JLB

Hepatitis C virus (HCV)-induced suppressor of cytokine signaling (SOCS) 3 regulates proinflammatory TNF-α responses

Aideen S. Collins,[*,1] Suaad Ahmed,[*,1] Silvia Napoletano,[*] Martina Schroeder,[†]
James A. Johnston,[‡] John E. Hegarty,[§] Cliona O'Farrelly,[*,‖,2] and Nigel J. Stevenson[*,2,3]

*School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, and ‖School of Medicine, Trinity College Dublin, Ireland; †Institute of Immunology, National University of Ireland, Maynooth, Ireland; ‡Centre for Infection and Immunity, School of Medicine, Dentistry and Biomedical Science, Queen's University of Belfast, Northern Ireland; and §National Liver Transplant Unit, St. Vincent's University Hospital, Dublin, Ireland

ABSTRACT

TNF-α is a proinflammatory cytokine, dramatically elevated during pathogenic infection and often responsible for inflammation-induced disease pathology. SOCS proteins are inhibitors of cytokine signaling and regulators of inflammation. In this study, we found that both SOCS1 and SOCS3 were transiently induced by TNF-α and negatively regulate its NF-κB-mediated signal transduction. We discovered that PBMCs from HCV-infected patients have elevated endogenous SOCS3 expression but less TNF-α-mediated IκB degradation and proinflammatory cytokine production than healthy controls. HCV protein expression in Huh7 hepatocytes also induced SOCS3 and directly inhibited TNF-α-mediated IL-8 production. Furthermore, we found that SOCS3 associates with TRAF2 and inhibits TRAF2-mediated NF-κB promoter activity, suggesting a mechanism by which SOCS3 inhibits TNF-α-mediated signaling. These results demonstrate a role for SOCS3 in regulating proinflammatory TNF-α signal transduction and reveal a novel immune-modulatory mechanism by which HCV suppresses inflammatory responses in primary immune cells and hepatocytes, perhaps explaining mild pathology often associated with acute HCV infection. *J. Leukoc. Biol.* **96:** 255–263; 2014.

Introduction

TNF-α is an important proinflammatory cytokine produced in response to infection. Excess levels of TNF-α are implicated in a range of inflammatory conditions, including rheumatoid arthritis, septic shock, inflammatory bowel disease, and viral hepatitis [1–3]. A key role for TNF-α in the pathology associated with chronic inflammation is emphasized by successful targeting of arthritis and inflammatory bowel disease with therapeutic anti-TNF-α [4]. The effects of TNF-α are mediated through TNFR1 and TNFR2, leading to proliferation, inflammation, and apoptosis [5]. Upon receptor binding, TNF-α signals through a variety of cytosolic proteins, including TRADD [6] and TRAF2 [7], leading to IκB degradation and the subsequent release and nuclear translocation of NF-κB. Binding of NF-κB to gene promoters initiates transcription of numerous proinflammatory cytokines, including IL-6, IL-8, TNF-α, and CXCL-10 [8, 9]. Interestingly, CXCL-10 can also be induced by TNF-α via the MAPK pathway [10]. TNF-α signaling is known to be regulated by the deubiquitinating enzyme, A20, which removes Lys-63-linked, nondegradative ubiquitin chains from the receptor-interacting protein and promotes its Lys-48-linked polyubiquitination, thus targeting it for proteasomal degradation [11]. However, unchecked TNF-α signaling results in chronic inflammation, cell death, and tumorigenesis, as demonstrated in A20-deficient mice [12]. Whereas ubiquitination is a critical regulatory mechanism of the TNF-α pathway, it is likely that this essential proinflammatory cytokine pathway is controlled by other unknown mechanisms to ensure an appropriate response to infection.

SOCS, a family of eight inhibitory intracellular proteins (CIS and SOCS1–7), are rapidly induced in response to numerous cytokines and microbial products, including IFN-γ, growth hormone, IL-2, fMLP, and LPS [13–15]. SOCS act in a negative-feedback loop to regulate inflammatory responses and were

discovered initially as inhibitors of the JAK/STAT pathway but are increasingly believed to regulate other pathways [16, 17].

Many patients (50–85%) fail to clear HCV, resulting in chronic infection [18]. Acute HCV disease progression is mediated by the expression of proinflammatory cytokines, such as IL-8, IL-6, and TNF-α; however, significant pathology is uncommon in these patients, meaning that infection often remains undetected for many years [19, 20]. The mechanism by which pathology is limited during HCV infection is unknown. A role for SOCS proteins during HCV infection has been suggested [21–24], which led us to investigate whether HCV-induced SOCS could regulate proinflammatory cytokine induction, thus contributing to the mild pathology associated with HCV infection [25].

In this study, we demonstrate that SOCS1 and SOCS3 are induced by TNF-α and inhibit its NF-κB signal transduction. We also found that SOCS3 levels are elevated in PBMCs from HCV-infected patients, which may be responsible for the observed reduction in TNF-α-mediated IκB degradation and proinflammatory cytokine production. Furthermore, HCV induced SOCS3 in Huh7 hepatocytes, which directly inhibited TNF-α-induced, proinflammatory IL-8 expression, possibly by blocking the NF-κB pathway through direct association with TRAF2. These findings suggest a novel mechanism by which HCV suppresses inflammatory responses, possibly explaining the mild pathology often associated with acute HCV infection.

MATERIALS AND METHODS

Patient samples

Twenty-one patients attending the HCV out-patient clinic at St. Vincent's University Hospital (Dublin, Ireland) were recruited for this study. All were infected with genotype 1 HCV, and no one had undergone IFN-α/ribavirin therapy. Detailed clinical information is provided in Supplemental Tables 1–3. Written, informed consent was obtained from each patient, and the study received ethical approval from the local Research and Ethics Committee at St. Vincent's University Hospital, in accordance with the guidelines of the 1975 Declaration of Helsinki. All patients tested positive for antibodies to HCV, using a third-generation enzyme immunoassay (Abbott Diagnostics, Lake Forest, IL, USA) and a third-generation recombinant immunoblot assay-3 (Chiron, Emeryville, CA, USA). Control blood was obtained from healthy volunteers.

PBMC isolation

Whole blood (30 ml) was obtained from each patient and control. PBMCs were isolated by density gradient centrifugation at 1580 g for 5 min over Ficoll (Amersham, Amersham, UK). The cells were then washed twice at 290 g for 5 min with RPMI medium before being resuspended in 1 ml and counted.

Cell culture

MEFs [a kind gift from Professor Aki Yoshimura (Keio University, Japan)], Huh7 cells [a kind gift from Professor Ralf Bartenschlarger (University of Heidelberg, Germany)], and 293T embryonic kidney cells (Invitrogen, San Diego, CA, USA) were grown in DMEM and PBMCs in RPMI, supplemented with 10% FCS, 2 mM L-glutamine, 250 U/ml penicillin, and 250 μg/ml streptomycin and cultured at 37°C in 5% CO_2 and 95% humidity. Cells were treated with 20 ng/ml TNF-α (PeproTech, Rocky Hill, NJ, USA), and SOCS1$^{-/-}$ MEFs were retrovirally infected with SOCS1 pMX-IRES-

EGFP (termed SOCS1+) and SOCS3$^{-/-}$ MEFs with SOCS3 pMX-IRES-EGFP (termed SOCS3+), as described previously [26].

Transfection

Huh7 cells, expressing a T7 polymerase, were transfected with 0.5 μg pBRTM/HCV1-3011 DNA construct (containing the structural and nonstructural proteins of HCV genotype 1b) [27] and a control EV (pBR322) and 0.5 μg shRNA SOCS3 and scrambled control shRNA for 24 h using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cells were then stimulated for 18 h with 20 ng/ml TNF-α (Pepro-Tech), and the supernatant was stored at -20°C until analysis. Levels of IL-8 and CXCL-10 were measured in the cell culture supernatants by ELISA, as directed by the manufacturer (BioLegend, San Diego, CA, USA). Transfection of 293T cells was carried out for 48 h with SOCS1, -2, or -3 constructs (3 μg) using Lipofectamine 2000.

RT-PCR analysis

Total RNA was isolated from cells using Trizol reagent (Invitrogen), following the manufacturer's protocol. RNA yields and quality were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RT-PCR was performed with 1 μg total cell RNA following the OneStep kit protocol (Qiagen, Hilden, Germany). Reactions were incubated for 30 min at 50°C, 95°C for 15 min, and subjected to 35 denaturing (94°C), annealing (58°C), and extending (72°C) cycles for 30 s, followed by a final extension step of 72°C for 10 min. All primers for real-time RT-PCR were designed using Primer3 software (http://primer3.sourceforge.net/) and commercially synthesized (Invitrogen). PCR amplification was performed using primer pairs (see Supplemental Figure 1). The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

For real-time qRT-PCR, 1 μg total RNA from each sample was reverse-transcribed into cDNA with oligo-dT primers using an Omniscript RT Kit, according to the manufacturer's instructions (Qiagen). A total of 40 ng cDNA was used for each real-time RT-PCR reaction. PCR amplification was performed using specific primer pairs (see Supplemental Figure 2). Each reaction was carried out in duplicate. Real-time RT-PCR was performed using a MX3000P qPCR system (Stratagene, La Jolla, CA, USA), with the following cycling parameters: 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s, followed by amplicon dissociation. All gene amplifications for human samples were normalized to ribosomal protein 15. GAPDH was used as the endogenous reference gene for all murine genes analyzed. Data analysis was carried out using the $2^{-\Delta\Delta}$ comparative threshold method [28].

Immunoblot analysis

Cells were harvested in radioimmunoprecipitation assay lysis buffer, supplemented with aprotinin (5 μg/ml), leupeptin (5 μg/ml), PMSF (1 mM), and Na_3VO_4 (1 mM) on ice for 15 min. Extracts were pelleted at 12,000 g at 4°C for 15 min. Total lysates were resolved by PAGE, and protein was transferred from the gel to a polyvinylidene difluoride membrane using semidry transfer. Primary antibodies were diluted 1:1000 in PBS with 5% milk and incubated at 4°C overnight. Primary antibodies SOCS1 (Invitrogen), SOCS2 (New England Biolabs, Ipswich, MA, USA), SOCS3 (Abcam, Cambridge, UK), IκB [a kind gift from Professor Ron Hay (University of Dundee, Scotland, UK)], NS2 [a kind gift from Professor Charles Rice (The Rockefeller University, New York, NY, USA)], β-actin (Sigma, St. Louis, MO, USA), GAPDH (Sigma), and γ-tubulin (Sigma) and infrared dye-labeled (Li-Cor, Lincoln, NE, USA) or HRP-labeled (New England Biolabs) secondary antibodies—goat anti-rabbit and goat anti-mouse—were used for detection. Proteins were analyzed on an Odyssey Infrared Imaging system using near-infrared fluorescence detection. Immunoblots analyzed using the Odyssey system were quantified using Li-Cor software, and immunoblots analyzed using photographic film were quantified using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA).

Flow cytometry

MEFs were surface-labeled with PE-conjugated TNFR or isotype control antibody (BD Biosciences, San Jose, CA, USA). Cells were fixed with 4% paraformaldehyde for 30 min on ice, washed 2× in PBS, and acquired using a Dako Cyan flow cytometer. Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Immunoprecipitation

HEK293T cells were transfected with 1 μg EV or myc-SOCS3 and Flag-TRAF2 for 24 h. Thereafter, cells were lysed in 600 μl lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EDTA, pH 8, 1% Nonidet P-40, and 0.5% sodium deoxycholate, supplemented with 1 mM PMSF, 1 mM dithiothreitol, and 1 mM NaVO₃) and left on ice for 20 min. Extracts were pelleted at 12,000 *g* at 4°C for 15 min. Next, cell lysates were incubated with 1 μg anti-SOCS3 antibody (Abcam), precoupled to 30 μl A/G Plus Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at 4°C with gentle shaking. The immune complexes were precipitated, washed, and subjected to immunoblotting analysis using an anti-myc and anti-Flag antibodies.

Reporter gene assays

HEK293T cells were plated into 96-well plates at a density of 5×10^4 cells/well. After 24 h, cells were cotransfected with vectors encoding the firefly luciferase reporter gene under the control of five NF-κB sites (80 ng/well) and a control Renilla luciferase construct (40 ng/well) and cotransfected with the expression vector, encoding full-length TRAF2 (50 ng/well), and an EV or increasing amounts of an expression vector, encoding full-length myc-SOCS3 (see Fig. 6), using TurboFect (Thermo Scientific), according to the manufacturer's instructions. A total amount of 230 ng/well DNA was kept constant by addition of the EV. After 24 h, medium was replaced, and the cells were cultured for an additional 24 h. Thereafter, reporter gene activity was measured using the dual-luciferase assay system (Promega, Madison, WI, USA). Data were expressed as the mean fold induction relative to the control.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Statistical analyses were performed using the nonparametric Mann-Whitney U-test and paired *t*-tests on data obtained using PBMCs and cell lines, respectively (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$).

RESULTS

TNF-α signaling is regulated by SOCS1 and SOCS3 via a negative-feedback loop

To investigate the regulation of TNF-α signal transduction, the effect of TNF-α on SOCS expression was examined in human PBMCs. We analyzed PBMC mRNA expression of all of the SOCS genes by RT-PCR over a time course of TNF-α stimulation. We found that CIS and SOCS4–7 mRNAs were constitutively expressed in PBMCs and were not up-regulated in response to TNF-α (**Fig. 1A**). However, expression of SOCS1, -2, and -3 mRNAs was transiently increased by TNF-α (Fig. 1B). SOCS1 and -3 protein induction mirrored the mRNA, but SOCS2 protein was constitutively expressed and showed no regulation by TNF-α (Fig. 1C). This immediate and transient up-regulation of SOCS1 and -3 suggests a novel role for these inhibitory proteins in controlling TNF-α signal transduction in immune cells. SOCS are known to regulate the JAK/STAT pathway, but only a few studies have documented their involve-

ment in NF-κB signaling [16, 17, 29]. Therefore, to investigate the effect of SOCS1 and -3 on TNF-α-induced NF-κB activation, we treated WT, SOCS1$^{-/-}$, SOCS3$^{-/-}$, SOCS1+, and SOCS3+ MEFs with TNF-α for 0, 10, and 20 min and measured IκB degradation, which occurred upon TNF-α treatment in WT, SOCS1 (SOCS1$^{-/-}$) and SOCS3 (SOCS3$^{-/-}$) null cells but was inhibited in SOCS1 (SOCS1+)- and SOCS3 (SOCS3+)-expressing cells (Fig. 1D). SOCS1 and -3 levels were confirmed by immunoblotting (Fig. 1E), and flow cytometric analysis found surface TNFR levels to be consistent between WT, SOCS1$^{-/-}$, SOCS3$^{-/-}$, SOCS1+, and SOCS3+ MEFs (Fig. 1F). To confirm further the effect of SOCS1 and -3 on TNF-α-mediated IκB degradation, SOCS1 and -3 constructs were expressed in HEK293T cells before being treated with TNF-α (20 ng/ml, 20 min). Ectopic expression of SOCS1 or -3 reduced TNF-α-mediated IκB degradation significantly compared with EV control cells (Fig. 1G and H), further indicating that SOCS1 and -3 inhibit TNF-α-induced NF-κB signaling. Interestingly, SOCS2 did not inhibit TNF-α-induced IκB degradation (Fig. 1I).

SOCS3 is elevated, and TNF-α-mediated signaling is reduced in PBMCs from HCV+ patients

During acute HCV infection, patients often only suffer mild, nonspecific symptoms, demonstrating the ability of HCV to suppress immune pathology [30]. Having observed that SOCS1 and -3 inhibited TNF-α signal transduction, we wondered if they were involved in suppressing its proinflammatory effect during HCV infection. Therefore, we initially analyzed endogenous SOCS1 and -3 mRNA levels in PBMCs from HCV+ and healthy individuals. We found significantly higher basal levels of SOCS3 mRNA in PBMCs from HCV+ patients, whereas SOCS1 mRNA levels were normal when compared with healthy controls (**Fig. 2A**). SOCS3 mRNA levels did not correlate with viral load (data not shown). Having observed elevated SOCS3 levels in HCV+ PBMCs, we next investigated SOCS3 mRNA and protein regulation following TNF-α stimulation. Interestingly, high basal levels of SOCS3, observed at the mRNA level, were reflected in SOCS3 protein expression (Fig. 2B and C), and SOCS3 mRNA (Fig. 2B) and protein (Fig. 2C) were poorly induced by TNF-α in HCV+ PBMCs compared with healthy controls.

Having observed elevated, endogenous SOCS3 levels in PBMCs of HCV+ patients, we wondered whether this was inhibiting TNF-α-induced NF-κB signaling. Therefore, we next analyzed TNF-α-mediated IκB degradation in PBMCs from HCV+ patients. Whereas IκB was degraded in PBMCs from healthy individuals, TNF-α treatment did not induce IκB degradation in PBMCs from HCV+ patients (Fig. 2D and E), indicating a block in the TNF-α signaling pathway. Taken together, these results strongly suggest that HCV-induced SOCS3 expression inhibits proinflammatory TNF-α signal transduction in primary immune cells.

TNF-α proinflammatory cytokine induction is attenuated in HCV+ PBMCs

As TNF-α signaling was reduced in PBMCs of HCV+ patients, we next investigated whether the loss in signal transduction
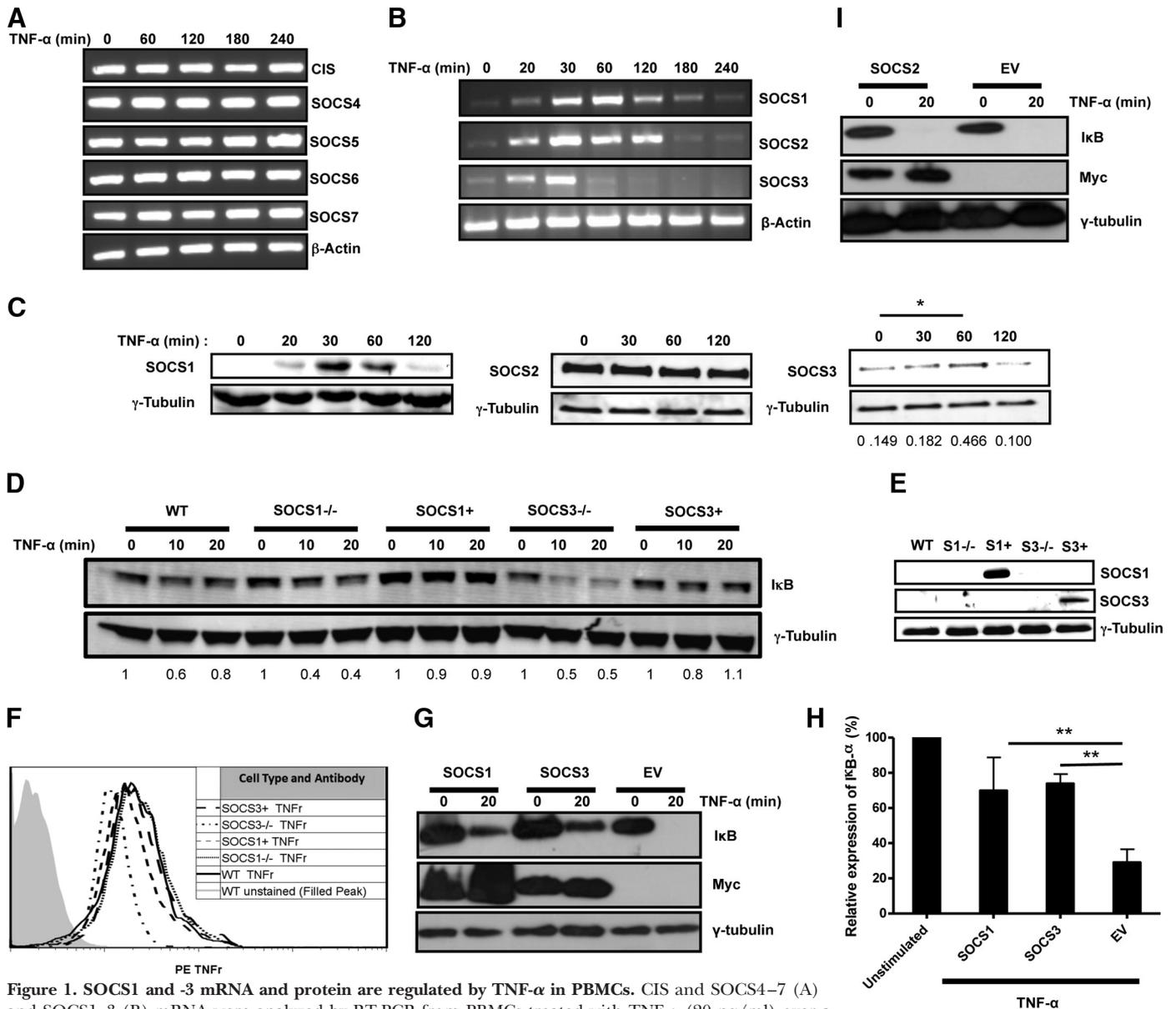
Figure 1. SOCS1 and -3 mRNA and protein are regulated by TNF-α in PBMCs. CIS and SOCS4–7 (A) and SOCS1–3 (B) mRNA were analyzed by RT-PCR from PBMCs treated with TNF-α (20 ng/ml) over a time course of 0–240 min ($n=3$; data are representative of a single sample). Protein lysates from PBMCs treated with TNF-α were analyzed by immunoblotting for SOCS1, -2, and -3 and γ-tubulin, using specific antibodies (C; $n=4$). SOCS1 Null (SOCS1$^{-/-}$) MEFs were retrovirally infected with SOCS1 pMX-IRES-EGFP (SOCS1+), and SOCS3 Null (SOCS3$^{-/-}$) MEFs were retrovirally infected with SOCS3 pMX-IRES-EGFP (SOCS3+). WT, SOCS1$^{-/-}$, SOCS1+, SOCS3$^{-/-}$, and SOCS3+ MEFs were treated ± TNF-α (20 ng/ml, 10 and 20 min) before IκB and γ-tubulin protein levels were quantified by immunoblotting and densitometric analysis (IκB band intensity was normalized to γ-tubulin band intensity, and all values are presented relative to unstimulated control cells, which was normalized to 1; $n=3$; D). SOCS1 (S1) and -3 (S3) protein levels were verified by Western blotting protein lysates using SOCS antibodies and γ-tubulin antibody (E). Flow cytometric analysis of surface TNFR levels of WT, SOCS1$^{-/-}$, SOCS1+, SOCS3$^{-/-}$, and SOCS3+ MEFs stained with TNFR antibody and WT MEFs unstained with antibody ($n=2$; F). Protein lysates from 293T cells, transfected with myc-tagged SOCS1 and -3 constructs and treated ± TNF-α (20 ng/ml, 20 min), were analyzed for IκB and γ-tubulin by immunoblotting ($n=3$; G). Quantification of IκB expression in HEK293T transfected with SOCS1 or -3 or EV (band intensity was normalized to γ-tubulin band intensity, and all values are presented relative to unstimulated control cells, which were normalized to 100%; H). Immunoblot of IκB expression from 293T cells transfected with myc-tagged SOCS2 or EV constructs and treated ± TNF-α (20 ng/ml, 20 min) γ-tubulin was used as a loading control ($n=3$; I).

affected proinflammatory cytokine induction. PBMCs from HCV+ and healthy individuals were treated with TNF-α for 0, 2, and 3 h, before analyzing mRNA of IL-6, TNF-α, IL-8, and CXCL-10. The mRNA level of these cytokines was up-

regulated by TNF-α in PBMCs from healthy individuals. However, while not statistically significant, IL-6 (2 h, $P=0.3555$; 3 h, $P=0.3132$), IL-8 (2 h, $P=0.2366$; 3 h, $P=3.408$), and TNF-α (2 h, $P=0.2502$; 3 h, $P=0.1658$) had
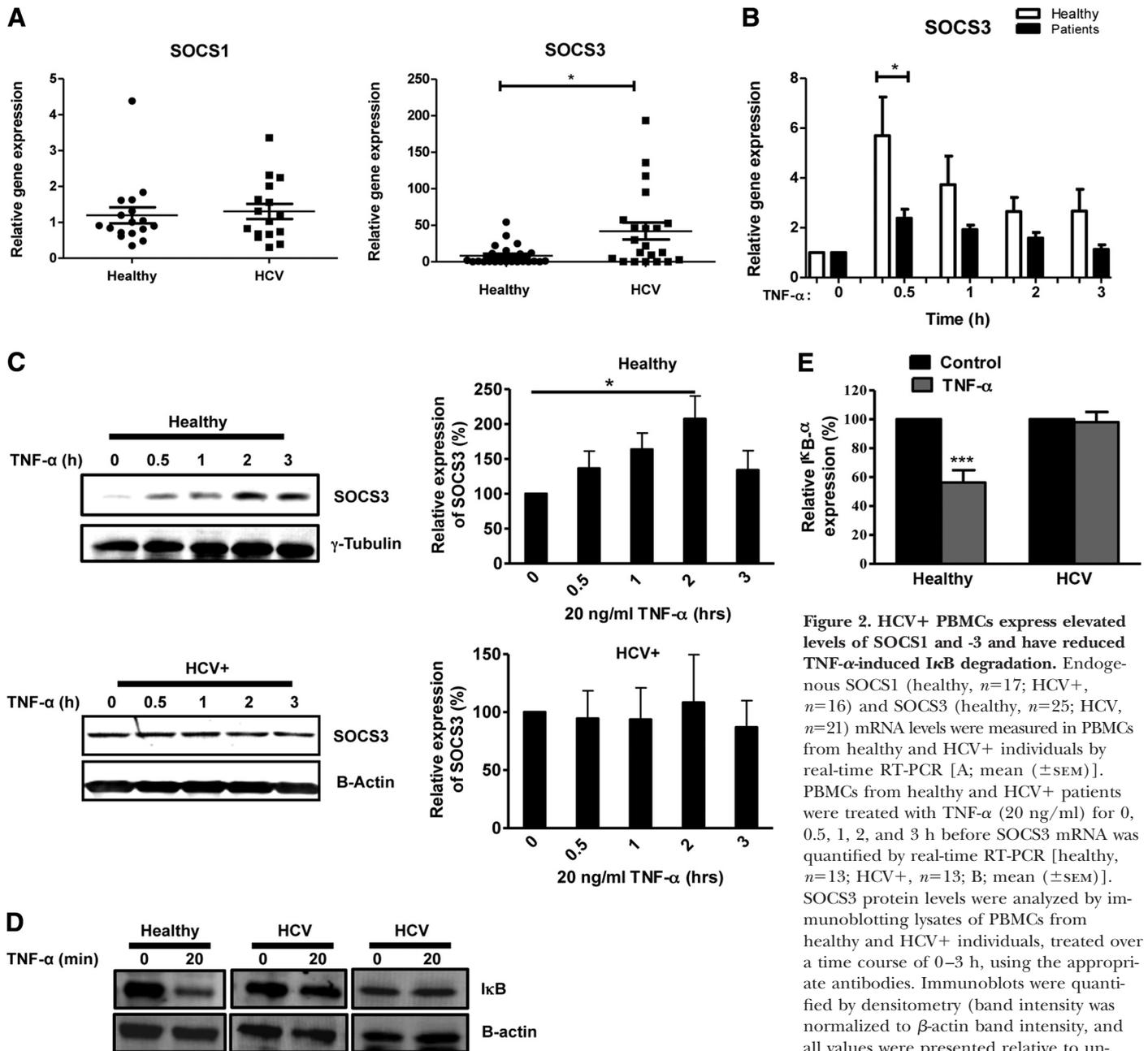
Figure 2. HCV+ PBMCs express elevated levels of SOCS1 and -3 and have reduced TNF-α-induced IκB degradation. Endogenous SOCS1 (healthy, $n=17$; HCV+, $n=16$) and SOCS3 (healthy, $n=25$; HCV, $n=21$) mRNA levels were measured in PBMCs from healthy and HCV+ individuals by real-time RT-PCR [A; mean ($\pm$SEM)]. PBMCs from healthy and HCV+ patients were treated with TNF-α (20 ng/ml) for 0, 0.5, 1, 2, and 3 h before SOCS3 mRNA was quantified by real-time RT-PCR [healthy, $n=13$; HCV+, $n=13$; B; mean ($\pm$SEM)]. SOCS3 protein levels were analyzed by immunoblotting lysates of PBMCs from healthy and HCV+ individuals, treated over a time course of 0–3 h, using the appropriate antibodies. Immunoblots were quantified by densitometry (band intensity was normalized to β-actin band intensity, and all values were presented relative to unstimulated control cells, which were normalized to 100%; $n=3$; C). IκB protein levels were measured by immunoblotting protein lysates of PBMCs from healthy ($n=6$) and HCV+ ($n=7$) patients, treated with TNF-α for 0 and 20 min (D). IκB expression in HCV and healthy PBMCs was quantifed by densitometric analysis (band intensity was normalized to β-actin band intensity, and all values are presented relative to unstimulated control cells, which were normalized to 100%; E).

less induction in HCV+ PBMCs. Interestingly, after 3 h of TNF-α stimulation, CXCL-10 mRNA levels were higher in HCV+ individuals ($P=0.4025$), possibly because CXCL-10 induction is not exclusively regulated by the NF-κB pathway (**Fig. 3**). Overall, these findings support our hypothesis that SOCS3-mediated inhibition of TNF-α signal transduction in HCV+ PBMCs may reduce the up-regulation of proinflammatory cytokines.

HCV-induced SOCS3 directly inhibits TNF-α-mediated IL-8 production

As the inflammatory response to HCV is also mediated through hepatocytes, we next examined the effect of HCV on SOCS3 and TNF-α-induced cytokine expression in the hepatocyte cell line, Huh7. First, we transfected Huh7 cells with HCV (pBRTM/HCV1-3011) or EV constructs for 24 h before analyzing SOCS3 mRNA and protein. We observed a statistically sig-

Figure 3. Induction of proinflammatory cytokines by TNF-α in healthy and HCV+ PBMCs. PBMCs from healthy (n=12) and HCV+ (n=12) individuals were treated with TNF-α (20 ng/ml) for 0, 2, and 3 h, before IL-8, IL-6, CXCL-10, and TNF-α mRNA levels were analyzed by real-time RT-PCR [mean (±SEM)].
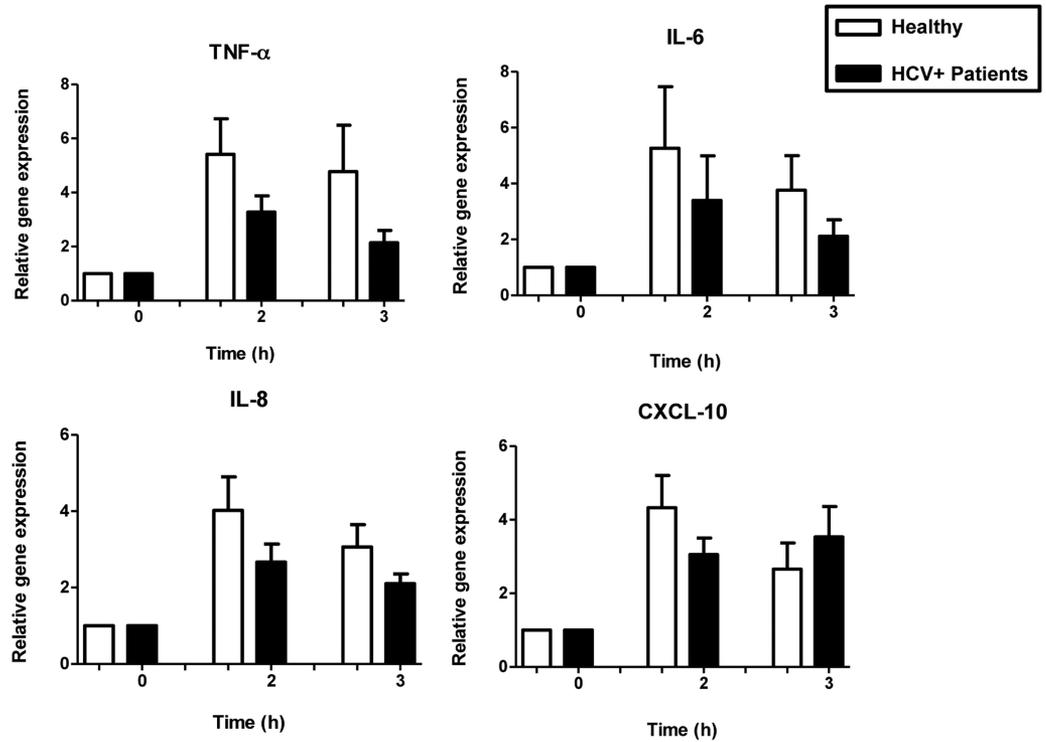
nificant increase in SOCS3 mRNA (**Fig. 4A**) and enhanced SOCS3 protein levels (Fig. 4B) upon HCV expression compared with EV controls. Next, we treated these Huh7 cells with TNF-α for 18 h and measured the secreted IL-8 and CXCL-10 protein levels by ELISA. We found that IL-8 expression was reduced significantly in Huh7 cells expressing HCV, whereas CXCL-10 induction was not reduced (Fig. 4C). These results indicate that as in PBMCs, HCV-induced SOCS3 expression may reduce TNF-α-mediated induction of proinflammatory cytokines.

To determine the direct involvement of SOCS3 in HCV-mediated immune subversion, we transfected Huh7 cells with SOCS3 shRNA or control shRNA for 24 h. After confirming SOCS3 knockdown by qRT-PCR (**Fig. 5A**), we transfected Huh7s with HCV or EV for 10 h, before stimulating with TNF-α for 18 h. We analyzed supernatants by ELISA and found that the reduction of IL-8 in Huh7 cells expressing HCV was restored when SOCS3 was silenced using shRNA, and CXCL-10 levels were unaffected (Fig. 5B). Collectively, these
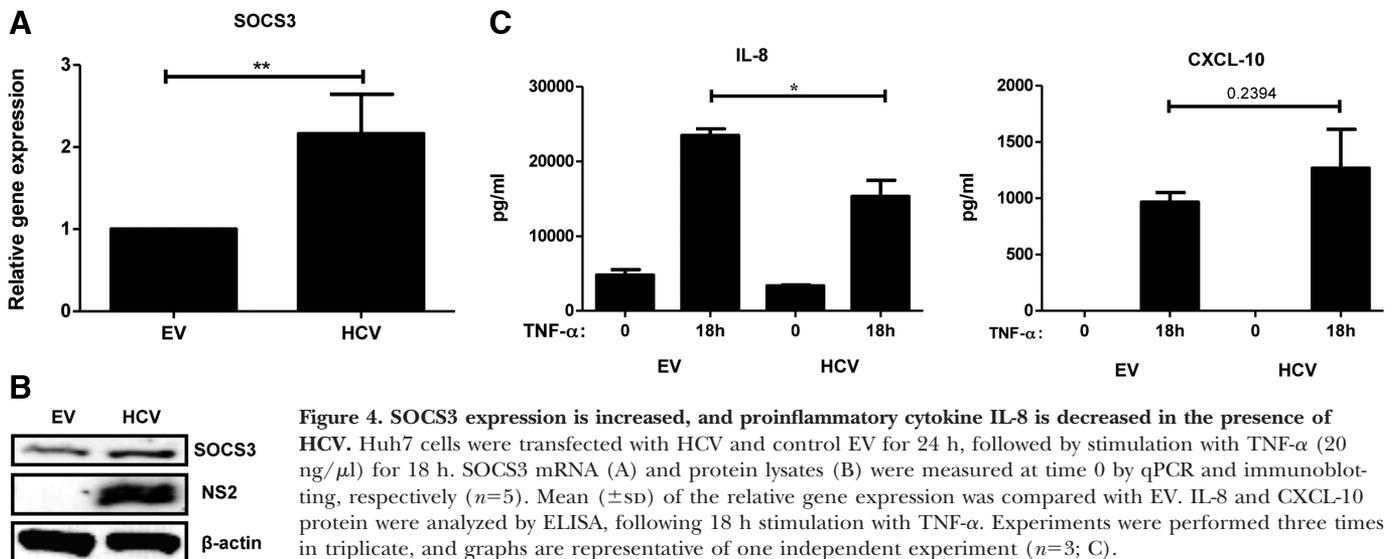

Figure 4. SOCS3 expression is increased, and proinflammatory cytokine IL-8 is decreased in the presence of HCV. Huh7 cells were transfected with HCV and control EV for 24 h, followed by stimulation with TNF-α (20 ng/μl) for 18 h. SOCS3 mRNA (A) and protein lysates (B) were measured at time 0 by qPCR and immunoblotting, respectively (n=5). Mean (±SD) of the relative gene expression was compared with EV. IL-8 and CXCL-10 protein were analyzed by ELISA, following 18 h stimulation with TNF-α. Experiments were performed three times in triplicate, and graphs are representative of one independent experiment (n=3; C).
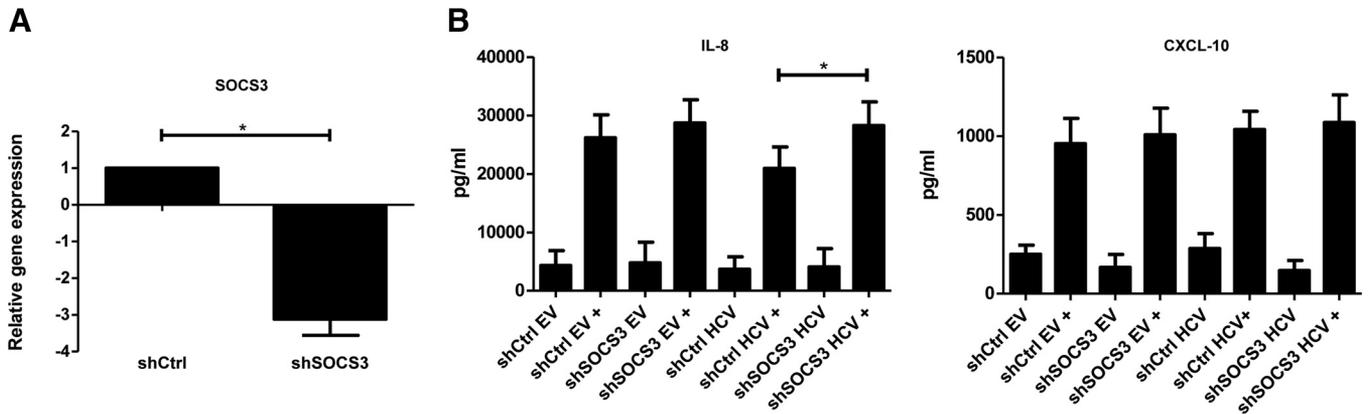
Figure 5. TNF-α-mediated induction of IL-8 is restored when SOCS3 is silenced in HCV-infected Huh7 cells. Huh7 cells were transfected with SOCS3 shRNA (shSOCS3) and shRNA scrambled control (shCtrl) for 24 h ($n=3$; A). SOCS3 mRNA was analyzed by real-time RT-PCR. Relative gene expression was compared with shRNA scrambled control (mean±SD). Huh7 cells were transfected with SOCS3 shRNA and shRNA for 24 h and then transfected with HCV and EV for 10 h, before being stimulated with TNF-α for 18 h (+). IL-8 ($n=5$) and CXCL-10 ($n=5$) protein was quantified by ELISA (B).

results indicate that HCV-induced SOCS3 expression directly inhibits proinflammatory TNF-α-mediated responses.

SOCS3 interacts with TRAF2 and inhibits TRAF2-induced NFκB promoter activation

Having found that HCV could induce SOCS3 that regulates proinflammatory responses to TNF-α, we next investigated the mechanism by which SOCS3 inhibits TNF-α signaling. To this end, we analyzed whether there was an association between SOCS3 and a key component of the TNF-α signaling pathway, TRAF2. We carried out immunoprecipitation assays on lysates from HEK293T cells transfected with myc-tagged SOCS3 and Flag-tagged TRAF2, immunoblotted for SOCS3 (myc) and TRAF2 (Flag), and found that indeed, SOCS3 interacted with

TRAF2 (**Fig. 6A**). Next, with the use of a NF-κB-dependent reporter gene assay, activated by ectopic expression of TRAF2, we investigated whether SOCS3 could inhibit activation of NF-κB signaling. We found that overexpression of SOCS3 significantly inhibited the TRAF2-dependent activation of the NF-κB reporter activity (Fig. 6B). These results are consistent with HCV-induced SOCS3 as having a role in inhibiting proinflammatory responses to TNF-α via TRAF2 of the NF-κB pathway.

DISCUSSION

In this study, we found that TNF-α transiently induced SOCS1 and SOCS3 in primary immune cells and that both proteins
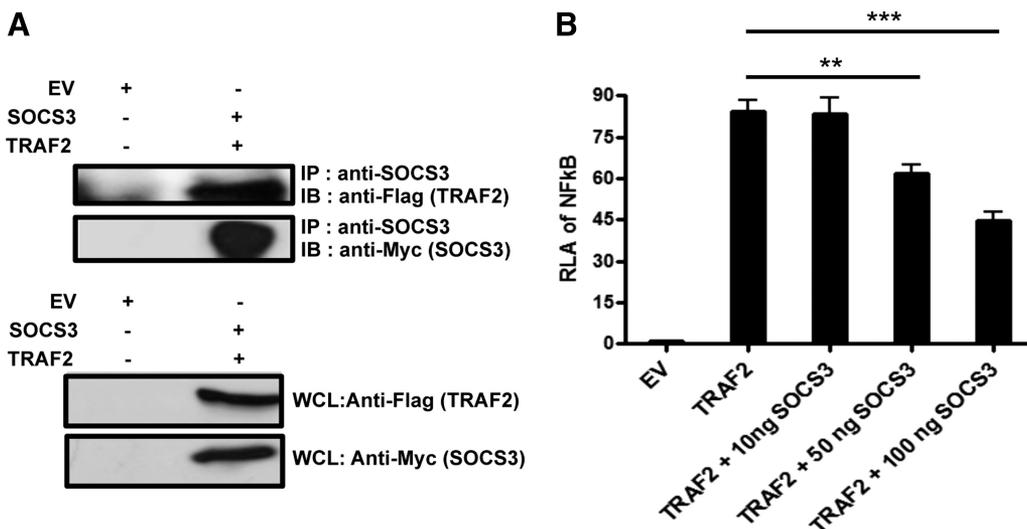

Figure 6. SOCS3 associates with TRAF2 and inhibits TRAF2-mediated NF-κB activity. HEK293T cells were cotransfected with myc-tagged SOCS3 and flag-tagged TRAF2 or EV. After 24 h, cells were harvested and lysed, and immunoprecipitation (IP) of SOCS3 was performed using anti-SOCS3 antibody. Immunocomplexes were separated by SDS gel electrophoresis, and immunoblotting (IB) was performed using anti-myc and anti-flag antibodies. Whole-cell lysates (WCL) were also analyzed for TRAF and SOCS3 expression using anti-flag and anti-myc antibodies, respectively ($n=3$; A). HEK293T cells were transfected with expression vectors encoding the reporter gene NF-κB and cotransfected with EV or Flag-tagged TRAF2 (50 ng) and increasing amounts of myc-tagged SOCS3 (10, 50, and 100 ng). Cell lysates were assessed for luciferase reporter gene activity and represented as relative luciferase activity (RLA) to the control ($n=3$; B).

inhibited TNF-α-induced NF-κB activation. Basal levels of SOCS3 were significantly higher in PBMCs from HCV-infected individuals, which correlated with a reduction in TNF-α-mediated IκB degradation and proinflammatory IL-6, IL-8, and TNF-α induction. Additionally, expression of HCV increased SOCS3 expression in Huh7 cells, which directly reduced proinflammatory IL-8 production. We also found that SOCS3 interacted with TRAF2 and blocked TRAF2-mediated NF-κB reporter gene activation, demonstrating a potential mechanism by which HCV-induced SOCS3 could block proinflammatory responses to TNF-α.

Whereas TNF-α is critical to successful clearance of viral infection, its regulation by SOCS in PBMCs has not been studied. Here, we have identified, for the first time, SOCS1 and -3 as inhibitors of TNF-α-induced NF-κB activation, supporting previous findings of SOCS-mediated control of TNF-α-regulated processes, including cell death in fibroblasts and sensitivity in pancreatic β cells [31]. Indeed, SOCS1$^{-/-}$ macrophages produce excessive amounts of IL-12 in response to TNF-α, and TNF-α-induced SOCS3 generates resistance to IFN-α [32], further suggesting an important role for SOCS in the control of TNF-α signal transduction and subsequent inflammation [33]. This study also reveals a possible mechanism by which the association of SOCS3 with TRAF2 inhibits TRAF2-dependent NF-κB activation, which is reminiscent of the findings by Frobøse et al. [34], where the interaction of SOCS3 with TRAF6 inhibited ubiquitin modification of TRAF6 during IL-1 signaling.

HCV has been documented to block several immune signaling pathways, including the cleavage of NF-κB signaling components [35] and the proteasomal degradation of JAK-STAT pathway components [36]; however, the effect of HCV upon TNF-α-mediated inflammatory responses is poorly understood. Our study has revealed a mechanism by which HCV-induced SOCS3 expression may block proinflammatory TNF-α signal transduction in PBMCs. The presence of HCV in Huh7 hepatocytes also significantly increased SOCS3 levels and consequently, reduced proinflammatory cytokine production. This effect was reversed when SOCS3 was silenced by shRNA, further supporting our hypothesis that HCV-induced SOCS3 negatively regulates the TNF-α signaling pathway to block proinflammatory immune responses. Previous studies have shown that overexpression of NS5A and NS5B inhibits TNF-α signal transduction by blocking TRADD-mediated and TRAF2- and IκB kinase-induced NF-κB activation, respectively [37]. Regulation of proinflammatory signals through the induction of SOCS is an important homeostatic mechanism used to generate a controlled response to infection. Our findings suggest that HCV-induced SOCS3 creates an environment that lacks strong antiviral inflammation. Indeed, a prominent characteristic of HCV infection is the prolonged nature of disease progression, often without any obvious signs of infection [38]. Our results, showing that HCV suppresses the inflammatory response to TNF-α via SOCS3 induction, might explain the "silent" progression of acute HCV infection to chronicity, as well as the relatively mild disease and slow progress of pathology seen in many chronically infected HCV patients.

In conclusion, these findings suggest a novel mechanism by which HCV-induced SOCS3 may regulate TNF-α signaling and thereby, control proinflammatory gene expression. Our results suggest that HCV manipulates this system by inducing SOCS3 expression to suppress TNF-α signaling. These discoveries may indicate a new regulatory mechanism used by HCV to suppress inflammation, perhaps explaining why HCV infection has limited pathology and can remain undetected for many years.

AUTHORSHIP

ACKNOWLEDGMENTS

REFERENCES

1. Bradley, J. (2008) TNF-mediated inflammatory disease. *J. Pathol.* **214,** 149–160.
2. Van der Poll, T., Lowry, S. (1995) Tumor necrosis factor in sepsis: mediator of multiple organ failure or essential part of host defense? *Shock* **3,** 1–12.
3. Sekiyama, K., Yoshiba, M., Thomson, A. (1994) Circulating proinflammatory cytokines (IL-1 β, TNF-α, and IL-6) and IL-1 receptor antagonist (IL-1Ra) in fulminant hepatic failure and acute hepatitis. *Clin. Exp. Immunol.* **98,** 71–77.
4. Kopf, M., Bachmann, M., Marsland, B. (2010) Averting inflammation by targeting the cytokine environment. *Nat. Rev. Drug Discov.* **9,** 703–718.
5. Wertz, I. E., Dixit, V. M. (2008) Ubiquitin-mediated regulation of TNFR1 signaling. *Cytokine Growth Factor Rev.* **19,** 313–324.
6. Jones, S., Ledgerwood, E., Prins, J., Galbraith, J., Johnson, D., Pober, J., Bradley, J. (1999) TNF recruits TRADD to the plasma membrane but not the trans-Golgi network, the principal subcellular location of TNF-R1. *J. Immunol.* **162,** 1042–1048.
7. Takeuchi, M., Rothe, M., Goeddel, D. (1996) Anatomy of TRAF2. Distinct domains for nuclear factor-κB activation and association with tumor necrosis factor signaling proteins. *J. Biol. Chem.* **271,** 19935–19942.
8. Yang, J., Lin, Y., Guo, Z., Cheng, J., Huang, J., Deng, L., Liao, W., Chen, Z., Liu, Z., Su, B. (2001) The essential role of MEKK3 in TNF-induced NF-κB activation. *Nat. Immunol.* **2,** 620–624.
9. Blonska, M., Shambharkar, P., Kobayashi, M., Zhang, D., Sakurai, H., Su, B., Lin, X. (2005) TAK1 is recruited to the tumor necrosis factor-α (TNF-α) receptor 1 complex in a receptor-interacting protein (RIP)-dependent manner and cooperates with MEKK3 leading to NF-κB activation. *J. Biol. Chem.* **280,** 43056–43063.
10. Lombardi, A., Cantini, G., Piscitelli, E., Gelmini, S., Francalanci, M., Mello, T., Ceni, E., Varano, G., Forti, G., Rotondi, M., Galli, A., Serio,

M., Luconi, M. (2008) A new mechanism involving ERK contributes to rosiglitazone inhibition of tumor necrosis factor-α and interferon-γ inflammatory effects in human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **28,** 718–724.

11. Wertz, I. E., Dixit, V. M. (2010) Signaling to NF-κB: regulation by ubiquitination. *Cold Spring Harb. Perspect. Biol.* **2,** a003350.

12. Wertz, I. E., O'Rourke, K. M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D. L., Ma, A., Koonin, E. V., Dixit, V. M. (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-κB signalling. *Nature* **430,** 694–699.

13. Kinjyo, I., Hanada, T., Inagaki-Ohara, K., Mori, H., Aki, D., Ohishi, M., Yoshida, H., Kubo, M., Yoshimura, A. (2002) SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity* **17,** 583–591.

14. Stevenson, N. J., Haan, S., McClurg, A. E., McGrattan, M. J., Armstrong, M. A., Heinrich, P. C., Johnston, J. A. (2004) The chemoattractants, IL-8 and formyl-methionyl-leucyl-phenylalanine, regulate granulocyte colony-stimulating factor signaling by inducing suppressor of cytokine signaling-1 expression. *J. Immunol.* **173,** 3243–3249.

15. Mansell, A., Smith, R., Doyle, S. L., Gray, P., Fenner, J. E., Crack, P. J., Nicholson, S. E., Hilton, D. J., O'Neill, L. A., Hertzog, P. J. (2006) Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. *Nat. Immunol.* **7,** 148–155.

16. Karlsen, A., Heding, P., Frobøse, H., Rønn, S., Kruhøffer, M., Orntoft, T., Darville, M., Eizirik, D., Pociot, F., Nerup, J., Mandrup-Poulsen, T., Billestrup, N. (2004) Suppressor of cytokine signalling (SOCS)-3 protects β cells against IL-1β-mediated toxicity through inhibition of multiple nuclear factor-κB-regulated proapoptotic pathways. *Diabetologia* **47,** 1998–2011.

17. Ryo, A., Suizu, F., Yoshida, Y., Perrem, K., Liou, Y., Wulf, G., Rottapel, R., Yamaoka, S., Lu, K. (2003) Regulation of NF-κB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Mol. Cell.* **12,** 1413–1426.

18. Zaltron, S., Spinetti, A., Biasi, L., Baiguera, C., Castelli, F. (2012) Chronic HCV infection: epidemiological and clinical relevance. *BMC Infect. Dis.* **12** (Suppl. 2), S2.

19. Moradpour, D., Cerny, A., Heim, M., Blum, H. (2001) Hepatitis C: an update. *Swiss Med. Wkly.* **131,** 291–298.

20. Thornton, L., Murphy, N., Jones, L., Connell, J., Dooley, S., Gavin, S., Hunter, K., Brennan, A. (2011) Determination of the burden of hepatitis C virus infection in Ireland. *Epidemiol. Infect.* **140,** 1461–1468.

21. Yao, Z., Prayther, D., Trabue, C., Dong, Z., Moorman, J. (2008) Differential regulation of SOCS-1 signalling in B and T lymphocytes by hepatitis C virus core protein. *Immunology* **125,** 197–207.

22. Walsh, M., Jonsson, J., Richardson, M., Lipka, G., Purdie, D., Clouston, A., Powell, E. (2006) Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1. *Gut* **55,** 529–535.

23. Imada, K., Leonard, W. (2000) The Jak-STAT pathway. *Mol. Immunol.* **37,** 1–11.

24. Naka, T., Fujimoto, M., Tsutsui, H., Yoshimura, A. (2005) Negative regulation of cytokine and TLR signalings by SOCS and others. *Adv. Immunol.* **87,** 61–122.

25. Guidotti, L., Chisari, F. (2001) Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu. Rev. Immunol.* **19,** 65–91.

26. Burrows, J., McGrattan, M., Rascle, A., Humbert, M., Baek, K., Johnston, J. (2004) DUB-3, a cytokine-inducible deubiquitinating enzyme that blocks proliferation. *J. Biol. Chem.* **279,** 13993–14000.

27. Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M., Rice, C. M. (1993) Expression and identification of hepatitis C virus polyprotein cleavage products. *J. Virol.* **67,** 1385–1395.

28. Livak, K. J., Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta C(T))}$ method. *Methods* **25,** 402–408.

29. Wang, L., Walia, B., Evans, J., Gewirtz, A., Merlin, D., Sitaraman, S. (2003) IL-6 induces NF-κ B activation in the intestinal epithelia. *J. Immunol.* **171,** 3194–3201.

30. Liang, T., Heller, T. (2004) Pathogenesis of hepatitis C-associated hepatocellular carcinoma. *Gastroenterology* **127,** S62–71.

31. Morita, Y., Naka, T., Kawazoe, Y., Fujimoto, M., Narazaki, M., Nakagawa, R., Fukuyama, H., Nagata, S., Kishimoto, T. (2000) Signals transducers and activators of transcription (STAT)-induced STAT inhibitor-1 (SSI-1)/suppressor of cytokine signaling-1 (SOCS-1) suppresses tumor necrosis factor α-induced cell death in fibroblasts. *Proc. Natl. Acad. Sci. USA* **97,** 5405–5410.

32. Hong, F., Nguyen, V., Gao, B. (2001) Tumor necrosis factor α attenuates interferon α signaling in the liver: involvement of SOCS3 and SHP2 and implication in resistance to interferon therapy. *FASEB J.* **15,** 1595–1597.

33. Chong, M., Metcalf, D., Jamieson, E., Alexander, W., Kay, T. (2005) Suppressor of cytokine signaling-1 in T cells and macrophages is critical for preventing lethal inflammation. *Blood* **106,** 1668–1675.

34. Frobøse, H., Rønn, S. G., Heding, P. E., Mendoza, H., Cohen, P., Mandrup-Poulsen, T., Billestrup, N. (2006) Suppressor of cytokine signaling-3 inhibits interleukin-1 signaling by targeting the TRAF-6/TAK1 complex. *Mol. Endocrinol.* **20,** 1587–1596.

35. Li, K., Foy, E., Ferreon, J., Nakamura, M., Ferreon, A., Ikeda, M., Ray, S., Gale, M. J., Lemon, S. (2005) Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl. Acad. Sci. USA* **102,** 2992–2997.

36. Stevenson, N. J., Bourke, N. M., Ryan, E. J., Binder, M., Fanning, L., Johnston, J. A., Hegarty, J. E., Long, A., O'Farrelly, C. (2013) Hepatitis C virus targets the interferon-α JAK/STAT pathway by promoting proteasomal degradation in immune cells and hepatocytes. *FEBS Lett.* **587,** 1571–1578.

37. Choi, S., Park, K., Ahn, B., Jung, G., Lai, M., Hwang, S. (2006) Hepatitis C virus nonstructural 5B protein regulates tumor necrosis factor α signaling through effects on cellular IκB kinase. *Mol. Cell. Biol.* **26,** 3048–3059.

38. Alter, M. (1997) The epidemiology of acute and chronic hepatitis C. *Clin. Liver Dis.* **1,** 559–568, vi–vii.