Hepatitis C virus targets the interferon-α JAK/STAT pathway by promoting proteasomal degradation in immune cells and hepatocytes

Nigel J. Stevenson a,n,⇑, Nollaig M. Bourke a,⇑, Elizabeth J. Ryan a, Marco Binder b, Liam Fanning c, James A. Johnston d, John E. Hegarty e, Aideen Long f,g, Cliona O’Farrelly a,g

a School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin (TCD), Dublin, Ireland
b Department of Infectious Diseases, Heidelberg University, Germany
Molecular Virology Diagnostic & Research Laboratory, University College Cork, Cork, Ireland
c Centre for Infection and Immunity, School of Medicine, Dentistry and Biomedical Sciences, Queen’s University Belfast, UK
d National Liver Transplant Unit, St. Vincent’s University Hospital, Dublin, Ireland
f Institute of Molecular Medicine, TCD, Ireland
g School of Medicine, TCD, Ireland

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Abstract
JAK/STAT signalling is essential for anti-viral immunity, making IFN-α an obvious anti-viral therapeutic. However, many HCV+ patients fail treatment, indicating that the virus blocks successful IFN-α signalling. We found that STAT1 and STAT3 proteins, key components of the IFN-α signalling pathway were reduced in immune cells and hepatocytes from HCV infected patients, and upon HCV expression in Huh7 hepatocytes. However, STAT1 and STAT3 mRNA levels were normal. Mechanistic analysis revealed that in the presence of HCV, STAT3 protein was preferentially ubiquitinated, and degradation was blocked by the proteasomal inhibitor MG132. These findings show that HCV inhibits IFN-α responses in a broad spectrum of cells via proteasomal degradation of JAK/STAT pathway components.

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1. Introduction

The anti-viral properties of interferon (IFN)-α have made it an obvious therapeutic agent to combat HCV infection, a major cause of chronic liver disease, hepatocellular carcinoma and liver transplantation [1]. Unfortunately, IFN-α therapy has a high failure rate, particularly in patients infected with genotype 1, with as few as 30% responding to treatment, suggesting that HCV might target this pathway [2]. Several molecular mechanisms by which HCV evades the immune response have already been described. HCV blocks adaptive immunity, by stimulating specific T regulatory cells [3], as well as inhibiting normal T cell migration and IL-2 secretion [4,5]. More recently, attention has turned to the innate immune response and HCV has been shown to target NK cell activity, dendritic cell function [6,7] and pathogen recognition pathways, including inhibition of Toll-Like Receptor (TLR)-3 and retinoic acid-inducible gene (RIG)-1 signalling [8]. However, the reasons for non-response to endogenous or therapeutic IFN-α remain poorly defined.

Anti-viral signalling commences upon IFN-α engagement with its receptor, thus activating tyrosine kinases, which phosphorylate receptor docking sites for cytoplasmic signal transducer and activator of transcription (STAT) proteins. STATs become phosphorylated, form dimers and translocate to the nucleus, where they bind specific DNA-recognition motifs, promoting gene transcription. Disruption of STAT genes in mice has confirmed that these proteins are essential in several biological processes, including cell growth and differentiation, as well as immune defence [9,10]. IFN-α signalling via STAT proteins leads to the upregulation of over 500 IFN Sensitive Genes (ISGs), including key anti-viral and pro-inflammatory mediators [11–14].

Signal transduction is often regulated by the homeostatic process of protein turnover via degradation of poly-ubiquitinated proteins through the 26S proteasome. Suppressor of cytokine signalling (SOCS) proteins associate with elongin B, elongin C, cul-lin (cul) and the RING finger-containing protein, to form functional Elongin B/C-cul-SOCS box (ECS)-like E3 ligase complexes, that catalyse ubiquitin transfer to target proteins [15]. Viral proteins regularly act as E3 ligase proteins; in fact, we and others have shown...
that Paramyxoviruses, such as Respiratory Syncytial Virus (RSV), target STAT proteins for degradation via the proteosome [16–18].

Since many HCV-infected patients do not respond to IFN-α therapy we investigated the status of the JAK/STAT pathway in both primary immune cells and hepatocytes from HCV infected patients. We found that STAT1 and STAT3 proteins were reduced in all major immune cell populations and hepatocytes from HCV+ patients. Furthermore, we discovered that STAT3 was preferentially ubiquitinated and targeted for proteosomal degradation in the presence of HCV. These findings demonstrate for the first time that essential anti-viral components of the IFN-α pathway are reduced in both immune cells and hepatocytes, revealing a novel mechanism by which HCV blocks responsiveness to IFN-α therapy.

2. Patients

Blood from 17 patients (15 female, 2 male, ranging between 32 and 71 years of age) who were PCR positive for HCV (Supplementary Table 1) and had not undergone IFN-α/Ribavirin standard treatment of care, were studied. All were informed and gave written consent. The study received ethical approval from the Research and Ethics Committee at SVUH, in accordance with the guidelines of the 1975 Declaration of Helsinki. Patients were tested for HCV antibodies using enzyme immunoassays (Abbott) and immunoblot assays (Chiron Corp., USA).

3. Materials and methods

3.1. PBMC isolation

PBMCs were isolated from 30 ml of blood by centrifugation (650g for 30 min) over Ficoll (Amersham, UK) and washed X2 (290g for 10 min) with RPMI.

3.2. HCV RNA detection in PBMCs

PBMCs (resuspended in stabilising solution RNAlater (Ambion, Amersham, UK)) were lysed according to the Magna Pure external lysis protocol (650/60°C for 30 min) over Ficoll (Amersham, UK) and washed X2 (290g for 10 min) with RPMI.

3.3. Cell culture

Huh7 cells were grown in DMEM supplemented with 6 μg/ml zeocin. PBMCs were cultured in RPMI containing 2% FCS, 250U/ml penicillin and 250 μg/ml streptomycin. Cells were treated with 1000 U/ml human IFN-α (Roche, Switzerland) and 10 μM MG132 (Calbiochem, USA).

3.4. Transfection

Huh7 cells expressing T7 polymerase were transfected for 16 h using Lipofectamine 2000 (Invitrogen, USA), with 0.75 μg (6 well plates) or 5 μg (9 cm diameter plates) of pBRTM/HCV1-3011 DNA construct (a kind gift from Prof. Charles Rice, The Rockefeller University) or EV (Invitrogen, USA) or with 0.1 μg of DNA in poly-o-lysine slides (Becton Dickson, USA). Huh7 cells were also transfected with 5 μg HA-tagged-Ubiquitin or EV for 48 h using Lipofectamine 2000.

3.5. Immunoprecipitation

Huh7 cells, transfected with EV/HCV (5 μg) +/−MG132 treatment, or transfected with HA-Ubiquitin and HCV, were harvested in mild lysis buffer (50 mM Heps, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40) or RIPA buffer, substituted with aprotenin [5 μg/ml], leupeptin [5 μg/ml], PMSF [1 mM], Na3VO4 [1 mM]). Lysates were immunoprecipitated with protein-A agarose beads and STAT3 antibody, before immunoblotting for HA (Sigma, USA), ubiquitin or STAT3 (Santa Cruz Biotechnologies, USA).

3.6. Immunoblotting

Cells were harvested in lysis buffer and analysed by immunoblotting using pSTAT1, pSTAT3, STAT1 (New England Biolabs, USA), STAT3, ubiquitin (Santa Cruz Biotechnologies, USA), HCV-N52, b-actin, γ-tubulin, HA (Sigma, USA), HCV-E2 (Acris, USA), HCV-Core (Abcam, UK) and secondary antibodies labelled with infrared dyes and the infrared Odyssey imager. Densitometry was calculated using LI-COR software. (LI-COR, Germany).

3.7. RT-PCR

RNA was isolated from cells following the Trizol manufacturer’s protocol (Invitrogen, USA). RT-PCR was performed using the One-step protocol (Qiagen, Germany). Cycling parameters: 30 min at 50°C, 95°C for 15 min and 35 cycles of 94°C, 58°C and 72°C for 30 s, 2 min for 10 min. qRT-PCR: as described before [19]. Human gene amplifications normalised to Ribosomal protein 15 (RPS15); Primers listed in Table 1.

3.8. HCV infection

Virus stock production as described previously [20]. In vitro transcribed Jc1 RNA was electro-transfected into Huh7.5 cells and supernatants collected 24, 48 and 72 h post transfection, pooled and infectious titer determined by TCID50. Huh7.5 cells were inoculated with infectious supernatants (MOI = 3) for 24 h, washed with DMEM and treated with MG132 or DMSO. After 2 h, cells were harvested and lysed in 50 μl RIPA.

3.9. Immunohistochemistry

Liver sections (8 μm) were cut from paraffin-embedded, formalin-fixed samples and microwaved in a pressure cooker in sodium citrate buffer (27 ml 0.1 M citric acid, 123 ml 0.1 M tri sodium citrate in 1500 ml dH2O, pH 6). Sections were stained using STAT3(Santa Cruz Biotechnologies, USA) and STAT1 (New England Biolabs) antibodies (1:80) for 1 h in antibody diluent (DAKO, Denmark) and detected using a polymer detection kit (Leica Microsystems, Germany) and analysed by microscopy.

3.10. Immunofluorescence

As described previously [19], antibodies included anti-STAT3 (Santa Cruz Biotechnologies, USA) labelled with Alexa 568 or 546.
Fig. 1. STAT1 and STAT3 protein levels are reduced in PBMCs from HCV+ patients. (A) Lysates from PBMCs of individual HCV+ or healthy subjects, treated –/+ IFN-α for 15 min, were analysed by immunoblotting for total or phosphorylated STAT1, STAT3 and β-actin (n = 3). STAT1 and STAT3 mRNA levels in PBMCs from HCV+ and healthy individuals were measured by (B) qualitative RT-PCR (HCV n = 6; healthy n = 4) and (C) quantitative qRT-PCR (healthy n = 7; HCV n = 12). (D) Ratio of STAT3: house-keeping protein optical density (OD) measured by densitometric analysis of immunoblots of STAT3 and β-actin or γ-tubulin protein levels in PBMCs from HCV+ (n = 17) and healthy (n = 8) individuals. (E) Immunoblot of HCV-NS2 and β-actin from healthy (H) (n = 3) and HCV+ patient PBMC lysates (n = 3). (F) qRT-PCR detection of HCV RNA in PBMCs from 5 HCV RNA+ individuals (patient numbers 1–5 inclusive), 2 HCV RNA negative patients (patient 6 spontaneously cleared HCV and patient 7 cleared HCV during IFN-α/ribavirin therapy) and 2 healthy controls (patient numbers 8 and 9).
(Invitrogen, USA); HCV-Core (Abcam, USA) and FITC secondary (Sigma, USA) were used. DNA was stained using Prolong gold anti-fade reagent containing DAPI (Invitrogen, CA, USA). HCV-E2 (Acris, USA) and Alexa 546 or 488 secondary; CD56, CD3 and Alexa 647 secondary and FITC-conjugated antibodies to: CD19, CD14, and HLA-DR (BD biosciences, USA) were used. Microscopy was performed on an Olympus FV1000 laser scanning confocal microscope, using an UPlanSAPO 60X/1.35 NA oil objective at room temperature. Cells were digitally sectioned by confocal laser scanning fluorescence microscopy at 0.3 μm per slice and analyzed using the Olympus FV1000 software.

3.11. Flow cytometry

PBMCs were surface labelled with FITC-conjugated antibodies to: CD45, CD19, CD14, CD16 and HLA-DR; PerCP-conjugated anti-CD3 or Alexa-Fluor 488-conjugated anti-CD56 (BD Biosciences, USA). Cells were fixed with 4% paraformaldehyde for 30 min on ice, washed X2 in PBS and resuspended in permeabilisation buffer (eBioscience). Cells were incubated with STAT1-PE and STAT3-APC (BD Biosciences, USA) for 30 min, washed in PBS and acquired using a Dako Cyan flow cytometer. Data was analysed using Flowjo software (Tree Star Inc, USA).

Fig. 2. STAT1 and STAT3 proteins are reduced in major immune cell lineages of HCV+ patients. PBMCs from healthy (n = 3) or HCV+ individuals (n = 7) were labelled with CD45, CD3, CD19, CD56, CD14, CD16 and HLA-DR antibodies. Cells were fixed, permeabilised and stained with (A) STAT1 and (B) STAT3 antibodies. Representative dot plots of a healthy and a HCV+ individual shown. (C) Confocal micrographs of PBMCs from HCV+ individuals labelled with HCV-E2 along with FITC labelled CD19, HLA-DR and CD14 or CD56 and CD3 primary antibodies (n = 2). Nucleus labelled with DAPI. Bar, 10 μm.

3.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, La Jolla, CA, USA). Groups were compared by paired Student's T-test for parametric samples with Gaussian distribution, non-parametric Mann-Whitney analysis for patient samples and ANOVA with Bonferroni's multiple comparison post-test analysis as appropriate. *P < 0.05, **P < 0.01, ***P < 0.001.

4. Results

4.1. STAT1 and STAT3 proteins are reduced in PBMCs from HCV+ individuals

To investigate the status of the JAK/STAT pathway in HCV patients, we initially analysed STAT1 and STAT3 tyrosine phosphorylation and protein levels in human PBMCs from HCV positive (+) and healthy individuals. We found that protein levels were lower in PBMCs from HCV+ patients compared to healthy controls (Fig. 1A), and IFN-α-induced tyrosine phosphorylation of both STAT1 and STAT3 proteins were lower, although mRNA levels did not differ significantly (Fig. 1B and C). Since this was the first demonstration of STAT3 protein reduction in HCV infected cells, protein levels were investigated in 16 additional treatment-naïve HCV+ (genotype 1) patients and 8 healthy controls. Densitometric analysis revealed that STAT3 protein levels were significantly lower in PBMCs from HCV+ patients compared to healthy controls (P < 0.001) (Fig. 1D). Interestingly, we found HCV-NS2 protein by immunoblotting (Fig. 1E) and HCV RNA by qRT-PCR (Fig. 1F) within PBMCs from HCV+ patients, indicating that intracellular HCV may directly act to promote loss of STAT proteins.

4.2. STAT1 and STAT3 proteins are reduced in all major immune cell populations

To determine if STAT1 and STAT3 in PBMCs were depleted from all major immune cell types, we analysed protein levels within specific immune cell populations using flow cytometry. Normal levels of STAT1 (Fig. 2A) and STAT3 (Fig. 2B) were observed in all the major peripheral blood cell subsets from healthy controls, but were significantly reduced in all cell subsets from HCV+ patients, including monocytes (CD14+), T cells (CD3+), B cells (CD19+), natural killer (NK) cells (CD56+), and HLA-DR+ antigen presenting cells (APCs), demonstrating that loss of STAT protein expression was not limited to any particular cell type (Fig. 2A and B). Furthermore, HCV was detected by confocal microscopy in all major immune cells types, demonstrating that its potential to directly target intracellular STAT expression (Fig. 2C).

4.3. HCV-mediated loss of STAT1 and STAT3 in hepatocytes and liver infiltrating leukocytes

To determine the effect of HCV upon STAT1 and STAT3 levels in liver cells, we next analysed their expression in Huh7 hepatocytes transfected with a HCV DNA construct. We found considerably reduced levels of STAT1 and STAT3 protein (Fig. 3A) in HCV transfected hepatocytes and as in PBMCs, mRNA levels were not significantly different (Fig. 3B). We further analysed STAT1 and STAT3 in primary human liver tissue by immunohistochemistry and found STAT1 and STAT3 levels greatly reduced in hepatocytes and infiltrating leukocytes of HCV+ livers, compared to healthy controls (Fig. 3C). Loss of STAT1 and STAT3 proteins in PBMCs, Huh7 hepatocytes, primary hepatocytes and liver infiltrating leukocytes demonstrates a conserved reduction across a spectrum of cell types and reveals STAT3, for the first time, as a target for HCV’s immune evasion mechanisms.

4.4. HCV promotes STAT3 degradation via the proteasome

We and others have shown that the ubiquitin/proteasome system is used by viruses to degrade STAT proteins [17,18] and since STAT1 had already been shown to be proteasomally degraded by HCV [21], we investigated the molecular mechanism responsible for the absence of STAT3 by blocking proteasomal activity with the inhibitor, MG132. Using immunoblotting and confocal microscopy, we found that absent STAT3 protein could be recovered in PBMCs from HCV+ patients following proteasomal inhibition (Fig. 4A and B). To determine if the loss of STAT3 in HCV transfected hepatocytes was also proteasome-dependent, we transfected Huh7 hepatocytes with a HCV construct and treated with MG132. We observed that HCV-mediated degradation of STAT3 was blocked by proteasomal inhibition (Fig. 4A and B). While HCV infection with cell cultured HCV (HCVcc) had no effect on STAT3 expression (Fig. 4C and D). While HCV infection in cell cultured HCV (HCVcc) had no effect on STAT3 mRNA expression (Fig. 4E), it promoted STAT3 protein degradation, which was inhibited by MG132, further confirming a HCV-controlled mechanism of STAT3 degradation via the proteasome (Fig. 4F).

4.5. HCV enhances ubiquitination of STAT3

Since ubiquitinated proteins are targeted for proteasomal degradation, we analysed protein ubiquitination in HCV expressing Huh7s. We found that endogenous total protein ubiquitination in
HCV promotes STAT3 ubiquitination and proteasomal degradation. (A) STAT3 immunoblot and (B) confocal micrograph of PBMCs from HCV+ and healthy individuals treated −/+ MG132 for 4 h (n = 3). STAT3 and the nucleus labelled with FITC and DAPI, respectively. Bar, 10 µm. (C) Immunoblot of STAT3, γ-tubulin and HCV-NS2 protein expression, −/+ HCV-DNA transfection of Huhe7 hepatocytes, −/+ MG132 (n = 3). (D) Confocal micrograph of STAT3, HCV-Core and the nucleus (N), labelled with FITC, Alexa 568 and DAPI respectively, −/+ HCV construct expression, alongside MG132 treatment (n = 3). Bar, 10 µm. (E) STAT3 mRNA from Huhe7 cells mock or HCVcc infected (n = 6). (F) Immunoblot of STAT3, HCV-Core and γ-tubulin proteins from Huhe7 cells mock or HCVcc infected and treated −/+ MG132 for 4 h (n = 4). (G) Immunoblot of Huhe7 cells mock or HCVcc infected and treated −/+ MG132 for 4 h, whole cell lysates (WCLs) probed with ubiquitin and γ-tubulin antibodies (n = 3). Values below the blot represent densitometric analysis. Ubiquitinated protein band intensity was normalised to γ-tubulin band intensity and all values are presented relative to uninfected control cells (lane 1), which was normalised to 1. (H) Huhe7 cells mock or HCVcc infected treated with MG132 for 4 h were lysed and immunoprecipitated for STAT3 and immunoblotted for ubiquitin. (I) Huhe7 cells transfected −/+ HCV-DNA, Ubiquitin-HA or EV constructs and treated with MG132 for 4 h were lysed, immunoprecipitated for STAT3 and immunoblotted for HA and STAT3. WCLs probed for HCV-E2 and β-actin (n = 3).
HCVcc infected cells was enhanced, compared to mock infected controls and treatment of HCV infected cells with MG132 further increased protein ubiquitination (Fig. 4G). To determine if STAT3 ubiquitination was specifically enhanced by HCV, lysates from Huh7 cells infected with HCVcc infected hepatocytes, –/+ MG132, were immunoprecipitated for STAT3 and immunoblotted for ubiquitin. While proteasomal inhibition with MG132 increased levels of ubiquitinated STAT3 (Fig. 4H, lane 4), HCV infection further enhanced levels of ubiquitinated STAT3, as seen by the presence of multiple bands increasing in molecular weight, characteristic of polyubiquitination (Fig. 4H, lane 3). To further analyse the specific ubiquitination of STAT3, we over-expressed hemagglutinin (HA)-tagged ubiquitin, –/+ transfection with HCV DNA construct, in Huh7 cells, before immunoprecipitating for STAT3 and immunoblotted for ubiquitin. Once again, STAT3 ubiquitination was enhanced in HCV expressing cells, compared to controls (Fig. 4I). These results indicate that HCV promotes the direct ubiquitination of STAT3, revealing the novel mechanism by which HCV promotes STAT3 protein degradation via the proteosome.

5. Discussion

This study describes the loss of STAT1 and STAT3 from HCV-infected immune cells and hepatocytes, and the mechanism by which HCV promotes STAT3 degradation through the proteosome, a process also used by other viruses, such as RSV, to evade immunity [17].

Since HCV is hepatotrophic, previous studies have focused on signalling pathways within hepatocytes [22]. However, because circulating and liver infiltrating immune cells play such a vital role in fighting HCV infection [23] and HCV has also been identified in immune cells [24], we investigated the IFN-α JAK/STAT pathway in the whole PBMC population and the major individual immune cell subgroups. In fact, we found that STAT1 and STAT3 proteins were absent from circulating and liver infiltrating immune cells of HCV patients, demonstrating widespread disruption of the anti-viral IFN-α pathway, which identifies the broad immune evasion strategy of HCV and widens our understanding of this virus’s spectrum of target cells.

Although STAT3 is known to be activated by IFN-α, its role in this pathway remains poorly understood. Several studies, using transformed or primary hepatocytes, suggest that HCV either activates STAT3 [25–29], has no impact [30–32] or blocks DNA binding [33]. However, this study indicates that HCV decreases STAT3 protein in PBMCs, liver infiltrating leukocytes and hepatocytes from infected patients, as well as Huh7 hepatocytes transfected or infected with HCV. These data support the results of studies showing decreased STAT3 mRNA and protein in the HCV replicon system [34,35]. However, unlike these previous reports, we discovered that HCV does not target STAT3 mRNA. Rather, we found that STAT3 mRNA in Huh7 cells was elevated upon HCV expression, suggesting a homeostatic mechanism that attempts to replace the STAT3 protein lost during HCV-mediated proteosomal degradation.

STAT3 is well known to promote cell proliferation and thus tumour progression [36]; properties that are demonstrated in the liver, with increases in hepatic STAT3 activation linked to liver regeneration, fibrosis and hepatocellular carcinoma [25,37]. However, even though STAT1 is well documented as an anti-viral mediator [38], as evidenced by STAT1 KO mice lacking IFN-α responsiveness and exhibiting increased susceptibility to viral infection [9,39], STAT3’s role in anti-viral signalling is disputed. Interestingly STAT3 was ruled out as an anti-viral mediator because STAT3 homo-dimers remain inducible in STAT1 +/- mice that succumb to viral infections. However, STAT1:STAT1 homo-dimers and STAT1:STAT3 dimers also could not form in STAT1 +/- mice [9], indicating a possible role for this hetero-dimer complex in anti-viral signalling. The functions of these STAT1:STAT3 hetero-dimers are not known, however the loss of STAT1 and STAT3 in HCV-infected cells may further indicate their important role during anti-viral signalling.

In conclusion, we have shown that both STAT1 and STAT3 are reduced in primary immune cells and hepatocytes of HCV+ patients and upon in vitro HCV expression or infection of hepatocytes. We have also provided evidence of HCV-mediated ubiquitination and proteasomal degradation of STAT3, which outlines a basic viral immune evasion mechanism used by HCV to block the IFN-α JAK/STAT pathway in immune cells and hepatocytes. This broad HCV-mediated immune evasion strategy may explain poor responsiveness to therapeutic IFN-α and highlights a potential target pathway for therapeutic restoration of anti-viral immune responses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.f礼拜i.2013.03.041.

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