Published in final edited form as:

Gastroenterology. 2017 August ; 153(2): 566–578.e5. doi:10.1053/j.gastro.2017.04.021.

Tumor Necrosis Factor Inhibits Spread of Hepatitis C Virus Among Liver Cells, Independent from Interferons

Stephen M. Laidlaw^{1,2}, Svetlana Marukian³, Rachel H. Gilmore³, Siobhán B. Cashman^{1,2}, Volodymyr Nechyporuk-Zloy¹, Charles M. Rice³, and Lynn B. Dustin^{1,2,3,4}

¹Kennedy Institute of Rheumatology, The University of Oxford, Oxford, UK

²Peter Medawar Building for Pathogen Research, The University of Oxford, Oxford, UK

³Laboratory of Virology and Infectious Disease, The Rockefeller University, New York, NY, USA

Abstract

Background & Aims—Tumor necrosis factor (TNF) an inflammatory cytokine expressed by human fetal liver cells (HFLCs) following infection with cell culture-derived hepatitis C virus. TNF has been reported to increase entry of HCV pseudoparticles into hepatoma cells and inhibit signaling by interferon alpha (IFNA), but have no effect on replication of HCV RNA. We investigated the effects of TNF on HCV infection of and spread among Huh-7 hepatoma cells and primary HFLCs.

Methods—Human hepatoma (Huh-7 and Huh-7.5) and primary HFLCs were incubated with TNF and/or recombinant IFNA2A, IFNB, IFNL1, and IFNL2 before or during HCV infection. We used 2 fully infectious HCV chimeric viruses of genotype 2A in these studies: J6/JFH (Clone 2) and Jc1(p7-nsGluc2A) (Jc1G), which encodes a secreted luciferase reporter. We measured HCV replication, entry, spread, production, and release in hepatoma cells and HFLCs.

Results—TNF inhibited completion of the HCV infectious cycle in hepatoma cells and HFLC in a dose-dependent and time-dependent manner. This inhibition required TNF binding to its receptor. Inhibition was independent of IFNA, IFNB, IFNL1, IFNL2, or JAK signaling via STAT. TNF reduced production of infectious viral particles by Huh-7 and HFLC, and thereby reduced numbers of infected cells and size of foci. TNF had little effect on HCV replicons and increased entry of HCV pseudoparticles. When cells were incubated with TNF before infection, the subsequent anti-viral effects of IFNs were increased.

Conclusion—In a cell culture system, we found TNF to have antiviral effects independently of, as well as in combination with, IFNs. TNF inhibits HCV infection despite increased HCV

⁴Corresponding author: lynn.dustin@kennedy.ox.ac.uk, Peter Medawar Building for Pathogen Research, South Parks Road, Oxford, OX1 3SY, UK.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors declare that no conflict of interest exists.

Authorship: Study concept and design: SML, SM, LBD. Data acquisition: SML, SM, RHG, SBC. Data analysis/statistics: SML, SM, RHG, SBC, VN-Z, CMR, LBD. Drafting of the manuscript: SML, LBD. Funding and supervision: LBD, CMR.

envelope glycoprotein-mediated infection of liver cells. These findings contradict those from other studies, which reported that TNF blocks signal transduction in response to IFNs. The destructive inflammatory effects of TNF must be considered along with its antiviral effects.

Keywords

viral infection; immune regulation; innate immunity; liver disease

Introduction

Years of intensive study of the hepatitis C virus (HCV) have now borne fruit in an array of highly effective, direct-acting antiviral drugs (DAAs) that target specific steps in HCV replication and virion assembly. While DAAs have the potential to cure almost everyone who is treated, the majority of the estimated 130–200 million people worldwide who have chronic HCV infection^{1–3} have poor access to modern health care, and have not yet been diagnosed, let alone treated^{4, 5}. Issues such as screening, cost, comorbidities, and advanced liver disease may affect treatment uptake. Genotype-specific differences in drug sensitivity and the emergence of resistant strains may also influence treatment efficacy. Patients cured by DAA treatment are not reliably protected against re-infection⁶. A prophylactic vaccine is not yet available⁷. In addition, HCV remains an important model for studies of acute and chronic viral infection.

Many studies have shown reduced IFN α / β induction and response in HCV-infected cells. HCV may dampen IFN α / β induction through viral protease-mediated cleavage of MAVS and TRIF, key signal transducers involved in viral RNA recognition (reviewed in^{8, 9}). HCV may counteract the antiviral actions of exogenous IFNs through a number of interactions with effector molecules^{8, 9}. Despite this multi-layered attack on innate immunity, HCV induces expression of IFN λ s and IFN-stimulated genes in infected primary liver cells^{10, 11}. Hundreds of IFN-stimulated genes that are expressed in the infected liver partially control but do not eliminate HCV during acute infection (reviewed in¹²).

Tumor necrosis factor- α (TNF α) is a pleiotropic cytokine produced by a variety of cell types including cytotoxic T cells, natural killer cells, endothelial cells, dendritic cells, monocytes, and macrophages. TNF α is a type II transmembrane protein trimer; a soluble form of TNF α can be released after proteolytic processing. Both cell-associated and cell-free TNF α are biologically active. TNF α can bind to one of two receptors with different downstream effects^{13, 14}. Most cell types express TNFR1, while TNFR2 expression is restricted to T cells and a few other cell types¹³. TNFR1 recruits the TNFR1-associated death domain protein, TRADD, leading to activation of NF κ B, c-Jun N-terminal kinase (JNK), p38, and cell death pathways; integration of multiple downstream signals determines the effects of TNFR1 signal transduction¹⁴.

While TNF α is considered an inflammatory cytokine, it may contribute to control of viral infections by induction of antiviral gene expression or apoptosis. TNF α can mediate antiviral activities by itself^{15, 16} and in synergy with IFNs (reviewed in¹⁷). In hepatocytes, TNF α was reported to inhibit hepatitis B virus (HBV) gene expression and replication, and to synergize with IFN γ ^{18–20}.

Hepatocytes also express TNF α following HCV infection, as we reported for cultured human fetal liver cells (HFLC) infected with HCV¹⁰. Others have reported that HCV induced TNF α transcription and protein release in Huh-7 hepatoma cells and primary human hepatocytes²¹. Hepatocyte induction of TNF α may occur independently of viral replication, as both UV-inactivated virus and poly I:C (a TLR3 agonist) stimulated TNF α expression and release in a manner dependent on TLR signaling^{10, 21}. TNF α has long been associated with viral hepatitis: patients with acute or chronic viral hepatitis, including HCV and HBV, had increased serum TNF α protein^{22, 23}; hepatocytes from HCV and HBV patients expressed TNF α mRNA and protein²⁴.

Initial studies of the impact of TNF α on HCV replication were not promising: in a subgenomic replicon system, TNF α had no effect on HCV replication²⁵. Work from Fletcher and colleagues suggested that TNF α enhanced HCV entry in a hepatoma cell line expressing CD81²⁶. TNF α has been reported both to support²¹ and to antagonize²⁷ IFN α/β signalling in Huh-7 hepatoma cells. Here, we report that TNF α inhibits HCV infection in Huh-7 hepatoma cells and in primary HFLC. TNF α potentially inhibits a late step in HCV infection via a mechanism independent of IFN binding or signal transduction.

Materials and Methods

Tissue culture

Unless otherwise indicated, tissue culture media and additives were purchased from Life Technologies. Huh-7 and Huh-7.5 hepatoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (Huh-7.5) or 10% (Huh-7) heat-inactivated FBS (Biosera) and 1 $\times$ non-essential amino acids.

Human fetal liver cells (HFLC)

De-identified fetal livers (16 to 20 weeks gestation) were procured through the Human Developmental Biology Resource at the Institute of Child Health, University College, London. Tissue collection was approved by The National Research Ethics Service, REC Reference 08/H0712/34+5. Cells were prepared and cultured as described previously²⁸. Culture medium was aspirated and replaced every 2 days.

Replicon assay

Huh-7 cells were prepared in T225 flasks, of which half were pretreated with TNF α for 16 hours. Cells were trypsinized and washed 3 $\times$ with cold PBS followed by electroporation with in vitro-transcribed subgenomic or full-length HCV RNA as indicated (5 μ g electroporated into 1.2 $\times 10^7$ cells). Electroporated cells were seeded at 2 $\times 10^4$ in replica 96-well plate wells. Cells were washed at 6 hours and subsequently at each harvesting timepoint. No drug selection was applied to replicon-bearing cultures.

Production and quantitation of HCVcc

We used two fully infectious HCV chimeric viruses of genotype 2A in these studies: J6/JFH (Clone 2)²⁹ is highly infectious but does not encode a reporter. Jc1(p7-nsGluc2A) (Jc1G)³⁰ encodes a secreted *Gaussia princeps* luciferase reporter. Viral stocks were prepared

essentially as described previously³¹. Briefly, in vitro-transcribed viral RNA (5 µg) was electroporated into Huh-7.5 cells (1.2×10^7). Virus was collected in DMEM containing 1.5% heat-inactivated FBS and 1% non-essential amino acids, and was concentrated in a pressurized stirred cell using Amicon Ultracel-100K filters (Millipore). Infectivity titers were determined by limiting dilution titration on naïve Huh-7.5 cells³² and are presented as median tissue culture infectious dose (TCID₅₀).

HCVcc infection

Unless otherwise indicated, Clone 2 was used at MOI of 0.01 TCID₅₀/cell for Huh-7 and Huh-7.5 cells, and 3 or 10 (depending on the availability of high-titer virus preparations) TCID₅₀/cell for HFLC. Jc1G was used at an MOI of 0.1 TCID₅₀/cell for Huh-7 and Huh-7.5 cells, and 3 or 10 TCID₅₀/cell for HFLC. At time 0, virus was added to cells for 6 hours; cells were then washed three times in complete medium. Medium, cytokines, and drugs were replaced and cultures continued from this point, which was considered +6 hours.

HCV pseudoparticles (HCVpp)

HCVpp were produced in 293T cells cotransfected with HCV E1E2 expression plasmids and pNL4-3.Gluc.R-E- as previously described³³.

Cytokines, drugs, and antibodies

Recombinant human TNFα, IFNα2a, IFNβ, and IFNλ1, and IFNλ2 were purchased from Peprotech and prepared according to the manufacturer's recommendations. Stocks were prepared as single-use aliquots and stored at -80°C. Ruxolitinib was purchased from Selleckchem and used at a final concentration of 3 µM. Danoprevir, an inhibitor of HCV's NS3-4A protease, was purchased from Selleckchem and used at 2 µM. Etanercept (Amgen) was kindly provided by the Kennedy Institute of Rheumatology and was stored in single-use aliquots at 4°C. Anti-CD81 (clone JS81, no azide-low endotoxin) was purchased from BD Biosciences. The viral IFN antagonists B18R³⁴ and 136R³⁵ were purchased from Affymetrix and Biotechne respectively. Cytokines, antagonists, and drugs were replaced at each media change unless otherwise indicated.

Measurement of cell-free and intracellular infectious virus

Cell-free HCV was collected from the supernatants of infected cultures. To prepare intracellular infectious virus, infected cells were washed three times with serum-free media; an aliquot of serum-free media was then added to the well and plates were subjected to three freeze-thaw cycles to release intracellular virus. Lysates were centrifuged ($480 \times g$) to remove cell debris. Infectious titers were determined as described³².

HCV RNA quantitation

Total RNA was extracted from tissue culture supernatants and freeze-thawed cell lysates using High Pure Viral RNA kits (Roche) or from washed cells using RNeasy minikits (Qiagen). RNA was eluted in 40 µl. 5 µl of RNA (one-eighth of the total) was used for RNA quantitation. HCV RNA was detected by quantitative RT-PCR using the Eragen Multicode-RTx method³⁶ (Eragen Biosciences) using primers to the 5' untranslated region of the HCV

genome (5'-GGCTCCATCTTAGCCC-3' and 5'-/56-FAM//iMe-isodC/GCTCACGGACCTTTCA-3'), and quantified using a synthetic HCV RNA standard (Apath LLC).

mRNA quantitation

Total cellular RNA was prepared from cultured Huh-7 or HFLCs using RNeasy minikits. cDNA synthesis, primers, real-time qRT-PCR, and analysis were as we have previously reported¹⁰.

Gaussia luciferase replication assay

To measure Jc1G replication, Huh-7 cells were plated in a 96-well plate and infected at an MOI of 0.1 for 6 h. Cells were washed, media replaced, and supernatants harvested for a 6-hour background reading. Supernatants were harvested at the indicated times post infection, with extensive washing at each timepoint. Secreted luciferase was measured using the Renilla Luciferase Assay System (Promega) and a Berthold TriStar2 multimode reader LB 942.

Flow cytometry

Immunostaining for HCV NS5A was performed as previously described³⁰ using Alexa 647-conjugated mAb 9E10³¹. Cells were analysed on a BD LSRII. Data were analysed using FlowJo version 10.

Assaying viral spread

Huh-7.5 cells expressing an HCV-dependent fluorescent reporter³⁷ were pretreated (16 h) with TNF α or control medium, then infected for 6 hours with Clone 2 (MOI = 0.01). Infections were carried out in 6-well plates, using a different plate for each timepoint. Infected cells were washed, media and TNF α replaced, and plates returned to culture. From this point until cells were fixed, care was taken to prevent plate movement and minimize mixing; thus, media and cytokines were not replaced during the assay. At each timepoint, an infected plate was selected, washed with PBS, and fixed (10 minutes) with 3.7% formaldehyde. Wells were washed 3 $\times$ with PBS and permeabilized (5 minutes) with 0.2% Triton X-100, washed 3 $\times$ with PBS, stained (10 minutes) with 2 ng/ml DAPI, and washed 3 $\times$ again. Cells were viewed using an Olympus inverted microscope (IX73). DAPI and RFP were excited using 400nm/565nm LED laser sources and detected using the 405/561 fluorescence filters. Images were taken at 10 $\times$ magnification and processed using cellSens (Olympus) and CellProfiler (cellprofiler.org). The CellProfiler workflow was as follows: DAPI⁺ nuclei were identified, and a 5-pixel perinuclear space defined. Mean RFP fluorescence intensities were measured for each nucleus and perinuclear space. The ratio of the mean nuclear:perinuclear space intensities was calculated for each cell. Cells with a ratio >1 were counted as infected.

Statistics

Graphing and statistical analysis were performed with GraphPad Prism 6.0h. Data are presented as mean $\pm$ SD of at least triplicate samples, and all experiments repeated a

minimum of three times. Statistical significance was determined by using Mann-Whitney test, One-Way ANOVA, or Two-Way ANOVA, as indicated, and corrected for multiple comparisons using Tukey's post test. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, ns = not significant.

Results

TNF α inhibits HCV infection in Huh-7 cells

Huh-7 hepatoma cells were treated overnight with medium or TNF α and then infected with fully infectious HCV genotype 2 chimeric viruses; culture was continued in the presence of either medium or TNF α . Supernatants were collected at 24-hour intervals to measure reporter expression. TNF α inhibited reporter expression in a dose-dependent manner (Fig. 1A). IC₅₀ values averaged below 1 ng/ml (range 0.1–1.74 ng/ml); IC₉₀ values averaged 19.14 ng/ml (range 1.38–37.86 ng/ml) in three experiments. Flow cytometric analysis revealed that fewer cells were infected in TNF α -treated cultures, although infected cells expressed comparable levels of NS5A (Fig. 1B). Inhibition was time-dependent, with the degree of inhibition increasing over time. The duration of TNF α exposure also influenced the degree of inhibition (Fig. 1C). While cells treated with TNF α beginning at or after virus addition showed little change in reporter expression, those treated with TNF α beginning 18 or 24 hours before HCV infection showed a ten-fold decrease in reporter expression; those treated starting 40 hours before virus addition showed a 2-log decrease in reporter expression (Fig. 1D). It was not possible to determine whether new gene expression or protein synthesis was required for the inhibitory effect, as Huh-7 cells could not be cultured for long periods with Actinomycin D or cycloheximide (data not shown). Huh-7 cells remained viable and proliferated to a similar extent in the presence or absence of TNF α (Supplemental Fig. 1). To confirm that HCV growth inhibition was due to the effects of TNF α rather than a contaminant, we carried out cultures in the presence of the TNF α antagonist, etanercept. Inclusion of this inhibitor restored the growth of HCV in the presence of exogenous TNF α (Fig. 1E). TNF α also inhibited HCV growth in primary HFLC, and this effect was also blocked by etanercept (Fig. 1F). Levels of viral RNA were reduced in TNF α -treated cultures (Supplemental Fig. 2). Together, these results show that TNF α inhibits HCV infection or growth in liver cells, that the inhibition is dose- and time-dependent, and that the inhibition is dependent on TNF α binding to its receptor on liver cells.

TNF α inhibits HCV spread

The observation that TNF α inhibits HCV infection was unexpected given published reports from other groups using different systems. To understand the basis for this difference, we measured the effects of TNF α on different stages of HCV infection. To ascertain whether TNF α inhibited HCV RNA replication, we electroporated subgenomic JFH1 luciferase reporter replicons into TNF α -treated Huh-7 cells. The results of this experiment showed that replication was not inhibited by TNF α (Fig. 2A). As a control, no replication was seen using a replicon containing a mutation within the NS5B polymerase (GNN). These results were consistent with a previous report that TNF α did not affect replication of an HCV subgenomic replicon²⁵. Expression of full-length genotype 1 replicons was also not inhibited for at least 5 days; however, expression of a full-length genotype 2A (J6/JFH)

replicon, which may produce infectious virus³¹, was inhibited by approximately 25% (Fig. 2B).

Consistent with a report showing that TNF α enhanced HCV envelope glycoprotein-mediated infection of HepG2 hepatoma cells expressing CD81²⁶, we observed that TNF α enhanced infection by lentiviral pseudoparticles bearing HCV glycoproteins (Fig. 2C, *left* and *center*). TNF α did not overcome the block to entry caused by the addition of anti-CD81 mAb JS81. Of note, TNF α also enhanced infection by pseudoparticles bearing an unrelated glycoprotein from the feline endogenous retrovirus RD114 (Fig. 2C, *right*).

Because TNF α reduced the number of infected cells (Fig. 1B) without inhibiting replication or entry (Fig. 2), we investigated its effects in an assay of viral spread (Figs. 3 and 4). We used Huh-7.5 cells expressing the HCV-dependent fluorescent reporter described in reference³⁷. Cells were infected with Clone 2, and the spread of infection monitored microscopically over the ensuing days. While HCV spread rapidly in control cultures, TNF α -treated cultures showed reduced viral spread (Fig. 3 and Fig. 4A) and reduced focus size (Fig. 3 and Fig. 4B). Note that these cultures were left undisturbed (and unfed) to prevent mixing that would accelerate viral spread.

One possible explanation for reduced viral spread is that TNF α prevents virus assembly or egress from infected cells. To ascertain whether TNF α affects production or release of infectious virus, we measured virus levels over time in cultures treated with TNF α or medium before and during infection with HCVcc Clone 2 (MOI=0.1). TNF α -treated cultures had profoundly reduced levels of infectious virus in both cell-free and cell-associated fractions (Fig. 4C). A similar result was obtained with primary HFLC, although overall production of infectious virus was low (Fig. 4D); we were unable to measure intracellular infectious virus in HFLC cultures.

TNF α inhibits HCV infection independently of IFN α/β and IFN λ

We hypothesized that TNF α acted by triggering autocrine IFN production. To define the role of IFN signalling in the effects of TNF α , we treated cells with a variety of inhibitors of IFN signalling or binding (Fig. 5A). We used two poxvirus-derived soluble IFN antagonists, B18R (which blocks IFN α/β receptor binding) and 136R (which preferentially blocks IFN λ receptor binding) to antagonize IFN signalling in Huh-7 cell cultures. B18R blocked the antiviral effects of IFN α but not those of TNF α . Similarly, 136R blocked the antiviral effects of IFN λ but not those of TNF α . A mixture of B18R + 136R also did not reverse the antiviral effect of TNF α . The Jak1/Jak2 antagonist, Ruxolitinib, blocked the effects of IFN α and IFN λ but not those of TNF α . TNF α 's inhibitory effect was blocked by etanercept, as also seen in Fig. 1E. As shown in Fig. 5B, HFLC cultures treated with Ruxolitinib remained sensitive to TNF α . These results indicate that TNF α mediates an antiviral effect independent of type I or type III IFN binding or Jak signalling. TNF α did not induce expression of type I or type III IFNs (Fig. 5C), or of typical IFN-stimulated genes such as OAS1, IFIT1, or ISG15 (Supplemental Fig. 3).

Interactions between TNF α and IFNs

While the results above indicate that TNF α can work independently of IFN binding or Jak-mediated IFN receptor signalling, they do not address the effects of TNF α stimulation on IFN responses, or vice-versa. To examine the effects of TNF α on IFN responses, we performed crosswise dose-response experiments in which Huh-7 hepatoma cells were treated simultaneously with TNF α , IFN β , IFN λ , or combinations of TNF α and an IFN. The presence of TNF α increased the antiviral effects of suboptimal levels of IFNs (Fig. 6A). We also tested the effects of sequential treatment with TNF α and IFNs in different combinations as shown in Fig. 6B. Cells were pretreated overnight (Cytokine 1), then washed and infected with HCVcc in the presence of Cytokine 2. Cytokine 2 treatment was continued throughout the course of infection, and HCV growth estimated by luciferase measurement. Cells treated with TNF α as Cytokine 1 showed reduced HCV growth, even when Cytokine 2 was absent (Fig. 6B, Cytokine 1 = TNF α vs. Cytokine 1 = None). TNF α -pretreated cells showed increased sensitivity to IFN α as Cytokine 2 (Fig. 6B, Cytokine 1 = TNF α vs. Cytokine 1 = None when Cytokine 2 = IFN α). Likewise, IFN α pretreatment enhanced the antiviral effects of TNF α as Cytokine 2. These results indicate that TNF α pretreatment increases the antiviral effects of IFNs, and that IFN pretreatment increases the antiviral effects of TNF α .

Discussion

TNF α , a cytokine with diverse roles in inflammation and immunity, is produced by a variety of cell types including monocytes/macrophages and cytotoxic T cells. We and others have demonstrated that cultured liver cells express TNF α following HCV infection^{10, 21}. Here we show that TNF α mediates antiviral activity against HCV. IFN binding and Jak-mediated signal transduction are not required for this antiviral effect, suggesting that TNF α can act independently of IFNs. TNF α acts at a late stage of the HCV infection cycle. Thus, TNF α does not inhibit and indeed may modestly enhance HCV pseudoparticle entry, suggesting that it does not inhibit viral entry. Persistence of HCV replicons that do not spread from cell to cell is largely unaffected by TNF α . TNF α inhibits a step after viral entry and translation of incoming RNA, possibly by reducing or blocking assembly and spread of infectious virus. Both intracellular and cell-free infectious virus levels are reduced. Of note, TNF α had little effect against yellow fever virus infection in Huh-7 cells (Supplemental Fig. 4), and enhanced yellow fever virus infection in dendritic cells from 2/3 donors (Supplemental Fig. 5). Studies are ongoing to determine how TNF α affects infection by other virus families in addition to those reported by other groups^{15, 16, 18, 19}.

These results may be considered unexpected given previous results from other labs. The differences between our observations and those of others highlight the evolution of HCV study systems. Frese and colleagues reported that TNF α did not affect replication of a subgenomic HCV RNA in a replicon-bearing cell line²⁵. Here, we observed that while replication of a subgenomic HCV replicon is not sensitive to TNF α , viral growth and spread were sensitive to this cytokine. This result was not attainable before the advent of systems permitting a complete infectious cycle in vitro. Similarly, Fletcher et al. showed that TNF α enhanced HCV and HCVpp entry into polarized HepG2 cells expressing CD81²⁶. We also find that TNF α treated Huh-7 cells show a modest increase in HCVpp entry. This increase in

entry is not HCV-specific, as we observed a similar result with pseudoparticles bearing glycoproteins from the unrelated feline endogenous retrovirus, RD114; this is consistent with the observations reported in²⁶ showing comparable increases in entry of pseudoparticles bearing non-HCV glycoproteins. Indeed, Fletcher and colleagues also observed that while TNF α -treated hepatoma cells supported increased HCV entry, there was no associated change in viral RNA replication²⁶. Later events, including HCV spread, were not measured in the aforementioned study²⁶. In our hands, TNF α -treated liver cells sometimes had an initial increase in HCV reporter expression at early time points (for example in primary HFLC, Fig. 4D). This transient increase is consistent with enhancement of initial viral entry (Fig. 2C) followed by inhibition of viral spread (Fig. 3–4).

Interactions between TNF α and antiviral cytokines such as IFN α have been the subject of conflicting reports. While TNF α is reported to potentiate the antiviral effects of IFNs against an array of DNA and RNA viruses^{15, 17, 38}, the mechanisms behind such activities are not well understood. It has been proposed that TNF α acts in part by inducing expression of IFN β in some cell types (reviewed in³⁸). We have demonstrated that TNF α mediates an antiviral effect against HCV in the presence of antagonists of IFN binding and signal transduction, suggesting that TNF α can act independently of IFNs.

In contrast, others have reported that TNF α inhibits IFN signal transduction in hepatoma cells by stimulating expression of the ubiquitin-specific peptidase, USP18²⁷. Importantly, the studies reported in²⁷ did not address the impact of such signalling effects on viral growth or replication. To examine this question, we treated cells either simultaneously or sequentially with IFNs and TNF α , as shown in Fig. 6. TNF α did not antagonize the antiviral effects of IFNs.

TNF α antagonists are widely used clinically, while TNF α agonists are used or in development for treatment of certain malignancies³⁹. Clinical therapy with TNF α antagonists appears to be generally safe in patients with autoimmune or inflammatory diseases and concurrent HCV infection⁴⁰, although dramatic ($>2 \log_{10}$) increases in viral load are observed in a minority of patients^{41, 42}. It is possible that TNF α plays a more important role in control of HCV in some patients than in others. Importantly, TNF α is active both as a transmembrane precursor and as a soluble protein released from producer cells^{43, 44}. The various TNF α antagonist drugs differ in (a) their affinity for soluble and transmembrane TNF α and (b) their effects on TNF α -producing cells, partially explaining their disparate clinical indications and contraindications (reviewed in⁴⁴). Certain antagonists may bind poorly to transmembrane TNF α , perhaps preserving the antiviral functions of TNF α -expressing cells.

The role of TNF α in control of HCV infection in the human liver remains uncertain. Elevated peripheral blood levels of TNF α have been reported in humans with hepatitis of various etiologies^{22, 23}; the cellular origins of the cytokine are not known, and may differ in different disease settings. Plasma levels of TNF α are significantly increased in patients with chronic HCV infection (Supplemental Fig. 6). Importantly, local concentrations of TNF α are likely to be higher in the infected liver²⁶. Innate immune cells in the liver, including Kupffer cells, macrophages, and dendritic cells, express TNF α in response to pathogen-

associated molecular patterns including those associated with HCV²⁶. T cell recognition of HCV-infected cells is key to spontaneous recovery, while exhaustion of HCV-specific T cells is a hallmark of persistent HCV infection. The polyfunctional T cells required for HCV clearance produce TNF α as part of their immune program. Loss of TNF α expression is a feature of T cell exhaustion⁴⁵. It is not straightforward to estimate the local concentration of cell-associated TNF α in the infected liver. Studies are ongoing to define the impacts of T cell-associated and macrophage-associated TNF α on HCV infection. TNF α , by mediating antiviral activities alone and by potentiating the antiviral effects of IFNs, may contribute to the partial control of viral load that is a feature of even chronic HCV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by NIH R01 AI089957 and R01 AI060561 to L.B.D., and R01 CA057973 and R01 AI072613 to C.M.R. Supported in part by grant # UL1 TR001866 from the National Center for Advancing Translational Sciences (NCATS, National Institutes of Health (NIH) Clinical and Translational Science Award (CTSA) program. The human embryonic and fetal material was provided by the Joint MRC/Wellcome Trust (grant #099175/Z/12/Z) Human Developmental Biology Resource (www.hdbi.org). We are grateful for the assistance of Benjamin Haxton and Dr. Patricia Faria-Shayler.

References

1. Mohd Hanafiah K, Groeger J, Flaxman AD, et al. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology*. 2013; 57:1333–42. [PubMed: 23172780]
2. Hajarizadeh B, Grebely J, Dore GJ. Epidemiology and natural history of HCV infection. *Nat Rev Gastroenterol Hepatol*. 2013; 10:553–62. [PubMed: 23817321]
3. Westbrook RH, Dusheiko G. Natural history of hepatitis C. *J Hepatol*. 2014; 61:S58–68. [PubMed: 25443346]
4. Rehermann B. HCV in 2015: Advances in hepatitis C research and treatment. *Nat Rev Gastroenterol Hepatol*. 2016; 13:70–2. [PubMed: 26790365]
5. Cox AL. MEDICINE. Global control of hepatitis C virus. *Science*. 2015; 349:790–1. [PubMed: 26293940]
6. Sulkowski MS, Gardiner DF, Rodriguez-Torres M, et al. Daclatasvir plus sofosbuvir for previously treated or untreated chronic HCV infection. *N Engl J Med*. 2014; 370:211–21. [PubMed: 24428467]
7. Honegger JR, Zhou Y, Walker CM. Will there be a vaccine to prevent HCV infection? *Semin Liver Dis*. 2014; 34:79–88. [PubMed: 24782261]
8. Horner SM, Gale M Jr. Regulation of hepatic innate immunity by hepatitis C virus. *Nat Med*. 2013; 19:879–88. [PubMed: 23836238]
9. Li K, Lemon SM. Innate immune responses in hepatitis C virus infection. *Semin Immunopathol*. 2013; 35:53–72. [PubMed: 22868377]
10. Marukian S, Andrus L, Sheahan TP, et al. Hepatitis C virus induces interferon-lambda and interferon-stimulated genes in primary liver cultures. *Hepatology*. 2011; 54:1913–23. [PubMed: 21800339]
11. Sheahan T, Imanaka N, Marukian S, et al. Interferon lambda alleles predict innate antiviral immune responses and hepatitis C virus permissiveness. *Cell Host & Microbe*. 2014; 15:190–202. [PubMed: 24528865]
12. Dustin LB, Cashman SB, Laidlaw SM. Immune control and failure in HCV infection—tipping the balance. *J Leukoc Biol*. 2014; 96:535–48. [PubMed: 25015956]

13. Faustman D, Davis M. TNF receptor 2 pathway: drug target for autoimmune diseases. *Nat Rev Drug Discov.* 2010; 9:482–93. [PubMed: 20489699]
14. Brenner D, Blaser H, Mak TW. Regulation of tumour necrosis factor signalling: live or let die. *Nat Rev Immunol.* 2015; 15:362–74. [PubMed: 26008591]
15. Wong GH, Goeddel DV. Tumour necrosis factors alpha and beta inhibit virus replication and synergize with interferons. *Nature.* 1986; 323:819–22. [PubMed: 2430188]
16. Mestan J, Brockhaus M, Kirchner H, et al. Antiviral activity of tumour necrosis factor. Synergism with interferons and induction of oligo-2',5'-adenylate synthetase. *J Gen Virol.* 1988; 69(Pt 12): 3113–20. [PubMed: 2462015]
17. Barte E, McFadden G. Cytokine synergy: an underappreciated contributor to innate anti-viral immunity. *Cytokine.* 2013; 63:237–40. [PubMed: 23693158]
18. Gilles PN, Fey G, Chisari FV. Tumor necrosis factor alpha negatively regulates hepatitis B virus gene expression in transgenic mice. *J Virol.* 1992; 66:3955–60. [PubMed: 1583737]
19. Biermer M, Puro R, Schneider RJ. Tumor necrosis factor alpha inhibition of hepatitis B virus replication involves disruption of capsid Integrity through activation of NF-kappaB. *J Virol.* 2003; 77:4033–42. [PubMed: 12634363]
20. Pasquetto V, Wieland SF, Uprichard SL, et al. Cytokine-sensitive replication of hepatitis B virus in immortalized mouse hepatocyte cultures. *J Virol.* 2002; 76:5646–53. [PubMed: 11991993]
21. Lee J, Tian Y, Chan ST, et al. TNF-alpha Induced by Hepatitis C Virus via TLR7 and TLR8 in Hepatocytes Supports Interferon Signaling via an Autocrine Mechanism. *PLoS Pathog.* 2015; 11:e1004937. [PubMed: 26023919]
22. Torre D, Zeroli C, Giola M, et al. Serum levels of interleukin-1 alpha, interleukin-1 beta, interleukin-6, and tumor necrosis factor in patients with acute viral hepatitis. *Clin Infect Dis.* 1994; 18:194–8. [PubMed: 8161626]
23. Tilg H, Wilmer A, Vogel W, et al. Serum levels of cytokines in chronic liver diseases. *Gastroenterology.* 1992; 103:264–74. [PubMed: 1612333]
24. Gonzalez-Amaro R, Garcia-Monzon C, Garcia-Buey L, et al. Induction of tumor necrosis factor alpha production by human hepatocytes in chronic viral hepatitis. *J Exp Med.* 1994; 179:841–8. [PubMed: 7509363]
25. Frese M, Barth K, Kaul A, et al. Hepatitis C virus RNA replication is resistant to tumour necrosis factor-alpha. *J Gen Virol.* 2003; 84:1253–9. [PubMed: 12692291]
26. Fletcher NF, Sutaria R, Jo J, et al. Activated macrophages promote hepatitis C virus entry in a tumor necrosis factor-dependent manner. *Hepatology.* 2014; 59:1320–30. [PubMed: 24259385]
27. MacParland SA, Ma XZ, Chen L, et al. Lipopolysaccharide and Tumor Necrosis Factor Alpha Inhibit Interferon Signaling in Hepatocytes by Increasing Ubiquitin-Like Protease 18 (USP18) Expression. *J Virol.* 2016; 90:5549–60. [PubMed: 27009955]
28. Andrus L, Marukian S, Jones CT, et al. Expression of paramyxovirus V proteins promotes replication and spread of hepatitis C virus in cultures of primary human fetal liver cells. *Hepatology.* 2011; 54:1901–12. [PubMed: 22144107]
29. Catanese MT, Loureiro J, Jones CT, et al. Different requirements for scavenger receptor class B type I in hepatitis C virus cell-free versus cell-to-cell transmission. *J Virol.* 2013; 87:8282–93. [PubMed: 23698298]
30. Marukian S, Jones CT, Andrus L, et al. Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. *Hepatology.* 2008; 48:1843–1850. [PubMed: 19003912]
31. Lindenbach BD, Evans MJ, Syder AJ, et al. Complete replication of hepatitis C virus in cell culture. *Science.* 2005; 309:623–6. [PubMed: 15947137]
32. Lindenbach BD. Measuring HCV infectivity produced in cell culture and in vivo. *Methods Mol Biol.* 2009; 510:329–36. [PubMed: 19009272]
33. McKeating JA, Zhang LQ, Logvinoff C, et al. Diverse hepatitis C virus glycoproteins mediate viral infection in a CD81 dependent manner. *J Virol.* 2004; 78:8496–505. [PubMed: 15280458]
34. Symons JA, Alcamì A, Smith GL. Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity. *Cell.* 1995; 81:551–60. [PubMed: 7758109]

35. Huang J, Smirnov SV, Lewis-Antes A, et al. Inhibition of type I and type III interferons by a secreted glycoprotein from Yaba-like disease virus. *Proc Natl Acad Sci U S A*. 2007; 104:9822–7. [PubMed: 17517620]
36. Mulligan EK, Germer JJ, Arens MQ, et al. Detection and quantification of hepatitis C virus (HCV) by MultiCode-RTx real-time PCR targeting the HCV 3' untranslated region. *J Clin Microbiol*. 2009; 47:2635–8. [PubMed: 19535519]
37. Jones CT, Catanese MT, Law LM, et al. Real-time imaging of hepatitis C virus infection using a fluorescent cell-based reporter system. *Nat Biotechnol*. 2010; 28:167–71. [PubMed: 20118917]
38. Barteo E, Mohamed MR, McFadden G. Tumor necrosis factor and interferon: cytokines in harmony. *Curr Opin Microbiol*. 2008; 11:378–83. [PubMed: 18595771]
39. Croft M, Benedict CA, Ware CF. Clinical targeting of the TNF and TNFR superfamilies. *Nat Rev Drug Discov*. 2013; 12:147–68. [PubMed: 23334208]
40. Brunasso AM, Puntoni M, Gulia A, et al. Safety of anti-tumour necrosis factor agents in patients with chronic hepatitis C infection: a systematic review. *Rheumatology (Oxford)*. 2011; 50:1700–11. [PubMed: 21690185]
41. Ferri C, Ferraccioli G, Ferrari D, et al. Safety of anti-tumor necrosis factor-alpha therapy in patients with rheumatoid arthritis and chronic hepatitis C virus infection. *J Rheumatol*. 2008; 35:1944–9. [PubMed: 18688917]
42. Li S, Kaur PP, Chan V, et al. Use of tumor necrosis factor-alpha (TNF-alpha) antagonists infliximab, etanercept, and adalimumab in patients with concurrent rheumatoid arthritis and hepatitis B or hepatitis C: a retrospective record review of 11 patients. *Clin Rheumatol*. 2009; 28:787–91. [PubMed: 19291350]
43. Idriss HT, Naismith JH. TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc Res Tech*. 2000; 50:184–95. [PubMed: 10891884]
44. Horiuchi T, Mitoma H, Harashima S, et al. Transmembrane TNF-alpha: structure, function and interaction with anti-TNF agents. *Rheumatology (Oxford)*. 2010; 49:1215–28. [PubMed: 20194223]
45. Wherry EJ. T cell exhaustion. *Nat Immunol*. 2011; 12:492–9. [PubMed: 21739672]

Figure 1. TNF α inhibits HCV infection

A, Dose-response experiment. Huh-7 cells (n = 3 wells/point) were incubated with the indicated doses of TNF α beginning 16 hours before addition of HCVcc (Jc1G), and treatment continued throughout the experiment. Media were sampled at the indicated times for luciferase. Raw data (mean $\pm$ SD, N=3) are shown in the top graph; percentages shown in the bottom graph were calculated from data at 96 hours after infection using the formula

$$\% \text{ of untreated} = 100 \times \left(\frac{RLU_{\text{treated}}}{RLU_{\text{control}}} \right).$$

B, Flow cytometric enumeration of infected

cells. Huh-7 cells (n = 3) were treated with the indicated doses of TNF α beginning 16 hours before infection with Jc1G (MOI= 0.1) or Clone 2 (MOI= 0.01) viruses. At the indicated times cells were harvested, fixed, and stained for NS5A. C, Schematic of pretreatment time course (results in panel D). D, Cells were treated with TNF α (20 ng/ml) or media beginning at the indicated times before addition of virus at time 0. Additional cells were treated with TNF α only after addition of virus. Supernatants were sampled for luciferase measurement. N = 3. E, Effects of TNF receptor blockade. Huh-7 cells (n=6 wells/condition) were treated with TNF α (20 ng/ml) $\pm$ 1 μ g/ml etanercept before and during infection with Jc1G. F, Effects of TNF α , etanercept, and danoprevir on HCV infection in HFLC. Cells (n=6 wells/condition) were treated with TNF α (20 ng/ml) $\pm$ etanercept (1 μ g/ml), danoprevir (2 μ M), or media control before and during infection with Jc1G. Secreted luciferase was measured 96 hours after the start of infection. Box-and-whiskers plot; whiskers represent minimum and maximum values. Values of p were calculated by two-way ANOVA (panel A, top, and panel D) and one-way ANOVA (panel A, bottom, and panels E and F). ***, p < 0.001; ****, p < 0.0001.

Figure 2. TNF α has little effect on HCV replication or entry

A, B, HCV replicons. Huh-7 cells were treated overnight with TNF α (20 ng/ml), IFN β (5 U/ml), or media as indicated, electroporated with 5 μ g of replicon or NS5B mutant (GNN) RNA, and culture continued in the presence or absence of TNF α /IFN β as indicated. A, Subgenomic JFH1 replicon. Secreted luciferase was measured at 24-hour intervals. B, Full-length HCV replicons (no reporter). NS5A⁺ cells were measured by flow cytometry at days 5 and 10. C, HCV pseudoparticle entry. Huh-7 cells (4 wells/point) were treated overnight with TNF α or media control. Anti-CD81 was added as indicated 2 hours before addition of

pseudoparticles bearing envelope proteins from HCV (H77 or Con1) or control RD114, as indicated. After 6 hours, cultures were washed 3× and culture continued in complete medium. Secreted luciferase was measured 72 hours after infection.

Figure 3.

Spread of infection in Huh-7.5 cells expressing an HCV-dependent fluorescent reporter. Cells were cultured in complete media $\pm$ 20 ng/ml TNF α as indicated. See Methods for details. Cells were fixed and stained with DAPI at the indicated times after infection. RFP localizes to the nucleus in infected cells. Magnification = 10 $\times$; scale bars = 100 μ m.

Figure 4. Effects of TNFα on HCV spread and infectious virus production

A, Infected cells expressing the HCV-dependent fluorescent reporter were counted, as detailed in Methods, at the indicated times after the start of infection (n=6 wells/condition/ timepoint). B, Focus size was estimated by counting numbers of adjacent RFP⁺ nuclei in the experiment shown in panel A (n=6). Infected foci were not counted after 48 hours as they had begun to merge together. C, Levels of infectious extracellular and intracellular virus in Huh-7 cells cultured without or with 20 ng/ml TNFα. Cells (1.4×10⁵/well) were cultured in 6-well plates and treated with TNFα for 16 hours before the start of infection. HCVcc Clone

2 was added to wells at MOI = 0.01. Wells were washed (3×) 6 hours after the start of infection. Virus was collected from supernatants and lysed cells at the indicated times. E, HCV growth and levels of infectious virus in HFLC cultured with or without 20 ng/ml TNFα. Cells (2×10⁵/well) were grown in collagen-coated 24 well plates. After 5 days, cells were treated for 16 hours with medium ± 20 ng/ml TNFα. Virus (Jc1G, *left*; Clone 2, *right*) was then added at MOI = 10 for 6 hours; cells were then washed extensively and culture continued in medium without or with TNFα. Supernatants were collected at 24-hour intervals for measurement of luciferase (*left*) and infectious virus (*right*). Fluids were pooled for the TCID₅₀ assay. We were unable to measure infectious virus in cell lysates.

Figure 5. TNF α can act independently of IFNs

A, Effects of IFN α (100 U/ml), TNF α (20 ng/ml), IFN λ 1 (10 ng/ml), and antagonists on HCV infection in Huh-7 cells. Cells (n= 4 wells/point) were cultured without or with cytokines $\pm$ the antagonists B18R, 136R, ruxolitinib, etanercept, or B18R + 136R, as indicated. HCVcc Jc1G was added at MOI = 0.1 for 6 hours, cultures washed and media $\pm$ cytokines $\pm$ antagonists replaced. Culture was continued for 96 hours and supernatants sampled at 96 hours for measurement of luciferase. Values of p were calculated by one-way ANOVA using “cytokine only” for each graph as control; ****, $p < 0.0001$. B, HCV

infection in HFLC treated with 3 μ M ruxolitinib $\pm$ 20 ng/ml TNF α . Cells were infected with HCVcc Jc1G or clone 2 (MOI = 10) for 6 hours, then washed extensively and culture was continued in the presence of ruxolitinib $\pm$ TNF α as indicated. Supernatants were collected at 24-hour intervals for measurement of luciferase and cell-free infectious virus. C, Expression of IFN genes. HFLC (n= 3 wells/point/timepoint) were treated with TNF α (10 ng/ml), IFN β (10 U/ml), IFN λ 1 (0.1 ng/ml), or polyI:C (5 μ g/ml) as indicated. RNA was prepared at the indicated times and subjected to quantitative RT-PCR.

Figure 6. TNF α increases the effects of IFNs

A, Effects of simultaneous treatment with TNF α and cytokines. Cells ($n = 2$ wells/point) were treated with TNF α at the indicated doses without or with IFNs as indicated. HCVcc (Jc1G, MOI = 0.1) was added for 6 hours; cells were then washed extensively and cytokines replaced. Supernatants were collected for luciferase measurement 72 hours post infection. Values of p were calculated by 2-way ANOVA and refer to the interaction between TNF α and each IFN. B, Effects of sequential treatment with TNF α and IFNs. Cells ($n = 5$ wells/point) were pretreated for 16 hours with no cytokine, TNF α (20 ng/ml), or IFN α (100 U/

ml), as indicated (Cytokine 1). They were infected with HCVcc (Jc1G, MOI= 0.1) for 6 hours, washed extensively, and then cultured with no cytokine (white bars), TNF α (red bars), IFN α (black bars), or TNF α + IFN α (red/black striped bars) as indicated (Cytokine 2) for the remainder of the experiment. Media were sampled for luciferase measurement after 96 hours. Fold change was calculated using the equation:

$$\text{Fold change} = \left(\frac{RLU_{\text{treated}}}{RLU_{\text{untreated}}} \right).$$
 Values of p were calculated by 2-way ANOVA and refer to the interaction between cytokines 1 and 2.