Tumour necrosis factor α (TNF)–TNF receptor 1-inducible cytoprotective proteins in the mouse liver: relevance of suppressors of cytokine signalling

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TNF (tumour necrosis factor α) induces tolerance towards itself in experimental liver injury. Tolerance induction has been shown to be dependent on TNFR1 (TNF receptor 1) signalling, but mechanisms and mediators of TNF-induced hepatic tolerance are unknown. We investigated the TNF-inducible gene-expression profile in livers of TNFR2^{-/-} mice, using cDNA array technology. We found that, out of 793 investigated genes involved in inflammation, cell cycle and signal transduction, 282 were expressed in the mouse liver in response to TNF via TNFR1. Among those, expression of 78 genes was induced, while expression of 60 genes was reduced. We investigated further the cellular expression of the 27 most prominently induced genes, and found that 20 of these

genes were up-regulated directly in parenchymal liver cells, representing potentially protective proteins and possible mediators of TNF tolerance. *In vitro* experiments revealed that overexpression of SOCS1 (silencer of cytokine signalling 1), a member of the SOCS family of proteins, as well as of HO-1 (haem oxygenase-1), but not of SOCS2 or SOCS3, protected isolated primary mouse hepatocytes from TNF-induced apoptosis. The identification of protective genes in hepatocytes is the prerequisite for future development of gene therapies for immune-mediated liver diseases.

Key words: apoptosis, cytokine, haem oxygenase-1, inflammation, microarray, tumour necrosis factor (TNF).

INTRODUCTION

In mice, induction of apoptotic liver damage by soluble recombinant murine TNF (tumour necrosis factor α), or by TNF-inducing agents, e.g. LPS (lipopolysaccharide), is dependent on transcriptional inhibition [1–3]. A widely used tool to block transcription specifically in hepatocytes is the administration of GalN (D-galactosamine), which depletes the hepatocellular pool of uracil nucleotides [4]. The resulting GalN/TNF-induced liver damage is characterized by the formation of mitochondrial permeability transition pores and release of cytochrome *c*, as well as by the activation of caspases [5–7]. On the other hand, administration of TNF alone, without simultaneous application of GalN, does not induce liver damage, but even protects mice from apoptotic liver injury subsequently induced by GalN/TNF treatment [8,9]. Using TNFR1^{-/-} and TNFR2^{-/-} (TNF receptors 1 and 2) mice, it has been shown that TNF-mediated damage, as well as protection from GalN/TNF-induced liver injury, is mediated by TNFR1, but not by TNFR2 [10–12]. Therefore GalN treatment seems to block the TNF–TNFR1-induced expression of anti-apoptotic proteins.

To investigate alterations in gene expression, microarray technology is a useful and highly specific tool. We used custom-designed cDNA microarrays, containing 793 defined genes, known to play important roles in the cell cycle, signal transduction or inflammation-related processes. We found that, out of 23 selected genes which were significantly up-regulated by TNF–TNFR1 signalling, 18 were expressed in hepatocytes. Those genes represent candidate hepatoprotective factors and maybe include the mediator(s) of TNF tolerance. In an attempt to define actually protective proteins in this group, we investigated the protective potential of three members of the SOCS (silencer of cytokine

signalling) family of genes in comparison with HO-1 [haem oxygenase 1; Hsp32 (heat-shock protein 32)]. Members of the SOCS family have been shown to attenuate cytokine-dependent apoptotic processes *in vitro* in pancreatic β -cells and fibroblasts [13,14] as well as *in vivo* [15]. HO-1 is a known mediator of protection against apoptotic processes in the liver [16]. We found that overexpression of SOCS1 or HO-1, but not of SOCS2 or SOCS3, protected isolated primary mouse hepatocytes from apoptotic damage induced by TNF in combination with the transcriptional inhibitor Act.D (actinomycin D).

EXPERIMENTAL

Animals

C57Bl/6 and C57Bl/6-TNFR2^{-/-} mice [17], kindly provided by Dr H. Bluethmann (Hoffmann-La Roche, Basel, Switzerland), received humane care according to the guidelines of the National Institutes of Health as well as to the legal requirements in Germany. They were maintained under controlled conditions (22°C, 55% humidity, and a 12-h day–night rhythm) and fed a standard laboratory chow.

Dosage and application routes

Recombinant murine TNF (Innogenetics, Brussels, Belgium), dissolved in saline/0.1% (w/v) HSA (human serum albumin), was administered to mice at 10 μ g/kg with or without 30 min pre-treatment with GalN (Roth, Karlsruhe, Germany). GalN was administered intraperitoneally at 700 mg/kg in pyrogen-free saline. For induction of liver damage by GalN/TNF in an 8-h model, TNF was applied at 4.5 μ g/kg.

Abbreviations used: Act.D, actinomycin D; ATF3, activating transcription factor 3; GADD45, growth arrest and DNA-damage-inducible protein of 45 kDa; GalN, D-galactosamine; HO-1, haem oxygenase-1; HRP, horseradish peroxidase; HSA, human serum albumin; Hsp, heat-shock protein; IAP1, inhibitor of apoptosis protein 1; ICAM1, intercellular adhesion molecule 1; IL-6, interleukin 6; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NF- κ B, nuclear factor κ B; RT, reverse transcriptase; SOCS, silencer of cytokine signalling; TNF, tumour necrosis factor α ; TNFR, TNF receptor.

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Analysis of liver enzymes

Hepatocyte damage was assessed 8 h after GalN/TNF administration by measuring plasma enzyme activity of ALT (alanine aminotransferase) [18] using an automated procedure. Cytotoxicity in primary hepatocyte cultures was measured by LDH (lactate dehydrogenase) activity in culture supernatants (S) and the remaining cell monolayer (C) after lysis with 0.1 % (v/v) Triton X-100. The percentage of LDH release was calculated from the ratio of S/(S + C).

Microarray experiments

C57Bl/6-TNFR2^{-/-} mice were treated with TNF dissolved in HSA or with HSA alone. At 1 h after treatment, mice were killed, and isolation of total RNA from liver tissue was carried out using the NucleoSpin[®] RNA II Kit (Macherey-Nagel, Düren, Germany). For each of three independent cDNA arrays, three mice per treatment group were used. Qualitative integrity tests of purified total RNA and quantitative measurements were performed by capillary electrophoresis using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, U.S.A.) and a spectrometer. Microarray production, sample preparation, hybridization, scanning and data analysis were performed by Memorec Biotec GmbH (Köln, Germany) according to the MIAME (Minimum Information About a Microarray Experiment) guidelines. Exact procedures have been described previously [19]. For array layout and complete data, see the supplementary data at <http://www.BiochemJ.org/bj/385/bj3850537add.htm> and the Memorec Biotec GmbH homepage at <http://www.memorec.com/>.

Detection of mRNA by RT (reverse transcriptase)-PCR

To verify altered gene expression detected by microarrays, mRNA was transcribed into cDNA using SuperScript[™] II RNaseH RT (Invitrogen GmbH, Karlsruhe, Germany). Oligonucleotides for subsequent PCRs were also obtained from Invitrogen (Table 1). Real-time RT-PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics GmbH, Mannheim, Germany) and the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics). Reactions were performed in a 10 µl volume. To confirm amplification specificity, PCR products were subjected to a melting-curve analysis and gel electrophoresis.

Cloning and sequencing

Full-length expression clones were obtained using the pcDNA3.1/V5-His-TOPO[®] TA Expression Kit (Invitrogen) in combination with the AccuPrime[™] Taq Polymerase System (Invitrogen). Sequence analysis for all clones was carried out using the ABI PRISM[™] 377 DNA sequencer (Applied Biosystems, Weiterstadt, Germany). Each expression clone contained the V5-tag following the overexpressed protein in the same reading frame. The V5-tag was used to detect the overexpressed recombinant proteins in Western blot analysis.

Primary hepatocyte cultures and transfection

Primary mouse hepatocytes were isolated by the two-step collagenase perfusion method of Seglen [20]. Cells were plated in 200 µl of RPMI 1640 medium containing 10 % (v/v) FCS (foetal calf serum; Invitrogen) in 48-well plates at 5 × 10⁴ cells/well. Cells were allowed to adhere to culture plates overnight. Transfection was performed using LIPOFECTAMINE[™] 2000 (Invitrogen), according to the manufacturer's instructions. For induction of cellular damage, 80 nM Act.D (Sigma Chemical Co., Taufkirchen, Germany) was added 30 min before TNF (at 40 ng/ml).

Table 1 Primer sequences for real-time RT-PCR

Gene	Sequence	Product size (bp)	Accession No.
<i>β-Actin</i>	5'-TGG AAT CCT GTG GCA TCC ATG AAA-3' 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'	347	X03765
<i>ATF3</i>	5'-GCA GAG CTA AGC AGA GGT G-3' 5'-TCC TGG AAT TGG TGA GAC AAA G-3'	233	U19118
<i>BCL10</i>	5'-GAG AGC ATC CAC TGT CAT G-3' 5'-GGA GAA ACA TCT CAC TTG AG-3'	229	NM_009740
<i>BCL2A1</i>	5'-CTT CCA CAA GAG CAG ATT GC-3' 5'-CCA GAT CTG TCC TGT CAT C-3'	188	NM_001058
<i>BCL3</i>	5'-CAA CAT CTA CAC CTG CCT TC-3' 5'-CAG TAG TAT GTG CAT GTG CC-3'	222	AF067774
<i>CCL9</i>	5'-CTC ACA ACC ACG GAC CTAC-3' 5'-TGT GTA GTC ATT GAG GTC AGC-3'	262	NM_011338
<i>CD14</i>	5'-CCA GTC AGC TAA ACT CGC TC-3' 5'-ATC TCC TAG GAG CAA AGC C-3'	290	BC057889
<i>CD83</i>	5'-CCA GTT ACC TCC CCA AGC-3' 5'-AGG AGG TTG ACC AGA TAG C-3'	309	NM_009856
<i>CXCL9</i>	5'-CTT GAG CCT AGT CGT GAT AAC-3' 5'-CCA GCT TGG TGA GGT GTG CC-3'	187	BC003343
<i>CXCL10</i>	5'-CCA CGT GTT GAG ATC ATT GC-3' 5'-AGT AGC AGC TGA TGT GAC C-3'	276	BC030067
<i>GADD45</i>	5'-CTG GAG GAA GTG CTC AGC-3' 5'-CAT TGT GAT GAA TGT GGG TTC-3'	337	BC011141
<i>H0-1</i>	5'-GAG ATA GAG CGC AAC AAG CAG-3' 5'-CTT GAC CTC AGG TGT CAT CTC-3'	398	NM_010177
<i>Hsp70.1</i>	5'-TTC GAC ATC GAC GCC AAC-3' 5'-CAC CTT CTT CTT GTC AGC C-3'	278	M35021
<i>IAP1</i>	5'-GGA CCT GGA GAT AGA GTG G-3' 5'-ACA CCT TGG AAA CCA CTT GG-3'	365	NM_007464
<i>ICAM-1</i>	5'-GCT CGG AGG ATC ACA AAC G-3' 5'-GAG TGG TAC AGT ACT GTC AG-3'	354	M31585
<i>IKBA</i>	5'-CAG TGT AGC AGT CTT GAC GC-3' 5'-CCC ACA CTT CAA CAG GAG C-3'	246	BC046754
<i>NFKB1</i>	5'-CTC ATG GAC AAC TAT GAG GTC-3' 5'-TGC GGA AGG ATG TCT CCA C-3'	252	NM_008689
<i>NFKB2</i>	5'-GGT TGA GAA GCC TGG TGG-3' 5'-TCT GTG CTG GGC AGC TTG-3'	149	NM_019408
<i>NFKB3</i>	5'-CTG GCA TCT GTG GAC AAC-3' 5'-TTA GGA GCT GAT CTG ACT C-3'	257	M61909
<i>NRF2</i>	5'-GTC CAA GGA GCA ATT CAA TG-3' 5'-TTT CAC ATC ACA GTA GGA AG-3'	436	BC026943
<i>RELB</i>	5'-GAT CAT GAC AGC TAC GGT G-3' 5'-GGC AAA GCC ATC GTC CAG-3'	272	M83380
<i>SCYB1</i>	5'-GTG TCA ACC ACT GTG CTA G-3' 5'-AAG GGA AGC GTC AAC ACG-3'	199	NM_008176
<i>SELE</i>	5'-GGT AGT TGC ACT TTC TGC GG-3' 5'-GCA GCA TGT TCT TCA TAC TG-3'	382	M87862
<i>SOCS1</i>	5'-CCT GGG TCG GAG GGA GTG-3' 5'-TGG TTT GTG CAA AGA TAC T-3'	207	NM_009896
<i>SOCS2</i>	5'-GAG CTC AGT CAA ACA GGA TG-3' 5'-CGC AGT CGA CAG AAA TGC TG-3'	385	NM_007706
<i>SOCS3</i>	5'-GCT TCG ACT GTG TAC TCA AG-3' 5'-GGA GCA TCA TAC TGA TCC AG-3'	324	NM_007707
<i>TNFSF6</i>	5'-CGC TGT TTT CCC TTG CTG CA-3' 5'-ACT GAG GTA GTT TTC ACT CCA-3'	900	NM_007987
<i>VCAM1</i>	5'-CAA GGG TGA CCA GCT CAT GAA-3' 5'-TGT GCA GCC ACC TGA GAT CC-3'	518	BC029823

Western blot analysis

Livers were homogenized in lysis buffer containing 0.5 % (v/v) NP40 (Nonidet P40), 137 mM NaCl, 2 mM EDTA, 50 mM Tris/HCl, pH 8.0, 10 % (v/v) glycerol. Following centrifugation at 20 000 g for 10 min at 4 °C, supernatants were stored at -80 °C. For Western blot analysis, 10 µg of protein was fractionated by SDS/10 % PAGE and blotted on to a nitrocellulose membrane. Western Blots were developed using an ECL[®] (enhanced chemiluminescence) system (Amersham Biosciences, Freiburg,

Germany), according to the manufacturer's instructions. Semi-quantitative evaluation was performed using the Gel Doc 2000 System (Bio-Rad Laboratories GmbH, München, Germany).

Antibodies

For detection of SOCS proteins, and β -actin, antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.): SOCS1, sc-7005; SOCS2, sc-7007; SOCS3, sc-7009; and β -actin, sc-1615. The anti-HO-1 antibody was purchased from Stressgen Biotechnologies (SPA-896; San Diego, CA, U.S.A.). The secondary antibody for Western blots was HRP (horseradish peroxidase)-conjugated rabbit anti-goat (Dianova, Hamburg, Germany), except for HO-1, where it was HRP-conjugated goat anti-rabbit (Dianova) and streptavidin–peroxidase (Boehringer, Mannheim, Germany). To detect overexpressed recombinant proteins in primary hepatocyte cultures, the anti-V5 antibody (Invitrogen) was used.

Determination of caspase 3 activity

Caspase 3 activity in transfected primary mouse hepatocytes was determined using the colorimetric caspase 3 assay kit (Sigma Chemical Co.), according to the manufacturer's instructions. Caspase 3 activity was detected 6 h after Act.D/TNF exposure.

Statistical analysis

The results were analysed using Student's *t* test if two groups were compared, and Dunnett's test if more groups were tested against a control group. If variances were inhomogeneous in Student's *t* test, the results were analysed using the Welsh test. All data in the present study are expressed as means $\pm$ S.E.M. $P \leq 0.05$ was considered significant.

RESULTS

TNF–TNFR1-induced protection from GalN/TNF-induced liver damage is present from 2 h after TNF pre-treatment

Pre-treatment with TNF protects mice against liver damage subsequently induced by, e.g., GalN/TNF. The underlying difference is hepatocellular transcriptional inhibition by GalN, meaning that some TNF-inducible genes must be able to protect hepatocytes against TNF-induced apoptosis. It has been shown previously that protection by TNF pre-treatment is mediated via TNFR1 [12]. In the present study, we show that TNF evokes significant protection from GalN/TNF challenge in TNFR2^{−/−} mice as soon as 2 h after pre-treatment, but protection is also inducible by longer pre-treatment with TNF (Figure 1).

TNF–TNFR1-induced gene expression in the mouse liver

In order to elucidate the nature of TNF-induced protective genes, we performed cDNA microarrays using livers of TNFR2^{−/−} mice, treated with either TNF or its solvent HSA for 1 h. This time point was chosen because 2 h of pre-treatment with TNF already resulted in full protection from liver damage (Figure 1). Microarrays were performed according to the MIAME guidelines. The full array layout and complete data are provided in the supplementary data at <http://www.BiochemJ.org/bj/385/bj3850537add.htm>. For each of three independent arrays, livers of three mice per treatment group, i.e. TNF or HSA, were pooled. Data analysis showed that, out of 793 investigated genes involved in cell cycle, inflammation and cellular signalling, 282 were expressed in the mouse liver using TNFR1. Of these genes, 78 were up-regulated and 60 were repressed, while the majority of genes was unaffected. To verify cDNA array data, we performed

Figure 1 Time course of TNF-induced tolerance against GalN/TNF-induced liver damage

C57Bl/6-TNFR2^{−/−} mice were treated with TNF for 1, 2, 4 or 12 h before the onset of liver injury by the administration of TNF in combination with GalN. The severity of liver injury was measured by determination of ALT (alanine aminotransferase) 8 h after the onset of liver injury. Results are means $\pm$ S.E.M. ($n = 4$; * $P \leq 0.05$ compared with HSA-pre-treated control mice). U/L, units/litre.

Table 2 TNF–TNFR1-inducible genes in the mouse liver

The Table includes genes found by microarray experiments to be > 2 -fold inducible by TNF–TNFR1 interaction in the livers of mice. Induction of all genes shown was verified by real-time RT-PCR. STAT, signal transduction and activators of transcription.

Gene inducible > 2 -fold via TNF–TNFR1 interaction	Expression in hepatocytes
<i>ATF3</i> : cAMP-dependent transcription factor ATF-3	Yes
<i>BCL10</i> : (<i>CIPER</i> ; <i>CLAP</i> ; <i>ME10</i>)	Yes
<i>BCL2A1</i> : (<i>BFL1</i> ; <i>GRS</i> ; <i>BCL2L5</i>) Bcl-2-related protein A1	No
<i>BCL3</i> : B-cell lymphoma 3-encoded protein	No
<i>CCL9</i> : (<i>SCYA9</i> ; <i>SCYA10</i> ; <i>MRP2</i>) small inducible cytokine A9 precursor	Yes
<i>CD14</i> : monocyte differentiation antigen CD14 precursor (LPS receptor)	Yes
<i>CD83</i> : antigen precursor (cell-surface protein HB15) (B-cell activation protein)	No
<i>CXCL10</i> : (<i>SCYB10</i> ; <i>INP10</i>) small inducible cytokine B10 precursor	Yes
<i>CXCL9</i> : (<i>MIG</i>) interferon- γ -induced monokine precursor	Yes
<i>GADD45</i> : (<i>GADD45A</i> ; <i>DDIT1</i>) growth arrest and DNA-damage-inducible protein	Yes
<i>HO-1</i> : (<i>HMOX1</i>) haem oxygenase 1	Yes
<i>Hsp70.1</i> : (<i>HSPA1A</i> ; <i>HSPA1</i> ; <i>HSPA1B</i>) heat-shock 70 kDa protein 1	Yes
<i>IAP1</i> : (<i>BIRC2</i> ; <i>API1</i> ; <i>MIHC</i>) baculoviral IAP repeat-containing protein 1	Yes
<i>ICAM1</i> : intercellular adhesion molecule 1	Yes
<i>IKBA</i> : (<i>NFKBIA</i> ; <i>NFKBI</i> ; <i>MAD3</i>) major histocompatibility complex enhancer-binding protein	Yes
<i>NFKB1</i> : nuclear factor NF- κ B p105 subunit	Yes
<i>NFKB2</i> : nuclear factor NF- κ B p100 subunit	Yes
<i>NFKB3</i> : (<i>RELA</i> or <i>NFKB3</i>) transcription factor p65 (nuclear factor NF- κ B p65 subunit)	Yes
<i>NRF2</i> : (<i>NFE2L2</i>) nuclear factor erythroid 2-related factor 2	No
<i>REL</i> : transcription factor RelB (<i>I-REL</i>)	Yes
<i>SCYB1_MOUSE</i> : (<i>GRO1</i> ; <i>GRO</i> ; <i>MGSA</i>) growth-regulated protein precursor	No
<i>SELE</i> : (<i>ELAM1</i>) E-selectin precursor	No
<i>SOCS1</i> : (<i>SSI1</i> ; <i>TIP3</i> ; <i>JAB</i> ; <i>CISH1</i>) suppressor of cytokine signalling-1	Yes
<i>SOCS2</i> : suppressor of cytokine signalling-2	Yes
<i>SOCS3</i> : STAT-induced STAT inhibitor-3 (protein EF-10)	Yes
<i>TNFRSF6</i> : (<i>APT1</i> ; <i>FAS</i>) FasL receptor precursor	Yes
<i>VCAM1</i> : (<i>L1CAM</i>) vascular cell-adhesion protein 1 precursor (CD106 antigen) (<i>INCAM-100</i>)	No

real-time RT-PCR analysis on the 27 most prominently induced genes, which were selected according to their significance and reproducibility of induction on the arrays. In a time-course experiment, TNF-inducible expression could be detected for all investigated genes (Figure 2, and results not shown; Table 2). For

Figure 2 Time course of gene expression following TNF exposure

C57Bl/6 mice were treated with TNF for 30 min, 1 h, 2 h or 4 h. Real-time RT-PCR was performed on cDNAs obtained from whole-liver homogenates. Results are means $\pm$ S.E.M. ($n = 4$; $*P \leq 0.05$ compared with HSA-treated control mice).

most genes investigated, gene expression maxima varied 1–2 h after TNF exposure.

Hepatocellular expression of potentially cytoprotective proteins

To decide which of the investigated 27 TNF–TNFR1-inducible genes might play a role in cytoprotection of the liver, expression of these genes in hepatocytes was investigated. Therefore mice were treated with TNF, in combination with the hepatocyte-specific transcriptional inhibitor GalN for 1 h, and gene induction was determined by real-time RT-PCR. Subsequently, the data

obtained were compared with gene induction after 1 h of TNF treatment alone. Significantly reduced gene expression in the presence of GalN revealed those genes which were expressed by hepatocytes and should include candidate hepatocyte protective factors. We found that, out of the 27 selected genes, 20 were expressed by hepatocytes (Figure 3 and Table 2). Expression of seven genes, i.e. *BCL2A1*, *BCL3*, *CD83*, *NRF2*, *SCYB1*, *SELE* and *VCAM1* (vascular cell-adhesion molecule 1) was not significantly altered by GalN administration, indicating that these genes are TNF-inducible in non-parenchymal liver cells.

Figure 3 Gene expression following transcriptional inhibition in hepatocytes

C57Bl/6 mice were treated with TNF with or without pre-treatment with GalN. Real-time RT-PCR was performed on cDNAs obtained from whole-liver homogenates. Results are means $\pm$ S.E.M. ($n = 4$; * $P \leq 0.05$ for TNF-compared with GalN/TNF-treated mice).

Protein expression of SOCS1, SOCS2, SOCS3 and HO-1 following TNF–TNFR1 interaction

For four of the genes which were, according to gene-expression data, inducible by TNF–TNFR1 interaction in hepatocytes, i.e. *SOCS1*, *SOCS2*, *SOCS3* and *HO-1*, protein expression was determined. Figure 4 shows that all proteins were inducible in livers of mice treated with TNF for 4 or 12 h (Figures 4A–4D). Protein expression increased over time, except for *SOCS1*, which was more prominently expressed after 4 h than after 12 h (Figure 4A). Co-treatment with the hepatocyte-specific transcrip-

tional inhibitor GalN and TNF for 4 h abolished protein expression in all cases, reconfirming that all investigated proteins were expressed in hepatocytes (Figures 4E–4H).

SOCS1 and HO-1 protect primary mouse hepatocytes from TNF-induced apoptosis

We investigated further the protective potential of the SOCS proteins, as well as of *HO-1*. These genes were cloned into expression vectors containing the in-frame V5-tag, sequence analysis was

Figure 4 Protein expression of *SOCS1*, *SOCS2*, *SOCS3* and *HO-1* in livers and hepatocytes

C57Bl/6 mice were treated with TNF or solvent for 4 h with or without pre-treatment with GalN, or for 12 h with TNF alone. Protein expression in whole-liver homogenates was measured by Western blot analysis and semiquantitative evaluation was performed. Results are means $\pm$ S.E.M. ($n=3$; * $P \leq 0.05$ for TNF-compared with solvent-treated mice; # $P \leq 0.05$ for TNF-compared with GalN/TNF-treated mice). Western blots shown are a representative example of three independent experiments each.

performed, and expression of recombinant proteins in transfected primary mouse hepatocytes was confirmed by Western blot analysis (Figure 5A). We found that all proteins were expressed to almost the same degree. Overexpression of *SOCS1*, as well as of *HO-1*, but not of *SOCS2* or *SOCS3*, protected primary mouse hepatocytes from TNF-induced apoptosis, as determined by measurement of LDH (results not shown) and activated caspase 3 in cell lysates (Figure 5B).

DISCUSSION

In a large variety of experimental models of immune-mediated liver diseases, TNF is a key mediator of liver damage [3]. Direct injection of TNF into mice sensitized with the hepatocyte-specific transcriptional inhibitor GalN results in apoptotic liver damage. In contrast, the same dose of TNF injected into non-sensitized mice does not cause liver injury [8]. Moreover,

Figure 5 SOCS1 and HO-1 protect against TNF-induced cellular damage *in vitro*

Primary mouse hepatocytes were transiently transfected with SOCS1-, SOCS2-, SOCS3- or HO-1-encoding eukaryotic expression clones. (A) Western blot analysis of protein expression 24 h after transfection. For detection of proteins, an anti-V5 antibody was used, which recognizes a V5 tag, expressed in-frame with all recombinant proteins. (B) At 24 h after transfection, cellular damage was induced by Act.D/TNF exposure. Activation of the effector caspase 3 was measured 6 h later and is given as the x-fold induction, compared with transfected, but non-Act.D/TNF-treated control cells. Results are means $\pm$ S.E.M. ($n = 8$ wells; * $P \leq 0.05$ for untreated compared with Act.D/TNF-treated cells).

pre-treatment with low doses of TNF protects mice from liver damage, subsequently induced by GalN/TNF [8]. These observations indicate that transcriptional inhibition by GalN treatment interferes with the expression of TNF-inducible, liver-protective genes.

To detect TNF-inducible hepatoprotective proteins, we first investigated the TNF-dependent expression profile in mouse liver. Since TNF is known to be involved in numerous processes that do not contribute to protection, and tolerance has been shown to be mediated by TNFR1 [12], we tried to exclude irrelevant genes, e.g. by investigating exclusively the expression profile of TNFR1, using TNFR2^{-/-} mice. Among the 282 TNF–TNFR1-dependent genes that we found to be expressed in the liver, the majority, i.e. 144 genes, were not regulated. Of the remaining 138 regulated genes, 78 were up- and 60 were down-regulated. Down-regulation of genes by TNF treatment might contribute to hepatoprotection as well, but in the present study we focused on genes up-regulated specifically in hepatocytes, since those could mediate TNF-induced tolerance. We identified 20 genes, i.e. *ATF3* (activating transcription factor 3), *BCL10*, *CCL9*, *CD14*, *CXCL9*, *CXCL10*, *GADD45* (growth arrest and DNA-damage-inducible protein of 45 kDa), *HO-1*, *Hsp70.1*, *IAP1* (inhibitor of apoptosis protein 1), *ICAM1* (intercellular adhesion molecule 1), *IKBA*, *NFKB* subunits p105, p100 and p65, *RELB*, *SOCS1*, *SOCS2*, *SOCS3* and *TNFSF6*, which were expressed directly in hepatocytes. TNFSF6 (Fas; CD95; Apo-1) and BCL10 could be excluded as mediators of protection, since they both are key factors in apoptotic processes [21,22]. Concerning the chemo-

kines CCL9, CXCL9 and CXCL10, there are so far no reports connecting them with anti-apoptotic processes in the liver. Since those factors are released by hepatocytes to attract immune cells to specific sites within the liver [23], and liver-damaging processes are mediated by subsequently released cytokines [24], a liver-protective role of chemokines is questionable. Likewise, ICAM1, as an adhesion molecule for immune cells, is not likely to mediate protection from apoptotic liver damage. On the other hand, induction of NF- κ B (nuclear factor κ B) expression in hepatocytes, as seen here for NF- κ B1, NF- κ B2 and NF- κ B3, as well as RELB, underlines previous observations that NF- κ B is a major factor in TNF-induced tolerance induction [25,26]. In addition to NF- κ B, ATF3, a transcriptional repressor and member of the ATF/CREB (cAMP-response-element-binding protein) family of transcription factors [27], was induced. Overexpression of ATF, which occurs in response to a wide range of stress stimuli, has been shown to protect from TNF-induced apoptosis *in vitro*, possibly by interfering with the expression of the tumour suppressor p53 gene [28]. Other possible candidates for hepatoprotection might be IAP1 (inhibitor of apoptosis protein 1), GADD45, CD14 and Hsp70.1. For IAP1, it has been shown *in vitro* that overexpression protects leukaemic cells from TNF-induced apoptosis upstream of caspase 8 activation and a second apoptotic event, the loss of the mitochondrial membrane potential [29]. GADD45 has been shown to be induced by TNF via NF- κ B and interferes with the pro-apoptotic, TNF-inducible JNK (c-Jun kinase) pathway by an as yet unknown mechanism [30,31]. In the present study, we could show that CD14, a receptor for LPS that is mainly expressed on immune cells [32], is also induced in hepatocytes following TNF stimulation of TNFR1. CD14 has been shown to be involved in NF- κ B activation and clearance of apoptotic cells [32]. Its expression in hepatocytes might therefore point to its involvement in hepatocellular protection. Likewise, overexpression of Hsp70.1, a member of the Hsp70 family of proteins, seems to protect cells from stress-induced cell death, although the mechanism of protection is unknown [33]. We also found three members of the SOCS family of genes [34,35] to be TNF-inducible in hepatocytes. SOCS proteins repress signalling cascades of their inducing and other cytokines by a negative-feedback loop [35]. It has been shown previously, that SOCS expression is dependent on IL-6 (interleukin 6), since IL-6^{-/-} mice do not express SOCS in response to TNF [36]. *In vitro* studies showed that SOCS1 is able to protect pancreatic β -cells or fibroblasts from TNF-induced apoptosis [13,14]. *In vivo*, it has been shown that SOCS1 and SOCS3 seem to co-ordinate both pro- and anti-apoptotic cytokine production, and therefore might have influence on the severity of cytokine-dependent hepatitis [37]. Using primary mouse hepatocytes, we found that overexpression of SOCS1 seems to prevent TNF-induced cellular damage, while overexpression of SOCS2 or SOCS3 does not. But although SOCS1 seems to play a beneficial role in TNF-dependent apoptotic processes, it is not likely to represent the direct cause of TNF-induced tolerance, since expression of SOCS proteins is strictly dependent on IL-6 [36] and tolerance can be achieved in IL-6^{-/-} mice [38]. Likewise, HO-1, which we also found to be induced in hepatocytes via TNFR1, has been shown to exert anti-apoptotic properties *in vitro* as well as *in vivo* [39,40], but does not seem to be a direct mediator of TNF-induced tolerance [12]. TNF-induced tolerance therefore might very well be a concerted action of a number of cytoprotective proteins.

In summary, we were able to identify SOCS1 as a hepatoprotective and anti-apoptotic protein. To elucidate the nature of the mediators of TNF-induced tolerance, further investigation will be necessary. Our work suggests several potential liver-protective genes, which, e.g., overexpressed *in vivo* by recombinant

adenoviral or retroviral vectors, would represent powerful therapeutic tools against immune-mediated liver injury.

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