Down-regulation of the De-ubiquitinating Enzyme Ubiquitin-specific Protease 2 Contributes to Tumor Necrosis Factor-α-induced Hepatocyte Survival*

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Florian Haimerl1, Annette Erhardt1, Gabriele Sass1, and Gisa Tiegs*‡§1

From the †Institute of Experimental and Clinical Pharmacology and Toxicology, University of Erlangen-Nuremberg, Erlangen D-91054 and the ‡Division of Experimental Immunology and Hepatology, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, Hamburg D-20246, Germany

Tumor necrosis factor-α (TNFα) stimulation of hepatocytes induces either cell survival or apoptosis, which seems to be regulated by the ubiquitin-proteasome system. Here we investigated the role of TNFα-induced down-modulation of the de-ubiquitinating enzyme USP2 for hepatocyte survival. Inhibition of hepatocyte apoptosis by pre-treatment with TNFα (TNFα tolerance) was analyzed in the mouse model of galactosamine/TNFα-induced liver injury and in actinomycin D/TNFα-treated primary mouse hepatocytes. The role of USP2 for TNFα-induced hepatocyte survival was studied using small interference RNA or an expression clone. Injection of mice or preincubation of hepatocytes with TNFα caused a rapid down-regulation of hepatic USP2–41kD, the predominant USP2 isoform in the liver. In vitro an artificial knockdown of USP2 inhibited actinomycin D/TNFα-induced hepatocyte apoptosis, which was associated with elevated levels of the anti-apoptotic protein c-FlipL/S and a concomitant decrease of cellular levels of the ubiquitin-ligase Itch, a negative regulator of c-Flip. USP2–41kD overexpression abrogated TNFα tolerance in vitro, prevented accumulation of c-FlipL/S and resulted in elevated levels of Itch. Accordingly, c-FlipL/S protein levels were elevated in livers of TNFα-tolerant mice, which correlated to a switch from JNK and ERK to p38 signaling after galactosamine/TNFα re-challenge. Our results indicate that TNFα-induced USP2 down-regulation is an effective cytoprotective mechanism in hepatocytes. Hence, USP2 could be a novel pharmacological target, and specific USP2 inhibitors might be potential candidates for the treatment of inflammation-related apoptotic liver damage.

Tumor necrosis factor receptor 1 (TNFFR1)2 stimulation of hepatocytes results in simultaneous activation of antagonistic apoptosis pathways. The pro-apoptotic downstream signaling

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‡ To whom correspondence should be addressed. Tel.: 49-40-428-038-731; Fax: 49-40-428-037-150; E-mail: g.tiegs@uke.de.

§ The abbreviations used are: TNFR1, tumor necrosis factor receptor 1; Ab, antibody; Act.D, actinomycin D; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ERK, extracellular signal-regulated kinase; GaIN, galactosamine; JNK, c-Jun-activated kinase; MAPK, mitogen-activated protein kinase; mru, recombinant murine; TNFα, tumor necrosis factor-α; UPS, ubiquitin-proteasome system; USP, ubiquitin-specific protease; wt, wild type; siRNA, small interference RNA; E3, ubiquitin-protein isopeptide ligase; EGFP, enhanced green fluorescent protein; LDH, lactate dehydrogenase; RT, reverse transcription.
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is highly overexpressed and promotes tumor growth by stabilizing fatty acid synthetase; accordingly, a knockdown of USP2–69kD by siRNA enhanced apoptosis (13). Furthermore, mouse double minute 2 E3-ubiquitin ligase was identified as a substrate of USP2–69kD, which thereby indirectly controls p53 and apoptosis in H1299 cells (14). However, the function of USP2 in the liver is yet unknown.

In the present study we investigated the behavior of the de-ubiquitinating enzyme USP2 in murine liver following TNFα pre-treatment in vivo. The hepatocyte-specific transcriptional inhibitor galactosamine (GalN) (15–17) sensitizes hepatocytes toward TNFα-induced apoptosis in vivo, and GalN/TNFα-induced liver injury in mice serves as a model for fulminant hepatic failure (18, 19). Pre-treatment with TNFα has been shown to induce tolerance toward GalN/TNFα-induced fulminant hepatic failure (20). Therefore, genes that are regulated by TNFα are candidates to provoke cytoprotection (17). We found that USP2–41kD was strongly down-regulated after intravenous administration of TNFα in murine liver on mRNA and protein level. This finding prompted us to investigate the role of USP2–41kD in hepatocellular apoptosis. We found that an artificial knockdown of USP2 protected hepatocytes from TNFα-induced apoptosis by interference with NF-κB and c-Flip signaling.

EXPERIMENTAL PROCEDURES

Mice—BALB/C and C57BL/6 wild-type mice were obtained from the internal animal breeding house of the Institute of Experimental and Clinical Pharmacology and Toxicology, University of Erlangen-Nuremberg. Animals were maintained under controlled conditions (22 °C, 55% humidity, 12-h day/night rhythm) and fed standard laboratory chow. All mice received human care according to the guidelines of the National Institutes of Health and legal requirements in Germany.

Animal Treatment—For pre-treatment studies, murine (rmu)-TNFα (Innogenetics, Gent, Belgium) was dissolved in sterile saline/0.1% human serum albumin and injected intravenously into mice to a lateral caudal vein at a dose of 10 μg/kg body weight. Saline/0.1% human serum albumin was used as vehicle control. For induction of liver damage, rmuTNFα was administered in a dose of 5 μg/kg 30 min after intraperitoneal injection of 700 mg/kg GaLN (Roth, Karlruhe, Germany) dissolved in saline.

Isolation of Primary Hepatocytes, Transfection, and Activation Routes—For primary hepatocyte isolation and culture, William’s E + GlutaMAX™-1 medium (Invitrogen) was supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Biochrom AG seromed®, Berlin, Germany) and 1% l-glutamine (Invitrogen). Hepatocytes were isolated by a modification of the two-step collagenase perfusion method of Seglen (21, 22) followed by 10-min centrifugation in a 90% Percoll® gradient (GE Healthcare, Uppsala, Sweden) at 50 × g to obtain highly purified hepatocytes. Hepatocytes were transfected using LifeActin®-2000 (Invitrogen). To monitor the transfection efficiency, 4 × 10⁵ primary hepatocytes were transfected with either an EGFP plasmid (pEGFP-N1, Clontech, Mountain View, CA) or 25 nM of a fluorochrome-linked siRNA (AllStars Neg. siRNA AlexaFluor 488, Qiagen) and incubated for 36 and 24 h, respectively. Cell samples were harvested, and either EGFP expression or AlexaFluor 488 fluorescence was measured by fluorescence-activated cell sorting analysis. Transfection efficiency was determined from the percentage of GFP- or AlexaFluor 488-positive cells in each sample and found to be 27.4 ± 0.8% for EGFP and 23.5 ± 2.7% for siRNA in transfected cells compared with 1.26 ± 0.04% and 0.55 ± 0.08% in non-transfected controls, respectively. Although transfection efficiency seems to be low in the case of siRNA (probably due to the fluorochrome), reduction of mRNA and protein expression were 73.5 ± 5% and ~52%, respectively, in case of siUSP2 (cf. Figs. 5B and 7B, lower panel).

For induction of apoptosis hepatocytes were incubated with Act.D/TNFα as previously described (21). For TNFα prestimulation in vitro rmuTNFα was added in a final concentration of 40 ng/ml for 6 h. For accumulation of ubiquitinylated protein, the proteasome inhibitor MG132 (23) was added to the culture medium at the doses indicated.

Analysis of Liver Enzymes and Celluar Damage—In vitro cytotoxicity assays (LDH activity) and serum ALT activity in plasma were determined as described previously (21).

Cloning and Sequencing—Cloning was carried out as described previously (21). Sequencing of USP2–41kD expression clone was performed by GATC Biotech AG Company (Konstanz, Germany) in pred20 quality via forward and reverse runs.

RNA Isolation and cDNA Synthesis RT-PCR—RNA isolation and cDNA synthesis procedures were performed as described recently (21). To determine the USP2–69kD and the USP2–45kD mRNA fractions from overall USP2 mRNA in murine liver, USP2–69kD and -45kD specific primers and USP2 overall primers, which detect all USP2 isoforms, were used. Primer efficiency calibration curves were recorded using an USP2 expression clone in 1:10 dilutions steps from 10⁻² to 10⁻¹⁰. Together with three murine liver cDNA samples, USP2 expression clone dilutions were analyzed with the USP2–69kD and -45kD specific primer and the USP2-overall primers in separate master mixes, but in the same RT-PCR run (24).

Primers and siRNA—Following primers were used for RT-PCR analysis: β-Actin (X03765, fwd 5′-TGGAAATCCTGTGG-CATCCATGAAA-3′, rev 5′-TAAAGCCGACCTGATACAGTGCCG-3′), USP2 all isoforms (NM016808.2, fwd 5′-AGAACATATGGATG GTG-3′, rev 5′-GGCGATAGGGCTG-TATAGTTG-3′), USP2 45kD isoform (NM198091.2, fwd 5′-CTGCTGCTTCCACCTTC-3′, rev 5′-CTCAATGGGCTCACCAC-3′), USP2 69kD isoform (NM198092.2, fwd 5′-CGCGAATTCCGCTAC-3′, rev 5′-GCCCTTTCGACCATA GTTTAC-3′), and c-Flip (NM207653.3, fwd 5′-CATTGATGATTATGGCTG-3′, rev 5′-CTACTGGTCCAAGGTGTATCG-3′). SiRNAs against the ubiquitin-specific-protease 2 (siUSP2, 5′-GGAAACCCGUCUCUAUGAACT-3′) and the enhanced green fluorescent protein (siEGFP, 5′-GCAUAAGUGGAAUCUUCAGA-3′) were obtained from Microsynth (Balgach, Switzerland).

Western Blot Analysis—Protein extraction and analysis were performed as previously described (21).

Immunoprecipitation—Cells were lysed in ice-cold radioimmune precipitation assay buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM K2HPO4, 10% v/v glycerol, 1% v/v
Triton X-100, 0.15% SDS), and centrifuged at 4 °C and 12,000 g for 5 min. Supernatant was adjusted to a protein concentration of 1.5 g/ml and precleared with protein-G-Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 1 h at 4 °C. Itch was immunoprecipitated by incubation with 5 ng of anti-Itch-IgG (Santa Cruz Biotechnology, Heidelberg, Germany) per microgram of protein at 4 °C for 6 h and subsequent addition of protein-G-Sepharose beads shaking overnight at 4 °C. Beads were washed four times with radioimmune precipitation assay buffer and boiled in Laemmli sample buffer prior to SDS-PAGE analysis.

Antibodies—USP2 protein was detected with USP2-S260 primary antibody (Abgent, San Diego, CA). Polyclonal rabbit anti-ubiquitin antibody Z0458 was purchased from Dako-Cytomation (Glostrup, Denmark). Anti-Flip polyclonal antibody H-202 was purchased from Santa Cruz Biotechnology. Anti-Itch antibody was purchased from BD Pharmingen (Heidelberg, Germany). Phospho-p38, caspase-3, phospho-JNK, and phospho-ERK antibodies were obtained from Cell Signaling (Danvers, MA).

Luciferase Assay—Cells were co-transfected in 24-well plates with either siEGFP or siUSP2 together with 0.4 mol of luciferase reporter pB2LUC, containing two NF-κB-binding sites, or with 0.4 mol of pGL3Basic, encoding only firefly luciferase without promoter or enhancer sequences as reference plasmid. 24 h later, transfected cells were harvested for preparation of whole cell extracts according to the manufacturer's instructions. Luciferase reporter activity was measured by a commercial assay (Luciferase Assay System, Promega, Mannheim, Germany).

Statistical Analysis—Results were analyzed using the Student's t test if two groups were compared. If more than one group was compared with a control group or if groups were compared among each other, the one-way analysis of variance test was used followed by Dunnett's and Bonferroni's tests, respectively. All data are expressed as a mean values ± S.E. A value of p ≤ 0.05 was considered significant.

RESULTS

TNFα Induces Cytoprotection toward Hepatocyte Death in Vivo and in Vitro—Administration of low doses of TNFα to mice does not induce apoptosis in the liver, but even protects

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FIGURE 1. TNFα pre-treatment desensitizes mice to GalN/TNFα induced apoptotic liver damage in vivo. A, three mice per group were pre-treated with a single dose (10 μg/kg) of muTNFα intravenously at the indicated times prior to challenge with GalN/TNFα. The mean serum ALT ± S.E. values were measured after 6 h for each group (n = 3, **, p < 0.01). B, primary hepatocytes were pre-treated with muTNFα (40 ng/ml) 6 h before induction of apoptosis with Act.D/TNFα. Cytotoxicity was assayed after 20 h via LDH activity (mean values ± S.E., n = 6, ***, p < 0.001).

FIGURE 2. TNFα down-regulates USP2 mRNA and protein in murine liver in vivo. Three mice per group were treated with TNFα or vehicle control intravenously. A, at the indicated times post TNFα injection, overall USP2 mRNA was quantified by RT-PCR analysis (n = 3, *, p < 0.05). B, 4 h after intravenous TNFα administration USP2–41kD protein was measured by Western blot analysis from liver tissue lysates. The bar graph shows densitometric Western blot quantification of USP2 protein in relation to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (n = 3, *, p < 0.05). C, 30-min prior to TNFα injection mice were treated with GalN or saline intraperitoneally. Two hours after intravenous TNFα injection, liver tissues were analyzed for USP2 mRNA by RT-PCR (n = 3, *, p < 0.05).
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**FIGURE 3.** USP2–41kD Is the predominant isoform in murine liver. **A,** liver cDNAs from three untreated animals were quantified via RT-PCR for the fractions of USP2–69kD and USP2–41kD in overall USP2 cDNA (n = 3, mean ± S.E.). B, proposed mean percentile distribution of USP2splice variant mRNA from three murine liver cDNAs. C, Western blot analysis of USP2expression in liver tissue. Each lane represents one animal. Only USP2–69kD and USP2–41kD proteins were detectable. C, the protein expression levels of the USP2 splice variants was analyzed in lysates from several murine tissues by Western blot.

mice from liver damage induced by GalN/TNFα re-challenge (TNF tolerance) (17). The time course of TNFα pretreatment shown in Fig. 1A indicates that TNF tolerance was inducible as early as 1–2 h after intravenous injection and persisted for at least 24 h. After 48 h, mice were again susceptible to GalN/TNFα-induced liver damage. TNF tolerance was also inducible in vitro (Fig. 1B), i.e. TNFα pre-treatment for 6 h prevented Act.D/TNFα-induced cell death of cultured primary mouse hepatocytes.

TNFα Down-regulates USP2 mRNA and Protein in Murine Liver—In view of the rapid hepatocellular desensitization upon TNFα pretreatment, we focused on a possible involvement of the UPS in the initiating mechanism of TNF tolerance. Recently performed cDNA array screening in TNFR2−/− mice for TNFR1-regulated genes in murine liver revealed both TNFα up-regulated as well as TNFα down-regulated mRNA gene transcripts (17). With regard to mediators assigned to the UPS, we found that the mRNA of the de-ubiquitinating enzyme USP2 was down-regulated by TNFα (17). Re-evaluation by quantitative real-time RT-PCR demonstrated that USP2 mRNA levels were substantially decreased in TNFα-treated mice compared with control animals (Fig. 2A). USP2 mRNA down-regulation occurred very rapidly and was restored 4 h after TNFα application. TNFα also decreased USP2 protein in murine liver lysates compared with control (Fig. 2B). The hepatocyte-specific transcriptional inhibitor GalN abolished TNFα-induced down-regulation of USP2 mRNA (Fig. 2C). Hence, USP2 seems to be regulated by TNFα in liver parenchyma and is potentially involved in GalN-induced sensitization of hepatocytes to TNFα.

USP2–41kD Is the Predominant Isoform in Murine Liver—USPs have N- and/or C-terminal extensions that allow the selective interaction with distinct substrates and determine the cellular localization of the de-ubiquitinating enzyme (25). Splicing isoforms of USP2 were shown to exhibit antagonistic biological roles (26), and recent studies attribute a tumor-promoting function to human USP2–69kD (13), but describe human USP2–41kD as a pro-apoptotic regulator (12). To further characterize the role of USP2 in the liver, we quantitatively analyzed USP2-spooling isoforms via real-time PCR (24) and found only small fractions of 45- and 69-kDa mRNA in overall USP2 mRNA (Fig. 3A). Hence it seems that USP2–41kD is the predominant isoform in murine liver on mRNA level (Fig. 3B). Western blot analysis confirmed USP2–41kD as the predominant isoform on the protein level, and only a very small fraction of USP2–69kD could be detected (Fig. 3C). USP2–45kD was not detectable on the protein level (Fig. 3C). Screening of different murine tissues for USP2 expression revealed remarkable USP2–69kD expression only in kidney lysates (Fig. 3D).

USP2–41kD Is a Pro-apoptotic Regulator in Hepatocytes in Vitro—To elucidate the role of USP2 in TNFα-induced apoptosis we studied the effect of an artificial USP2 knockdown on Act.D/TNFα-induced cell damage in primary hepatocytes. Compared with siEGFP-transfected primary hepatocytes siUSP2-treated cells showed significantly less cellular damage in response to Act.D/TNFα, as quantified by LDH release (Fig. 4A), as well as substantially reduced cleavage of caspase-3 into its catalytic active 17-kDa fragment, as determined 3.5 h after incubation with Act.D/TNFα (Fig. 4B). Hence, siUSP2 inhibited LDH release by 40 ± 5% at the end of the experiment (Fig. 4A) compared with 58 ± 6% reduction of LDH release by pre-incubation of hepatocytes with TNFα (Fig. 1B). Because the efficacy of the USP2 knockdown was 73.5 ± 5% with respect to mRNA (Fig. 7B, lower panel) and ~52% with respect to USP2 protein repression (Fig. 5B), the potency to block cytotoxicity of either siUSP2 or TNFα was in a comparable range. The role of down-modulation of USP2–41kD for TNF tolerance was confirmed by showing that hepatocytes were re-sensitized to Act.D/TNFα-induced apoptosis by USP2–41 overexpression (Fig. 4C).

Hepatocellular c-Flip and Itch Protein Levels Correlate with USP2 Expression in Vitro—To investigate the correlation between TNF-induced cytoprotection and down-modulation of the de-ubiquitinating enzyme USP2 we focused on c-Flip, which is an NF-κB-dependent TNFα-inducible survival protein,
that is also regulated by the UPS (27, 28). We studied whether artificial knockdown of USP2 would affect levels of c-Flip in hepatocytes. As shown in Fig. 5A, transfection of siUSP2 caused elevated levels of c-Flip protein. Because USP2 is a de-ubiquitinating enzyme, these results, showing that the lack of USP2 resulted in c-Flip stabilization, were unexpected and suggested an indirect effect of USP2 down-modulation and inhibition of c-Flip degradation. It has been shown recently that the ubiquitin E3-ligase Itch is a negative regulator of c-Flip (28). Itch knockout animals were resistant to TNFα-induced liver damage (28). Indeed, the USP2 knockdown resulted in a reduction of cellular levels of Itch (Fig. 5B) suggesting an interference of USP2 with the JNK-Itch pro-apoptotic pathway, which has been identified recently (28).

Proteasome inhibitors were shown to protect hepatocytes from TNFα-induced apoptosis by accumulation of c-Flip (28–30). The proteasome inhibitor MG132 enriched the fraction of ubiquitin-conjugated proteins in hepatocytes in vitro in a concentration- and time-dependent manner (data not shown). Western blot analysis revealed that a concentration of 5 μM MG132 generated a backup of ubiquitinated proteins within 24 h without completely abolishing proteasome function, as 200 μM MG132 resulted in much higher accumulation of ubiquitinated proteins within the same time frame (data not shown). Hence, proteasomal degradation of proteins is still possible in the presence of 5 μM MG132. The regimen of 24-h preincubation with 5 μM MG132 alleviated Act.D/TNFα-induced apoptosis of hepatocytes in vitro (Fig. 5C). Although MG132 alone showed low toxicity at this concentration, probably by its NF-κB-silencing activity (31), the overall effect seemed to be overcompensated by accumulation of c-Flip (Fig. 5D). To investigate whether USP2–41kD would interfere with MG132-induced accumulation of ubiquitinated proteins and c-Flip, we overexpressed USP2–41kD in primary hepatocytes. USP2–41kD transfection inhibited the accumulation of ubiquitinated proteins (Fig. 5D) as well as of c-Flip (Fig. 5E) in the presence of MG132. According to our results obtained with the USP2 knockdown we found that protein levels of Itch were elevated by USP2–41kD overexpression (Fig. 5E). Likewise, in contrast to hepatocytes transfected with a control vector, no ubiquitinated Itch protein could be immunoprecipitated from hepatocytes overexpressing USP2–41kD (Fig. 5F). Concordantly, USP2–41kD overexpression caused a mild increase of MG132 cytotoxicity, indicating that the de-ubiquitination by USP2–41kD stabilizes apoptotic factors when the supply of ubiquitinated substrates is elevated by MG132 (Fig. 5G). Because TNFα down-regulates USP2 and USP2 seems to negatively influence Itch ubiquitination, we incubated primary hepatocytes in vitro with rmuTNFα and analyzed Itch expression. We found that the cellular levels of Itch protein promptly declined following addition of TNFα and were almost restored after 24 h (Fig. 5H). Fig. 5H also shows that TNFα-induced down-modulation of Itch correlated to down-modulation USP2 mRNA expression in vitro (right bar graph).

TNFα Tolerance Is Accompanied by Elevated Levels of c-FlipLS Protein and a Switch from JNK to p38 Signaling in Vivo—The pro-apoptotic role of prolonged JNK activation during TNFα-induced apoptotic liver damage has recently been described (1). The role of p38MAPK signaling in TNFα-induced apoptosis is highly controversial, because inhibitors of p38MAPK enhanced (32, 33) or prevented (34, 35) TNFα-induced apoptosis depending on cell type and model of damage. Moreover, activation of ERK 1 and 2, i.e. MAPKs involved either in cell proliferation and survival or in apoptosis and tissue damage (36, 37), was
FIGURE 5. USP2–41kD expression alters cellular levels of c-Flip and Itch protein. A, hepatocytes were transfected with siUSP2 or control. After 24 h hepatocytes were harvested and analyzed for c-Flip expression via Western blot. Each lane represents three pooled wells. B, hepatocytes were treated like those described in A and analyzed for Itch protein via Western blot. Each lane represents three pooled wells. C, primary hepatocytes were treated with vehicle control, MG132 (5 μM), Act.D/TNFα, or both as indicated. After 20 h hepatocytes were assayed for cytotoxicity via LDH activity (% LDH release ± S.E., n = 6, *** p < 0.001). D, upper panel: primary hepatocytes were transfected with USP2–41kD expression clone or control vector. After 24 h medium was changed and supplemented with 5 μM MG132. After another 24 h cells were harvested and analyzed for ubiquitin-conjugated proteins via Western blot. One lane represents three pooled wells. Lower panel: primary hepatocytes were incubated at the indicated concentrations of MG132 in vitro for 24 h. C-Flip expression was analyzed via Western blot. Each lane represents three pooled wells. E, hepatocytes were transfected with USP2–41kD expression clone or control vector and challenged with 5 μM MG132 after 24 h. C-Flip and Itch protein expression was analyzed by Western blot analysis after another 24 h. One lane represents three pooled wells. F, hepatocytes were transfected with USP2–41kD expression clone. After 24 h cells were incubated with 5 μM MG132. After another 24 h cells were lysed and an anti-Itch immunoprecipitation was performed. The precipitates were analyzed using an anti-ubiquitin antibody. One lane represents three pooled wells. G, primary hepatocytes were treated as described for E. After another 24 h cell damage was evaluated by measurement of LDH (mean values ± S.E., n = 6, *, p < 0.05). H, hepatocytes were stimulated with 40 ng/ml rmuTNFα and lysed at the indicated time points. Itch expression was quantified using Western blot analysis. The upper bar graph shows densitometric Western blot quantification of Itch protein in relation to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. One lane represents three pooled wells. The right bar graph shows real-time RT-PCR analysis of the USP2-mRNA knockdown in vitro 2 h after administration of 40 ng/ml rmuTNFα to cultivated hepatocytes (mean values ± S.E., n = 3, **, p < 0.01).
of USP2, we further investigated whether an USP2 knockdown in primary hepatocytes would also modulate NF-κB signaling. As shown in Fig. 7A, a knockdown of USP2 substantially enhanced NF-κB-dependent expression of luciferase in a reporter assay compared with controls, indicating that USP2 knockdown caused enhanced translocation of NF-κB into the nucleus of hepatocytes. Quantitative real-time RT-PCR analysis of hepatocyte mRNA 24 h after transfection with siUSP2 confirmed the successful knockdown of USP2 mRNA and revealed a mild de novo synthesis of c-Flip mRNA, presumably due to the enhanced transcriptional activity of NF-κB (Fig. 7B).

**DISCUSSION**

In acute and chronic liver disease, overstimulation of the TNFα/TNFRI system is a major pathogenic factor (38). A single systemic challenge with TNFα causes NF-κB activation, hepatocyte survival, and TNF tolerance in the liver (17, 21). During inflammatory liver disease the anti-apoptotic TNFR1 signaling fades into the background and TNFα mediates inflammation and hepatocyte death. Hence, identification of novel targets for treatment of inflammatory liver disease that counteracts TNF-induced hepatocyte apoptosis will benefit from studies of the molecular mechanisms of hepatocyte survival induced by TNF tolerance.

In livers of TNFα-pretreated mice, we observed a switch from JNK and ERK to p38 signaling and elevated levels of the major cytoprotective factor c-FlipS/L. Recent studies showed that selective JNK inhibitors protected rat hepatocytes from Act.D/TNFα-induced damage (39) and that JNK knockout mice were protected from TNFα-mediated liver damage (28, 40). It seems that JNK1 mediates TNFα-induced activation of Itch and subsequent c-Flip degradation, whereas JNK2 seems to directly activate caspase-8 (28, 40). These observations together with our results showing abrogation of JNK signaling in TNF-tolerant mice underscore the hypothesis that prolonged JNK activation facilitates TNFRI-mediated liver apoptosis (1). Besides its effects on proliferation and cell cycle progression of cancer cells, ERK 1 and 2 have been implicated in apoptosis of neuronal and renal epithelial cells as well as of hepatoma cell lines, and inactivation of ERK has been shown to protect from tissue damage (37). The pro-apoptotic effect of ERK activation has been associated with activation of the mitochondrial pathway, direct activation of caspase-3 or down-

**FIGURE 6.** TNF tolerance includes a switch from JNK and ERK to p38 signaling and elevated levels of cytoprotective c-FlipS/L protein. Three mice per group were treated with rmuTNFα (10 μg/kg) intravenously at the indicated times prior to re-challenge with GalN/TNFα. A, serum ALT activity was measured after 6 h for each animal. Each bar represents one animal. B–E, C-Flip, phospho-p38, phospho-ERK, and phospho-JNK proteins were quantified via Western blot analysis of liver lysates. Each lane represents the liver protein expression of one animal in correspondence to its serum ALT value in Fig. 6A.
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**FIGURE 7.** Knockdown of USP2 resulted in enhanced NF-κB 24-h basal activity. A, primary hepatocytes were co-transfected with siRNA against USP2 and a luciferase-reporter plasmid with two NF-κB promoter binding sites or controls. As negative control a luciferase plasmid without promoter/enhancer sequences was used. After 24 h luciferase activity was determined from whole cell lysates via bioluminescence assay (mean values ± S.E., n = 3, **p < 0.01**). B, primary hepatocytes were transfected with siRNA against USP2. After 24 h mRNA was isolated and transcribed to cDNA for RT-PCR analysis. USP2 knockdown was verified and transcriptional status of c-Flip was analyzed via real-time PCR (mean values ± S.E., n = 4, *, p < 0.05).

modulation of the anti-apoptotic signaling molecule Akt (37). In prostate cancer cells, resistance to TNF-related apoptosis-inducing ligand correlated with deactivation of ERK1/2 signaling, again providing evidence for a pro-apoptotic effect of ERK (41). To our knowledge, we are the first to demonstrate that ERK1/2 is down-modulated as a result of resistance to TNFα (41). To our knowledge, we are the first to demonstrate that ERK1/2 is down-modulated as a result of resistance to TNFα-induced apoptotic liver damage, suggesting a pro-apoptotic effect of these MAPKs during TNFα signaling in primary hepatocytes. However, the concomitant increase of phosphorylated p38 in TNF-tolerant mice indicates that p38 might transduce survival signals in the liver. These results correspond to studies with embryonic mouse fibroblasts and hepatoblasts showing that JNK and p38 antagonistically control cell proliferation and regeneration (42). Moreover, in the human endothelial cell line ECV304 selective p38 inhibitors were shown to decrease expression of the cytoprotective factors cIAP1 and -2 (32). According to our results presented here, very recent data obtained with p38α knockout mice in a model of endotoxin-induced liver failure showed that p38 deficiency aggravated JNK activation and concomitantly decreased c-Flip levels thereby contributing to an exacerbation of TNFα-mediated apoptotic liver damage (43).

C-Flip is a well characterized anti-apoptotic protein. It is known to be a potent inhibitor of death receptor-induced apoptosis, as it antagonizes caspase-8 activation at the stage of TNFR-complex II formation (44, 45). During TNFR1- and CD95-induced apoptosis the ratio of c-Flip relative to procaspase-8 is known to critically control survival and death (46, 47). In general c-Flip is spliced into the two isoforms c-FlipL and c-FlipS (48). Both isoforms contain the death-editing domain, dual ubiquitin-editing functions, was shown to protect mice from GalN/TNFα-induced apoptosis by editing RIP ubiquitination and down-modulation of Bax protein (21, 51, 52). The UPS is indispensable for regulation and activation of NF-κB, a major cytoprotective transcription factor in the liver, because it coordinates the degradation of the NF-κB superrepressor IkBα (53). The proteasome inhibitor MG132 was shown to antagonize TNFα-induced IkBα degradation and NF-κB activation in a dose-dependent manner (31), which might have been one reason for its basal cytotoxicity observed in this study. Conditional p65-knockout mice (54) or mice transgenic for a non-phosphorylatable N-terminal deletion mutant of IkBα (N-1xIkBα) (55) are sensitized to TNFα-induced liver damage due to a selective silencing of the NF-κB activation. Nevertheless MG132 protected mouse hepatocytes from Act.D/TNFα-induced apoptosis, presumably due to accumulation of c-Flip. This dual effect of MG132 on hepatocytes underscores the involvement of the UPS in both pro-apoptotic as well as anti-apoptotic TNFR1 downstream signaling events in liver.

The rapid initiation of TNF tolerance upon TNFα pretreatment in vivo prompted us to screen for early TNF-regulated genes assigned to the UPS. We found the de-ubiquitinating enzyme USP2 to be substantially down-regulated in murine livers as early as 15 min after TNFα administration in vivo. USP2 mRNA depletion was abrogated by GalN, which suggests an involvement of USP2 in the sensitization of hepatocytes to TNFα by GalN. GalN might block the transcription of a TNFα-inducible USP2 repressor. Because USP2 saves its substrates from proteasomal degradation by de-ubiquitination, depletion
of USP2 should lead to enhanced proteasomal degradation of its substrates (25).

TNFα preincubation also induced USP2 down-modulation *in vitro* (although to a lesser extent than *in vivo*) and protected primary mouse hepatocytes from Act.D/TNFα-induced apoptosis. Accordingly, artificial knockdown of USP2 was an efficient cytotoxic protective anti-apoptotic mechanism *in vitro*. Degradation of pro-apoptotic factors upon USP2 depletion would explain these findings and suggest that the TNFα-mediated down-modulation of USP2 contributes to the rapid initiation of TNF tolerance in liver. Accordingly it seems that a USP2 knockdown blunts the c-Flip-degrading pathways by inducing Itch degradation and promotes the de novo synthesis of c-Flip protein by elevating NF-κB basal activity and c-Flip mRNA transcription. Hence, we consider the elevated levels of c-Flip protein during USP2 knockdown as one major cytoprotective mechanism.

In contrast to former reports, where an overexpression of human USP2-41kD induced apoptosis in HEK293 cells (12), mere overexpression of USP2 in hepatocytes could not elicit cell damage. Only in presence of proteasomal inhibition by MG132, USP2-41kD overexpression exacerbated cell damage. Only in presence of proteasomal inhibition by MG132, USP2-41kD overexpression exacerbated cell damage. Only in presence of proteasomal inhibition by MG132, USP2-41kD overexpression exacerbated cell damage. Only in presence of proteasomal inhibition by MG132, USP2-41kD overexpression exacerbated cell damage. Only in presence of proteasomal inhibition by MG132, USP2-41kD overexpression exacerbated cell damage.

NF-κB is an important transcription factor and positive regulator of c-Flip expression (44). Our findings regarding enhanced NF-κB basal activation and mRNA transcription in siUSP2-treated hepatocytes indicate that the number of USP2 substrates includes negative regulators of NF-κB activation or p65 processing.

Although USP2 overexpression prevented the formation of ubiquitinated Itch, it cannot be concluded from our experiments whether USP2-41kD directly interacted with Itch protein, or USP2 action involves an upstream modulator or signaling event or an additional Itch-enzyme modulator. However, as summarized in Fig. 8 USP2 seems to act as a constitutively active pro-apoptotic factor in the liver, which limits NF-κB basal activity and c-Flip protein levels. Its rapid down-regulation by TNFα enables a fast adaptation of hepatocytes to environmental inflammatory stimuli. Therefore, USP2 depletion in liver parenchyma enhances cytoprotective NF-κB signaling, c-Flip expression, and Itch degradation and initiates a fast desensitization against TNFα-induced apoptosis. Hence, the USP2-41kD enzyme is a potential target for the discovery of a new class of therapeutic drugs for treatment of apoptotic liver diseases (56).

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