggest a role for the PI3K/Akt pathway in the constitutive expression of $FLIP_L$. Therefore, experiments were performed to determine the role of constitutive PI3K in the turnover of $FLIP_L$ in the absence of activation with TNFα or LPS. The protein synthesis inhibitor cycloheximide (CHX, 10 μg/ml) was utilized to block new protein synthesis by macrophages. Following treatment with CHX, there was a steady loss of $FLIP_L$ when examined between 2 and 6 h (Fig. 7, *A–C*). Inhibition of the PI3K pathway with LY294002 in the presence of CHX reduced $FLIP_L$ turnover by about 19% at 6 h compared with CHX alone (Fig. 8*A*). In contrast, the proteasome inhibitor MG132 suppressed the reduction of $FLIP_L$ by 64% at 6 h compared with CHX alone (Fig. 8, *B* and *C*). These observations demonstrate that in the absence of activation with TNFα or LPS, the contribution of the PI3K pathway to the turnover of $FLIP_L$ is limited.

Akt Physically Interacts with $FLIP_L$ and Results in $FLIP_L$ Serine Phosphorylation—To determine the mechanism by which TNFα and LPS trigger the reduction of $FLIP_L$, experiments were performed to determine whether $FLIP_L$ and activated Akt are capable of physically interacting within the cell. Macrophages were co-infected with adenoviral vectors expressing activated Akt (HA-tagged Myr-Akt) and $FLIP_L$, and the cell lysates were immunoprecipitated with anti-FLIP (Fig. 9*A*) or anti-HA (Fig. 9*B*) antibodies and then subjected to immunoblot analysis. $FLIP_L$ and Akt were able to co-immunoprecipitate, suggesting that activated Akt physically interacts with $FLIP_L$ (Fig. 9, *A* and *B*), either directly or through an intermediary protein in a larger complex.

Because Akt is a serine/threonine kinase and its function is based on phosphorylation of down stream target molecules, experiments were performed to determine whether activated Akt was capable of phosphorylating $FLIP_L$. Macrophages were infected with an adenoviral vector expressing constitutively activated Akt (Myr-Akt) and then incubated i*n vivo* with [^{32}P]orthophosphate. The expression of the Myr-Akt in macrophages increased $FLIP_L$ phosphorylation (Fig. 9*C*). Employing the Scansite Web Program (44), a putative Akt phosphorylation site was detected at serine 273, which is unique for $FLIP_L$ and is not included in $FLIP_S$. Therefore, experiments were performed to determine whether stimulation with TNFα promoted $FLIP_L$ serine phosphorylation. Lysates from macrophages that had been incubated with TNFα in the presence or absence of LY294002 were immunoprecipitated with an anti-FLIP antibody, and the immunoblots were probed with anti-phosphoserine and anti-FLIP antibodies (Fig. 9*D*). TNFα significantly ($p < 0.01$) increased the serine phosphorylation of $FLIP_L$, which was prevented ($p < 0.02$) by suppression of the PI3K pathway (Fig. 9*E*). These observations suggest that the PI3K/Akt-1-mediated phosphorylation of

$FLIP_L$ may contribute to its reduction following macrophage activation.

$FLIP_L$ Serine 273 to Alanine Substitution (S273A) Results in Resistance to Reduction by LPS—To determine whether serine 273 was critical for the activation-induced reduction of $FLIP_L$, retroviral vectors expressing wild type or S273A $FLIP_L$ were employed to stably infect the RAW267.4 macrophage cell line. The ectopically expressed wild type and S293A mutant $FLIP_L$ were detected at comparable levels (Fig. 10*A*), and the expression of the endogenous $FLIP_L$ was much weaker than the ectopically expressed protein and cannot be seen on the blots at this exposure. Treatment with LPS for 2 h resulted in an approximately 40% reduction ($p < 0.01$) of the wild type $FLIP_L$ (Fig. 10). Although LPS reduced the S273A mutant $FLIP_L$ somewhat, the reduction observed with the wild type $FLIP_L$ was significantly greater ($p < 0.01$) than observed with the S273A mutant (Fig. 10*B*). These observations demonstrate that the LPS-induced reduction of $FLIP_L$ is mediated, at least in part, through the Akt-mediated phosphorylation of serine 273 of $FLIP_L$.

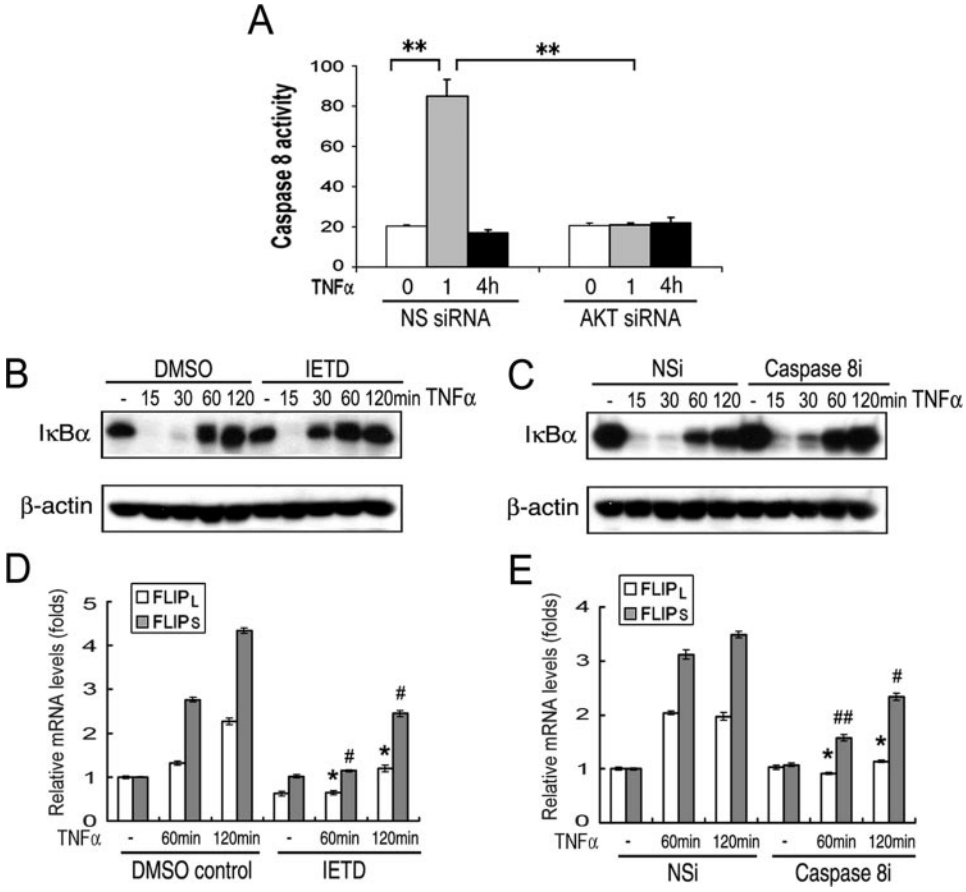

FIGURE 4. **Inhibition of caspase 8 activation attenuated NF-κB activation.** Macrophages were transfected with 100 nM Akt-1 or nonspecific (*NS*) siRNA for 48 h and then incubated with TNFα for 1 or 4 h, the cells were isolated, and the lysates were examined for caspase 8-like activity (*A*). Macrophages were incubated for 30 min with IETD (20 μM) or control DMSO (*B* and *D*), and then incubated with TNFα (10 ng/ml) for indicated time. The harvested samples were subjected to immunoblot (*B*) and quantitative PCR analysis (*D*). *C* and *E*, macrophages were transfected with 100 nM caspase 8 or nonspecific (*NS*) siRNA for 48 h and then incubated with TNFα. Cell samples were employed for immunoblot (*C*) and quantitative PCR analysis (*E*). The summaries of three (*A*) or two (*D* and *E*) independent experiments are presented. **, $p < 0.01$ in *A* compared with the indicated treatments. */#, $p < 0.05$; **/##, $p < 0.01$ in *D* and *E* compared the control group at the same time point.

DISCUSSION

The expression of FLIP is critical for macrophage survival because these cells express both Fas and FasL and are the principal source of TNFα (27). In the absence of an additional stimulus, the forced reduction of FLIP induced caspase activation and apoptosis of macrophages, mediated by Fas-FasL interactions (27). The current study demonstrates that TNFα not only activates NF-κB, which leads to the expression of *flip* mRNA resulting in an increase of both $FLIP_L$ and $FLIP_S$, but also activates the PI3K/Akt pathway, which leads to the reduction of $FLIP_L$ protein at 1–2 h. These observations identify a novel

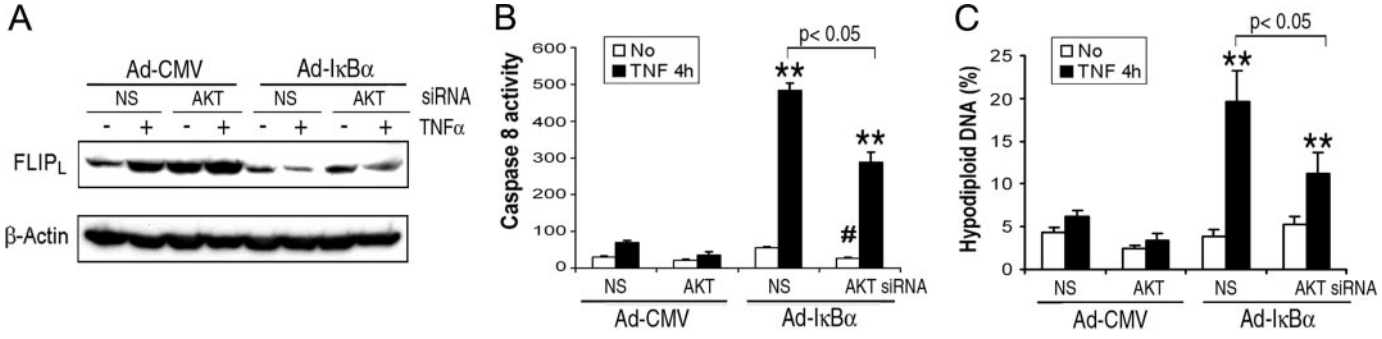

FIGURE 5. **The forced reduction of Akt reduced TNFα-induced caspase 8 activation and cell apoptosis.** *A*, macrophages were transfected with 100 nM Akt-1 or nonspecific (*NS*) siRNA for 48 h and then infected with a multiplicity of infection of 100 of Ad-IκBα or the Ad-CMV control vector for 24 h. After incubation with TNFα (10 ng/ml) or control medium for 4 h, the cells were harvested the lysates examined by immunoblot (*A*), and for caspase 8-like activity (*B*), and for apoptosis (hypodiploid DNA, *C*). The results are the means ± S.E. of three (*B*) or two (*C*) independent experiments, performed in duplicate. **, $p < 0.01$ *versus* control treatment; #, in *B*, $p < 0.05$ *versus* Ad- IκBα/NS siRNA/no TNFα.

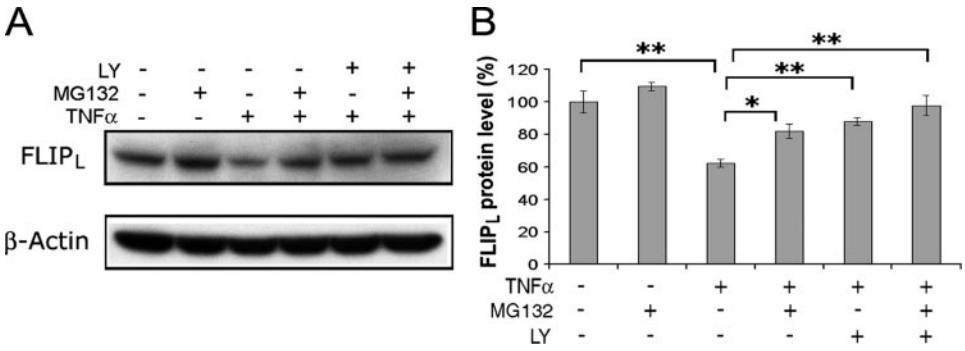

FIGURE 6. **The proteasomal pathway participates in TNFα-triggered reduction of FLIP$_L$.** Macrophages were preincubated with DMSO, LY294002 (20 μM) and MG132 (10 μM) for 30 min prior to the addition of TNFα (10 ng/ml) for 1 h. The cell lysates were subjected to immunoblot analysis. The results of a representative immunoblot (*A*) and the summary of 4 independent experiments (*B*) are presented. FLIP$_L$ assessed by densitometry was normalized with β-Actin. *, $p < 0.05$; **, $p < 0.01$ compared with the groups indicated.

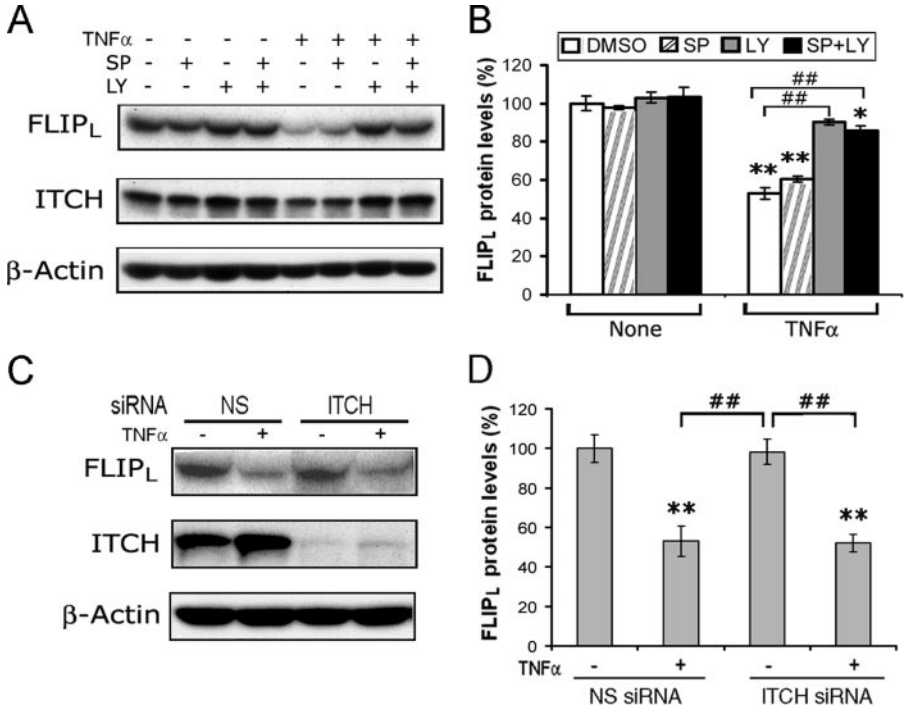

FIGURE 7. **The TNFα-induced reduction of FLIP$_L$ is not mediated through JNK-ITCH signaling.** *A* and *B*, JNK inhibition does not prevent TNFα induced FLIP$_L$ reduction. Macrophages were preincubated with DMSO, SP600125 (20 μM), or LY294002 (20 μM) as indicated for 30 min prior to the addition of TNFα (10 ng/ml) or control medium, which were incubated for 1 h. The cells were harvested, and the lysates were employed for immunoblot analysis employing antibodies to FLIP, Itch, and β-actin. In *A* is a representative blot, and *B* shows the means ± S.E. of four independent experiments. *C* and *D*, the forced reduction of Itch does not prevent the TNFα-induced reduction of FLIP$_L$. Macrophages were transfected with 50 nM Itch siRNA or nonspecific siRNA for 48 h, treated with TNFα (10 ng/ml) for 1 h, and harvested, and the cell extracts were examined by immunoblot analysis for FLIP$_L$, Itch, and β-actin. A representative immunoblot (*C*) and the means ± S.E. of three independent experiments (*D*) are presented. **, represents $p < 0.01$; *, $p < 0.05$ compared with DMSO or medium controls without TNFα; ##, $p < 0.01$ between the groups indicated.

mechanism whereby activation by TNFα initiates a potential death promoting signal through Akt and suggest that this effect is counteracted by the activation of NF-κB, which rescues the cell by the rapid induction of FLIP$_S$. Similarly, in short term activated T cells, the transient induction of FLIP$_S$ protected against death receptor-mediated apoptosis (45). Supporting the relevance of this process, the reduction of FLIP$_L$ by TNFα was accompanied by the short term induction of caspase 8-like activity, which promoted the activation of NF-κB and enhanced the expression of FLIP$_L$ and FLIP$_S$, which was associated with protection against apoptosis. The activation-induced reduction of FLIP$_L$ is not unique to TNFα, because the TLR4 ligand LPS also resulted in the Akt-mediated reduction of FLIP$_L$.

TNFα and LPS have been shown to induce the NF-κB-mediated expression of FLIP$_L$ and FLIP$_S$ in a variety of cell types, promoting inducible resistance to death receptor signaling (10, 11, 46). Consistent with these observations, our data with primary human macrophages demonstrate that TNFα and LPS result in the rapid induction of mRNA for both FLIP$_L$ and FLIP$_S$, which was mediated through NF-κB activation. Of interest, the expression of the super-repressor IκBα for 24 h in the absence of TNFα or LPS resulted in no reduction of FLIP mRNA or protein, despite the fact that NF-κB is constitutively activated in macrophages (47). Consistent with this observation, the basal expression of FLIP$_L$ mRNA and protein in RelA-deficient mouse embryonic fibroblasts and RelA-deficient hepatocytes is not different from the wild type controls (48, 49). These observations suggest that mechanisms other than NF-κB, such as PI3K/Akt, or MAPK/extracellular signal-regulated kinase (ERK) (14–16, 50–53), may contribute to basal expression of FLIP in macrophages.

Employing chemical inhibitors, DN-Akt and an Akt-specific siRNA, we demonstrate in this study that Akt mediated the TNFα-induced reduction of FLIP$_L$. Earlier observations, documenting the ability of Akt to up-regulate the expression of FLIP$_L$ (14–16, 53), appear to contradict this interpretation. However, the differences may relate to the timing and mechanism of activation of Akt. Neither LY294002 nor DN-Akt had an effect on the basal level of mRNA of FLIP$_L$ or FLIP$_S$ in macrophages, suggesting that the PI3K/Akt pathway, although constitutively activated in *in vitro* differentiated macrophages (29), does not regulate the constitutive transcription of *flip* in macrophages (data not shown). However, infection of macrophages with an adenoviral vector expressing activated Akt for 24 h resulted in the induction of mRNA and protein for FLIP$_L$ and FLIP$_S$, which was mediated through the activation of NF-κB (data

not shown), as previously described (54). In other studies, the activation of Akt employing bioactive lipids, endothelial growth factor, or Notch-1 resulted in the induction of $FLIP_L$, although the effects at early time points were not examined (15, 16, 53). These observations suggest that the PI3K/Akt pathway does not contribute to the basal expression of FLIP mRNA in macrophages, in contrast to the observations in a variety of tumor cells (14).

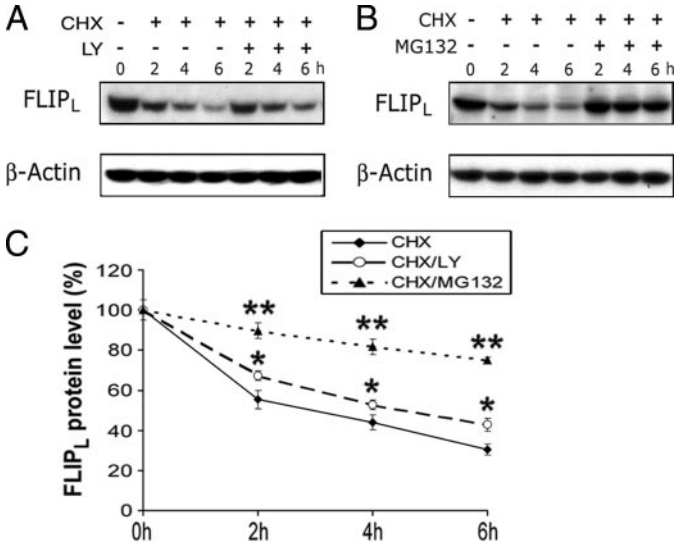

FIGURE 8. **In the absence of activation, inhibition of the PI3K pathway minimally affects $FLIP_L$ turnover.** Macrophages were incubated with CHX (10 μg/ml) alone or in the presence of LY294002 or MG132. The cells were harvested between 0 and 6 h, and cell extracts were employed for immunoblots that were probed with antibodies to $FLIP_L$ and β-actin (*A* and *B*). Changes in the levels of $FLIP_L$ were assessed by densitometry, which was normalized with β-actin, and the results (means $\pm$ S.E.) of two to four experiments are presented in *C*. *, represents $p < 0.05$; **, $p < 0.01$ compared with CHX alone.

Although the PI3K/Akt pathway does not contribute to the basal expression of FLIP mRNA in macrophages, in contrast to the observations in a variety of tumor cells (14).

The ability of the PI3K/Akt pathway to promote the degradation of $FLIP_L$ was, at least in part, mediated by the phosphorylation of $FLIP_L$ on serine 273 in the caspase 8-like domain. In macrophages, activated Akt physically interacted with $FLIP_L$, resulting in the serine phosphorylation of $FLIP_L$. Examination of the sequence of $FLIP_L$ revealed a single putative Akt phosphorylation site at serine 273 that is not present in $FLIP_S$. Supporting the importance of this site, mutation of serine 273 to alanine significantly suppressed the LPS-induced reduction of $FLIP_L$. Further supporting the importance of this site, TNFα and LPS resulted in the induction of $FLIP_S$ mRNA, without evidence of degradation at 1–2 h. Other studies have documented the phosphorylation of $FLIP_L$. The bile acid glycochenodeoxycholate phosphorylated $FLIP_L$ and $FLIP_S$ in a protein kinase C-dependent manner, decreasing the association of both isoforms with Fas-associated death domain, thereby decreasing recruitment to the TNF-related apoptosis-inducing ligand death-inducing signal complex, sensitizing the cells to TNF-related apoptosis-inducing ligand-induced apoptosis (55). In contrast, in a Fas ligand-resistant cell line, the phosphorylation of $FLIP_L$ by calcium/calmodulin-dependent protein kinase II resulted in increased recruitment of the p43 intermediate form of $FLIP_L$ to the Fas death-inducing signal complex, resulting in resistance to Fas ligand-mediated apoptosis (56). Together, these observations demonstrate that phosphorylation of $FLIP_L$ at different locations may affect its localization within the cell (55, 56) or its turnover.

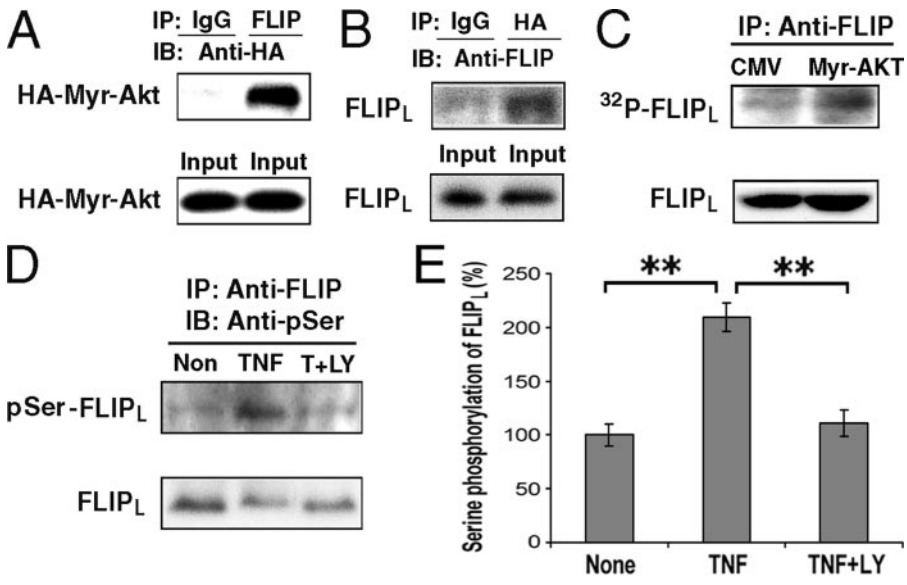

FIGURE 9. **Akt physically interacts with $FLIP_L$ and results in $FLIP_L$ serine phosphorylation.** *A* and *B*, $FLIP_L$ co-immunoprecipitates with Akt-1. Macrophages were co-infected with adenoviral vectors expressing HA-Myr-Akt and $FLIP_L$ (multiplicity of infection of 50 for each) for 24 h. Cell lysates were prepared and immunoprecipitated employing anti-FLIP (*A*) or anti-HA (*B*) antibodies or control IgG and then subjected to immunoblot analysis. The membranes were probed with anti-HA antibody (*A*) and anti-FLIP antibodies (*B*). *C*, activated Akt increases $FLIP_L$ phosphorylation. Macrophages were infected with CMV empty vector or a vector expressing Myr-Akt for 24 h. The cells were then incubated with [^{32}P]orthophosphate for 2 h, and $FLIP_L$ was immunoprecipitated with anti-FLIP antibody and examined following SDS-PAGE. Phosphorylated $FLIP_L$ was detected by autoradiography. The protein lysates of each sample, served as loading controls, following immunoblot analysis for $FLIP_L$ (*lower panel*). This result is representative of two independent experiments. *D* and *E*, TNFα increased $FLIP_L$ protein serine phosphorylation. Macrophages were treated with TNFα alone or TNFα plus LY294002 for 1 h. Cell lysates were immunoprecipitated with anti-FLIP antibody and examined by immunoblot analysis probing with an anti-phosphoserine antibody (*D*). The membranes were then stripped and reprobed with anti-FLIP antibody (*D*, *lower panel*). The results of three independent experiments quantified by densitometry are presented in *E*. **, $p < 0.01$ between the identified groups.

The reduction of $FLIP_L$ at 1–2 h was not due to processing by caspase activation because the addition of the caspase 8 inhibitor IETD or forced reduction of caspase 8 by specific siRNA did not prevent the reduction of $FLIP_L$. This observation is consistent with an earlier study that demonstrated that caspase inhibition did not prevent the reduction of $FLIP_L$ in endothelial cells induced by treatment with LPS in the presence of cycloheximide (57). Even though the caspase 8-like activity was detected 1 h after the addition of TNFα, our observations suggest that this activity was the result, rather than the cause, of reduction of $FLIP_L$. Numerous studies have documented the ubiquitinylation of $FLIP_L$, identifying this pathway as a key regulator of the level of

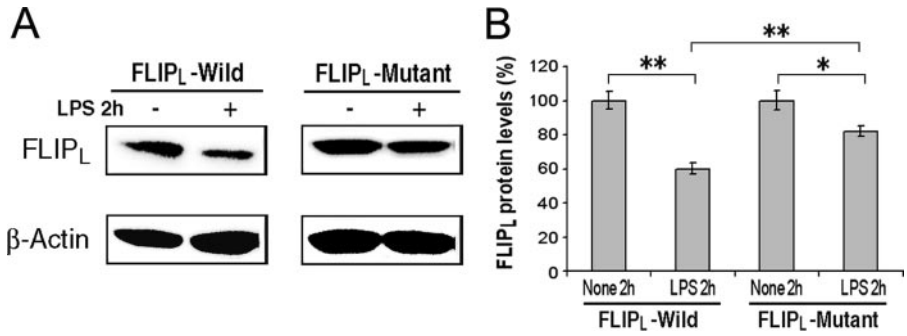

FIGURE 10. **Serine 273 to alanine mutation suppresses LPS-induced reduction of FLIP$_L$.** Wild type and S273A mutant FLIP$_L$ were stably expressed in RAW264.7 cells, which were treated with LPS (10 ng/ml) or control medium for 2 h. The cell lysates were employed for immunoblot analysis probing with antibodies to FLIP$_L$ and to β-actin (*A*). The summary (means ± S.E.) of three independent experiments quantified by densitometry is presented in *B*. *, represents $p < 0.05$; **, < 0.01.

FLIP$_L$ (17, 18, 21, 40, 41). Relevant to our observations, Fas ligation of an epithelial cell line, in the absence of an additional treatment, resulted in the degradation of FLIP$_L$ which was mediated through the ubiquitin-proteasome pathway (17). Our data suggest that activation of Akt promotes the proteasomal degradation of FLIP$_L$, because inhibition of the PI3K/Akt and the proteasomal pathways equally protected macrophages from TNFα-induced cell death.

Recently, TNFα was shown to promote the degradation of FLIP$_L$ through the activation of JNK1, which resulted in the phosphorylation and activation of the E3 ubiquitin ligase Itch, which then ubiquitinated FLIP$_L$ promoting its proteasomal degradation. In this system, the prolonged activation of JNK1 was necessary and was observed when the activation of NF-κB was prevented by genetic deletion, suppression of NF-κB activation, or suppression of protein synthesis with cycloheximide (21). The suppression of NF-κB resulted in inhibition of MAPK phosphatases (58), which were necessary for the normal attenuation of TNFα-induced JNK activation. Additionally, FLIP$_L$ was also shown to bind MKK7 and suppress the prolonged activation of JNK (48). In contrast, in human macrophages, the reduction of FLIP$_L$ induced by TNFα or LPS was observed even in the absence of inhibition of NF-κB or the use of cycloheximide, supporting the potential biological relevance of our observations. Further, we did not find evidence for the involvement of JNK or Itch because neither the JNK inhibitor SP600125 nor the forced suppression of Itch prevented the reduction of FLIP$_L$ in macrophages following stimulation with TNFα. The difference between the studies is likely due to the fact that the reduction of FLIP$_L$ observed in our study occurred in the absence of the inhibition of NF-κB, cycloheximide, or the prolonged activation of JNK. Additionally there may be important cell type-specific differences because our study focused on macrophages, whereas hepatocytes and mouse embryonic fibroblasts were the focus of the study that identified the JNK/Itch pathway (21).

Species variation may also account for the differences in the mechanism for the TNFα-mediated reduction of FLIP$_L$. Mouse embryonic fibroblasts (48) and murine hepatocytes (49) deficient in NF-κB p65 express normal levels of FLIP$_L$ but undergo apoptotic cell death following the addition of TNFα. The cell death is associated with the reduction of FLIP$_L$ (48, 49). However, in these cell types, apoptosis and the reduction of FLIP$_L$ is mediated through the activation of caspase 8 (48, 49). We also examined murine bone marrow-derived macrophages. In the absence of the inhibition of NF-κB, the addition of LPS resulted in the reduction of FLIP$_L$, which was mediated through caspase 8 activation, and suppression of the PI3K/Akt pathway did not prevent the reduction of FLIP$_L$ (data not shown). One potential explanation for the lack of PI3K/Akt involvement in murine cells may be that the murine FLIP$_L$ sequence does not contain a putative Akt phosphorylation site. In summary there are at least three mechanisms by which TNFα induces the post-translational degradation of FLIP$_L$, which employs the PI3K/Akt-, JNK1/Itch-, and caspase 8-mediated pathways, and they may be species-, cell type-, and context-specific.

The activation-induced reduction of FLIP$_L$ provides a potential mechanism for the initiation of cell death by which macrophages, which are not capable of adequately responding, may be eliminated following exposure to environmental stress. The post-translational reduction of FLIP$_L$ may be an integral event in the many studies that have characterized death receptor-mediated cell death when NF-κB activation is suppressed (59, 60). It is possible that the activation-induced reduction of FLIP$_L$ may be involved in macrophage apoptosis induced following microbial infection. Macrophages infected with *Yesinia enterocolitica*, *Mycobacterium tuberculosis*, or *Escherichia coli* may undergo apoptosis (61–64). Following infection, NF-κB activation was suppressed, and TNFα and TLR4 ligation contributed to the apoptosis observed (61–64), supporting a potential role for activation-induced reduction of FLIP$_L$ in this setting. It is also possible that the short term activation of caspase 8 may have a nonapoptotic function. The activation of caspase 8 contributes to differentiation of macrophages from monocytes and may also promote T cell receptor-mediated NF-κB activation of T cells and LPS-induced NF-κB activation of B cells (65–68). In macrophages the activation-induced reduction of FLIP$_L$ resulted in caspase 8 activation, which promoted NF-κB activation and the rapid induction of FLIP. In summary, this dynamic regulation of FLIP following a potential death-inducing signal determines the fate of the macrophage and may provide a therapeutic target in strategies directed at promoting death receptor-mediated apoptosis.

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