HHS Public Access

Author manuscript

Alcohol Clin Exp Res. Author manuscript; available in PMC 2019 October 18.

Published in final edited form as:

Alcohol Clin Exp Res. 2016 November ; 40(11): 2329–2338. doi:10.1111/acer.13226.

Acetaldehyde Disrupts Interferon Alpha Signaling in Hepatitis C Virus Infected Liver Cells by Up-Regulating USP18

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Abstract

Background: Alcohol consumption exacerbates the pathogenesis of HCV-infection and worsens disease outcomes. The exact reasons are not clear yet, but they might be partially attributed to the ability of alcohol to further suppress innate immunity. Innate immunity is known to be already decreased by HCV in liver cells.

Methods and Results: In this study, we aimed to explore the mechanisms of how alcohol metabolism dysregulates IFNα signaling (STAT1 phosphorylation) in HCV^+ hepatoma cells. To this end, $CYP2E1^+$ Huh7.5 cells were infected with HCV and exposed to the acetaldehyde generating system (AGS). Continuously produced acetaldehyde suppressed IFNα-induced STAT1 phosphorylation, but increased the level of a protease, USP18 (both measured by Western blot), which interferes with IFNα signaling. Induction of USP18 by acetaldehyde was confirmed in primary human hepatocyte cultures and in livers of ethanol-fed HCV transgenic mice. Silencing of USP18 by specific siRNA attenuated the pSTAT1 suppression by acetaldehyde. The mechanism by which acetaldehyde downregulates pSTAT1 is related to an enhanced interaction between IFNαR2 and USP18 that finally dysregulates the cross-talk between the IFN receptor on the cell surface and STAT1. Furthermore, acetaldehyde decreases ISGylation of STAT1 (protein conjugation of a small ubiquitin-like modifier, ISG15, Western blot), which preserves STAT1 activation. Suppressed ISGylation leads to an increase in STAT1 K48 polyubiquitination which allows pSTAT1 degrading by proteasome. We **conclude** that acetaldehyde disrupts IFNα-induced STAT1 phosphorylation by the upregulation USP18 to block the innate immunity protection in HCV-infected liver cells, thereby contributing to HCV-alcohol pathogenesis. This, in part, may explain the mechanism of HCV-infection exacerbation/progression in alcohol-abusing patients.

Keywords

alcohol; USP18; ISGylation; acetaldehyde; HCV; IFNα signaling

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INTRODUCTION

It is known that alcohol consumption exacerbates liver injury and worsens outcomes in patients infected with HCV. The suggested scenario of these events involves HCV and ethanol-induced suppression of immune responses and particularly, of the innate immunity that controls viral replication and HCV dissemination. The host antiviral protection is based on many factors, including interferon type 1 signaling in infected cells. Using the HCV-infected Huh 7.5 cells, we have shown previously that ethanol metabolites (acetaldehyde, Ach) decrease the downstream events of IFNα-induced signaling via the JAK-STAT1 pathway by suppressing STAT1 methylation required for STAT1 attachment to DNA followed by activation of anti-viral genes (Ganesan et al., 2015). Furthermore, in addition to inhibition of STAT1 phosphorylation by HCV (Lan et al., 2007, Kumthip et al., 2012, Lin et al., 2006), CYP2E1-mediated ethanol metabolism also suppressed IFNα signaling (McCartney et al., 2008). However, the mechanisms of additive/synergistic effects of HCV and ethanol metabolites on STAT1 phosphorylation are not clearly understood.

Multiple suppressive mechanisms regulate IFN-induced STAT1 phosphorylation. Most of them are related to induction of negative regulators of IFN signaling, such as SH2-containing phosphatases (SHP1 and SHP2) anchoring to the IFN receptor and dephosphorylating Tyr 701 (Stephanou and Latchman, 2003). In addition, there are suppressors of cytokine signaling 1 and 3 (SOCS1 and 3) that block STAT1 phosphorylation at the level of JAKs (i.e. upstream from STAT1 phosphorylation) (Wormald and Hilton, 2004). An enhancement of ubiquitin-specific protease 18 (USP18) is another possible mechanism for decreasing STAT1 activation. Most likely, USP18 reverses STAT1 posttranslational modification (ISGylation) by de-conjugation of a small ubiquitin-like protein, ISG15 that is increased in response to IFNα treatment. ISGylation stabilizes and prolongs the IFNα signal. Since ISGylation antagonizes ubiquitination to prevent degradation of phosphorylated (p) STAT1 via the ubiquitin-proteasome pathway (Desai et al., 2006), de-ISGylation of the substrate proteins by USP18 may direct the ubiquitinated pSTAT1 for cleavage by the 26S proteasome. In addition, USP18 can suppress IFNα signaling due to interactions with IFNαR2 by disrupting its JAK1 binding, which is upstream from STAT1 phosphorylation, (Malakhova et al., 2006). Overexpression of USP18 is observed in HCV-positive mice/cells and promotes HCV replication (Randall et al., 2006, Randall et al., 2007).

In this study, we hypothesize that in HCV-infected cells, Ach induces USP18, which suppresses IFN-induced STAT1 phosphorylation, thereby interfering with STAT1 ISGylation to destabilize pSTAT1. Here, we demonstrate that USP18 expression is increased in HCV^+ infected $CYP2E1^+$ Huh7.5 (RLW) cells exposed to Ach and that silencing of USP18 attenuates Ach-mediated suppression of pSTAT1. Induction of USP18 by ethanol metabolism was also reproduced in HCV^+ human hepatocytes and in the livers of HCV^+ transgenic (HCV-Tg) mice fed ethanol diet. The reduction of STAT1 phosphorylation by Ach, on one hand, occurs due to enhanced attachment of USP18 to IFNαR2, attenuating IFNα-induced STAT1 activation (pSTAT1) and on the other hand, is due to increased

pSTAT1 ubiquitination linked to a suppressed STAT1 ISGylation that marks pSTAT1 for proteasomal degradation.

MATERIALS AND METHODS

Reagents and Antibodies

High glucose Dulbecco's Modified Eagle Medium (DMEM), Williams Medium and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Human recombinant interferon alpha (IFNα), antibodies to the IFNαR2 and IFNαR2-PE were from Miltenyi Biotech Inc (MACS, CA). Antibody to phosphorylated STAT-1 (Tyr 701), K48 linked polyubiquitin, ISG15, humanUSP18 and TRIM25 were from Cell Signaling (Beverly, MA); antibodies to the STAT-1, mouse USP18, β-actin and IFNαR2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). USP18 siRNA, control siRNA and the transfection reagents were from OriGene (Rockville, MD). PCR reagents, probes and primers were from Life Technologies, Inc. Other reagents, all of analytical grade quality, were from Sigma (St. Louis, MO).

Hepatoma Cells

Huh7.5 cells (obtained from Dr. Charles M. Rice, The Rockefeller University, New York, USA) were transfected with pIV-G2 (CYP2E1) plasmid as previously described for other cell lines (Donohue et al., 2006, Osna et al., 2003) using Lipo TAXI (Invitrogen Corp., Carlsbad, CA). Recombinant cells were selected in culture medium containing G418 at 400μg/ml. Clones were expanded and screened for CYP2E1expression and activity. The clone of Huh7.5 cells with the highest $CYP2E1^+$ expression was designated as RLW cells. However, Huh7.5 cells do not express alcohol dehydrogenase (ADH) that metabolizes ethanol to acetaldehyde, and we were unable to transfect them with the ADH plasmid. To mimic ethanol metabolism by ADH, we used exogenously produced acetaldehyde generated by special *in vitro* system (AGS), which continuously make physiological amounts of Ach (for details, see (Ganesan et al., 2015)).

Human hepatocytes

Human hepatocytes were from Triangle Research Labs, LLC, Research Triangle Park, NC. Cells were attached to collagen-coated 6-well plates ($8x10^5$ cells/well) and then infected by serum from an HCV+ patient (IRB# 520-14-EX, HCV RNA is $3x10^6$ copies/ml). Infected cells were cultured in William's Medium with supplements (insulin, holo-transferin, L-ascorbic acid, dexamethazone, selenium and antibiotics) and 10% FBS for 3 days and then exposed to AGS for 48 hrs (total infection time is 5 days). The level of infection was confirmed by measurement of HCV RNA.

Animal Studies

C57Bl/6J mice transgenically expressing HCV structural proteins obtained from Dr. S. Weinman (Kansas University Medical Center, Kansas, USA) were characterized elsewhere (21, 30). Mice (6-8 weeks old) were divided into two groups (n=6 per group): Control and Ethanol. They were pair-fed control and ethanol Lieber De Carli Diets for 10 days; then were gavaged with PBS/maltose dextran or ethanol on day 11 and sacrificed 9 hrs after

gavaging as described in details for chronic-acute ethanol study (Bertola et al., 2013). Four hrs before sacrifice, each mouse was injected IP with mouse IFNα (1000 IU). Mice were treated according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals". Animal use and protocols of ethanol feeding were approved by the Institutional Animal Care and Use Biosafety Committees at VA Medical Center, Omaha, NE. We found no difference in liver weight between control and ethanol groups (control: 1.01±0.05 g; ethanol: 1.18±0.06g), and the body weight after 11 days of feeding was the same in both groups.

Cell Treatments

RLW cells were infected by JFH1 (HCV genotype 2a) virus (obtained from Dr. T. Wakita, Tokyo, Japan) at MOI=0.1 as previously described (Wakita et al., 2005) or were left uninfected. To study whether ethanol metabolites affected IFNα signaling, on day 2 post infection, cells were exposed for 48 hrs to AGS. AGS contains yeast alcohol dehydrogenase (ADH, 0.02 U/ml), 2 mM Nicotinamide adenine dinucleotide (NAD) and 50 mM ethanol (EtOH) and continuously (for at least 48 hrs) produces 50-200 μM of Ach as a result of ADH enzymatic reaction (Ganesan et al., 2015). After treatment, the signaling was induced by human IFNα (1000 IU) for one hour, and cells were lysed in phosphorylation buffer as described (Osna et al., 2005). Non-treated cells were used as a control. It is known that HCV by itself induces USP18 expression (Randall et al., 2006, Randall et al., 2007) Thus, to determine whether Ach further increases IFNα-induced USP18 in HCV^+ cells and how this increase affects HCV RNA levels, we compared both the expression of USP18 and subsequent intracellular content of HCV RNA in control and Ach-treated HCV-infected cells.

RNA isolation and Real-time PCR

Reagents for RNA isolation, cDNA synthesis and real time PCR were from Life Technologies (Carlsbad, CA). Total RNA was isolated from cells using Trizol Reagent. A 2 - step procedure was applied, in which 200 ng RNA was reverse-transcribed to cDNA using the high capacity reverse transcription kit. Then the cDNA was amplified using TaqMan Universal Master Mix- II with fluorescent-labeled primers (TaqMan gene expression systems). These were incubated in a Model 7500 qRT-PCR thermal cycler. The relative quantity of each RNA transcript was calculated by its threshold cycle (Ct) after subtracting that of the reference cDNA (GAPDH). Data are expressed as the quantity of transcript (RQ). The relative HCV RNA expression level in infected cells was quantified using the following primers and probe for this consensus sequence, which were designed using PrimerExpress Software v2.0 (Applied Biosystems): 5'UTRF GACCGGGTCCTTTCTTGGAT; 5'UTRR CCAACACTACTCGGCTAGCAGTCT; probe FAM-ATTTGGGCGTGCCCCCGC-NFQ.

Immunoblotting and Immunoprecipitation

Immunoblotting and Immunoprecipitation assays were performed as previously described (Ganesan et al., 2015): Briefly, cell lysates were prepared in 0.5M EDTA, 2MTris, 20mM Na3VO4, 200mM Na4P2O7, 100mM PMSF, 1M NaF, 20% Triton X-100 and Aprotinin, pH=7. The pre-clearing with protein G beads has been done to minimize the non-specific effects. Immunoprecipitations were done by incubating each Ag-Ab complex with protein G

sepharose (GE Healthcare Biosciences AB, Sweden) for overnight in a rotating shaker at 40°C, followed by washing and incubation with SDS-PAGE sample solubilizing buffer at 95°C for 8 mins. Isotype –specific IgG were used as a negative control. Complexes were subsequently subjected to denaturing SDS-PAGE in polyacrylamide gels. Immunoblots were developed using Odyssey® infrared imaging system, and protein band was quantified using Li-Cor software (Thomes et al., 2013) . Beta actin was used as the loading control to normalize the proteins.

Flow Cytometry

Cell surface IFNαR2 levels were quantified in HCV-infected RLW cells using directly conjugated anti-IFNαR2-Pycoerythrin (PE) antibody. Fluorescence was detected by flow cytometry (BD LSR-II-Green).

Transfection of siRNA

Transfection was done using the protocol from the manufacturer (OriGene, Rockville, MD) with control (scrambled) or specific siRNA. The efficacy of transfection was monitored in 48 hrs by real-time-PCR.

Statistical Analyses

Data from at least three independent experiments are expressed as mean values ± standard error. Comparisons among multiple groups were determined by one-way ANOVA, using a Tukey post-hoc test. For comparisons between two groups, we used Student's t-test. A probability value of 0.05 or less was considered significant.

RESULTS

AGS suppresses pSTAT1/STAT1 ratio in HCV-infected RLW cells

In hepatocytes, ethanol (EtOH) is metabolized by two major enzymes, CYP2E1 (to produce ROS and low amount of Ach) and ADH (to generate sustained and high amounts of Ach). For our experiments, we used Huh7.5-CYP (RLW) cells that are transfected with CYP2E1 but not ADH. Therefore, these cells do not produce measurable amount of Ach. To recapitulate ethanol metabolism in hepatocytes, we exposed the cells to an artificial AGS that continuously generates physiological amounts of Ach (Ganesan et al., 2015, Ganesan et al., 2016). HCV-infected cells were exposed either to ethanol (in which case, mainly ROS were generated via CYP2E1-mediated metabolism) or to AGS. In this way, cells produced ROS via CYP2E1-mediated EtOH catalysis (as EtOH is a part of AGS) and were also exposed to Ach generated from AGS, which mimics ethanol metabolism by hepatocytes. These treated cells were then activated with IFNα to induce STAT1 phosphorylation. While in HCV^+ RLW cells EtOH did not affect pSTAT1/STAT1 ratio, AGS treatment decreased this ratio by 29% (Fig. 1A, B), indicating the specific role of Ach in suppressing STAT1 activation. Next, in the same experiments, we measured the kinetics of pSTAT1 and USP18 expression for up to 24h following IFNα exposure. There was a reciprocal regulation of pSTAT1 and USP18 levels by IFNα (Fig. 1C–E), indicating that increasing USP18 may down-regulate STAT1 phosphorylation.

AGS increases USP18 expression in HCV$^+$liver cells

To elucidate the effects of Ach on USP18 expression, cells were exposed to AGS and then both USP18 mRNA and protein levels were determined by real-time PCR and immunoblotting, correspondingly. AGS induced USP18 mRNA and protein levels by about 2.5 fold (Fig. 2A–C). These upregulating effects of Ach on USP18 obtained in RLW cells were further confirmed *in vitro* by using HCV$^+$ human hepatocytes (infected as described in (Ganesan et al., 2015)). Infection of hepatocytes was confirmed by HCV RNA levels (Fig. 2E). In hepatocytes infection with HCV requires at least 5 days after treatment with virus. Since by that time the primary hepatocyte cultures lose their ethanol-metabolizing capacity, these cells were treated with AGS, but not with ethanol only (Fig. 2D). In addition to human hepatocytes, USP 18 was measured in the livers of HCV-Tg mice fed control and ethanol diets. Importantly, *in vivo* ethanol feeding also increased USP18 mRNA and protein levels in liver cells (Fig. 2F–G) and HCV core protein expression (Fig. 2 I).

Silencing of USP 18 attenuates the effects of AGS on pSTAT1

To prove that Ach reduces STAT1 phosphorylation by up-regulation of USP18, we blocked USP18 by specific siRNA. Then the cells were treated in the presence or absence of Ach to measure its effect on pSTAT1/STAT1 ratio. The efficacy of silencing was confirmed by a 60% decrease in USP18 mRNA expression (Fig.3A). While Ach increased USP18 mRNA up to 1.35 fold in cells transfected with control (scrambled) siRNA, the response of USP18 to Ach was almost totally abrogated by USP18 siRNA transfection. Further, AGS suppressed pSTAT/STAT1 by only 22% upon USP18 siRNA silencing vs 41% without USP18 silencing (Fig. 3B, C).

AGS, IFNαR2 and USP18

Analyzing the mechanisms by which AGS-induced increase in USP18 affects pSTAT1 levels, we quantified IFNαR2 expression in HCV-infected RLW cells by flow cytometry and by immunoblotting technique (Fig. 4A, B, C). AGS treatment did not affect the expression of IFNαR2, but enhanced the complex formation between IFNαR2 and USP18 (Fig. 4 D, E), as revealed in the results of immunoprecipitation experiments. Since USP18 binds to IFNαR2, but not JAK1, we assume that the dysregulation of STAT1 phosphorylation by USP18 attachment occurs at the level of the IFNαR2 docking site for STAT1, where activated JAK1 phosphorylates STAT1.

Effects of AGS on unconjugated ISG15 and TRIM25 expression

We measured the effects of AGS on free (unconjugated) ISG15 (both mRNA and protein levels) as well as on expression of ISG15 E3 ligase, Trim25. While AGS exposure to cells enhanced unconjugated ISG15 protein levels, it suppressed mRNA levels without affecting TRIM25 expression (Fig. 4F–I).

Suppression of STAT1 ISGylation by AGS

STAT1 ISGylation was measured on IFNα-stimulated control and AGS-treated cells by immunoprecipitation. AGS (but not EtOH per se) treatment suppressed STAT1 ISGylation (Fig. 5 A, B). In the same samples, we observed a 2.5 fold increase in STAT1 ubiquitination

on Lys48 (specific for targeting by proteasome for degradation) after AGS exposure (Fig. 5C, D).

AGS induces HCV RNA in RLW cells

The impairment of IFNα signaling by AGS exposure parallels the increase of HCV RNA levels in RLW cells (Fig. 6A).

DISCUSSION

Alcohol consumption worsens HCV-induced liver injury due to the amplification of HCV-infection by alcohol metabolism. The reduced phosphorylation of STAT1, the transduction factor necessary for interferon stimulated genes (ISG) activation by IFN in HCV-infected hepatocytes, plays an important role in limiting HCV-infection. Therefore, IFNα released from HCV-uninfected plasmacytoid dendritic cells in the liver (Takahashi et al., 2010) can cause antiviral protection in hepatocytes that sense IFN type I through the activation of the JAK-STAT pathway. The JAK-STAT signaling initiates upon the binding of IFN to its receptors on hepatocyte surface. HCV (Heim et al., 1999) and ethanol (Nguyen et al., 2000) are known to suppress the JAK-STAT pathway, but there are only a few studies addressing the mechanisms of the combined effects (Szabo et al., 2006, Plumlee et al., 2005) and even in these studies, ethanol metabolism is not taken into account.

Further, HCV increases the levels of de-ISGylating protease, USP18, in the biopsies of HCV patients unresponsive to IFNα therapy (Sarasin-Filipowicz et al., 2008, Dill et al., 2012), but nothing is known about the regulation of USP18 levels by ethanol. In this study, we demonstrated inverse effects of IFNα exposure to HCV-infected hepatoma cells on the kinetics of STAT1 activation and USP18 expression, suggesting that up-regulation of USP18 may decrease STAT1 phosphorylation. Furthermore, the product of ethanol metabolism, Ach, enhances USP18 and suppresses IFNα-induced STAT1 phosphorylation in Huh 7.5-CYP (RLW) cells. Silencing USP18 in the cells attenuated AGS-suppressed STAT1 phosphorylation, indicating that here, USP18 is AGS-induced negative regulator of IFNα signaling. Interestingly, this effect is Ach-specific since no induction of USP18 occurred when ethanol was metabolized only by CYP2E1, in the absence of Ach. The up-regulation of USP18 mRNA by Ach was validated using HCV-infected primary hepatocyte cell cultures. These *in vitro* results were corroborated by *in vivo* studies in which similar increases in USP18 levels were observed in livers of ethanol-fed HCV-Tg mice compared with mice pair-fed control diet. Collectively, the current study demonstrates that ethanol metabolites may suppress IFNα signaling in liver cells by upregulating USP18, which accompanies by increase in HCV RNA. Furthermore, these results also confirm our previous observation on the enhanced HCV RNA levels after 24 hrs of AGS treatment in RLW cells (Ganesan et al., 2015).

We then investigated the mechanism by which USP 18 suppresses IFNα-triggered STAT1 activation in HCV-infected cells in response to Ach. As recently shown, USP18 may act via non-enzymatic and enzymatic pathways (Ketscher and Knobeloch, 2015). As demonstrated earlier, USP18 prevents STAT1 phosphorylation due to the blockade of IFNαR2-JAK1 interactions (Malakhova et al., 2006). In fact, in our hands, AGS treatment enhanced the

interaction between IFNαR2 and USP18 in HCV^+ RLW cells without affecting the level of IFNαR2 as determined by both flow cytometry and IB. This indicates that Ach impairs the cross-talk between IFNαR2 and STAT1, thereby attenuating STAT1 phosphorylation.

We also considered the possibility that Ach affected IFNα-induced de-ISGylation of STAT1 by an ISG15-specific protease, USP18. ISGylation is an ISG15 conjugation system, which broadly targets newly sensitized proteins (Durfee et al., 2010), and its function in some studies is interpreted as a pro-viral for HCV-infection (Broering et al., 2010, Chen et al., 2011). In contrast, others have shown that silencing of USP18 promotes ISGylation and anti-viral effects of exogenous IFNα (Randall et al., 2006, Giannakopoulos et al., 2005, Ketscher and Knobeloch, 2015). We observed Ach-mediated increase of free ISG15 even when it suppressed ISG15 mRNA. This indicates that major source of free ISG15 is not Ach-enhanced ISG15 synthesis, but protein de-iSGylation executed by USP18. We indeed detected the reduction of STAT1-conjugated ISG15 after AGS exposure. The expression of Trim25, an E3 ligase for ISG15, was not affected by AGS in HCV^+ RLW cells. Thus, it is likely that the reduction in STAT1 ISGylation is entirely attributed to Ach-enhanced de-ISGylation by USP18. Importantly, lysine residues of STAT1 can be modified by either ISGylation or ubiquitination. Thus, the decreased protein ISGylation may concomitantly cause increased protein ubiquitination as suggested in previous models (Wood et al., 2011)). In fact, AGS induced an increase in STAT1 ubiquitination on K48 that specifically targets proteins for degradation by 26S proteasome. Since ISGylation preserves pSTAT1 and ubiquitination degrades pSTAT1, the interplay between STAT1 ISGylation and ubiquitination may regulate STAT1 activation. Thus, loss of p-STAT1 may occur via proteasomal degradation of the ubiquitinated non-ISGylated phosphorylated STAT1. The scheme of HCV- and ethanol-induced regulation of IFNα-induced STAT1 phosphorylation by USP18 is presented as Fig. 6B.

Nowadays, HCV-infection is successfully treated with direct antiviral agents (DAA), including Harvoni. However, this fact does not minimize the role of IFNα signaling as a protective innate immunity factor for viral infections because the events described here are induced by endogenous (not exogenous) IFNα released by liver (mainly, dendritic) cells and sensed by infected hepatocytes. We strongly believe that the restoration of innate immunity/IFNα signaling in hepatocytes is a necessary step in potentiation of DAA treatment efficiency in chronic hepatitis C and that by blocking viral replication, DAA will be almost equally effective in alcoholic and non-alcoholic HCV patients. The clinical trials to test the responsiveness to DAA treatment in HCV^+ alcohol consumers are in progress now.

We conclude that acetaldehyde-induced USP18 suppresses IFNα signaling by decreasing STAT1 phosphorylation due to enhanced complex formation between IFNαR2 and USP18. In addition, acetaldehyde can promote pSTAT1 degradation by proteasome due to its increased K48 polyubiquitination, which is in turn, promoted by intensive STAT1 de-ISGylation. Acetaldehyde-Impaired IFNα signaling activates HCV RNA replication and in part, may explain the mechanism of HCV-infection persistence in alcohol-abusing patients.

ACKNOWLEDGEMENTS:

We thank Dr. Steven Weinman for providing HCV^{+}Tg mice, Dr. C. Rice for Huh7.5 cells and Dr. T. Wakita for JFH1 virus. The authors declare that they have no conflict of interest.

Funding:

This work was supported by Merit Review BX001673 from the Department of Veterans Affairs, Office of Research and Development (Biomedical Laboratory Research and Development).

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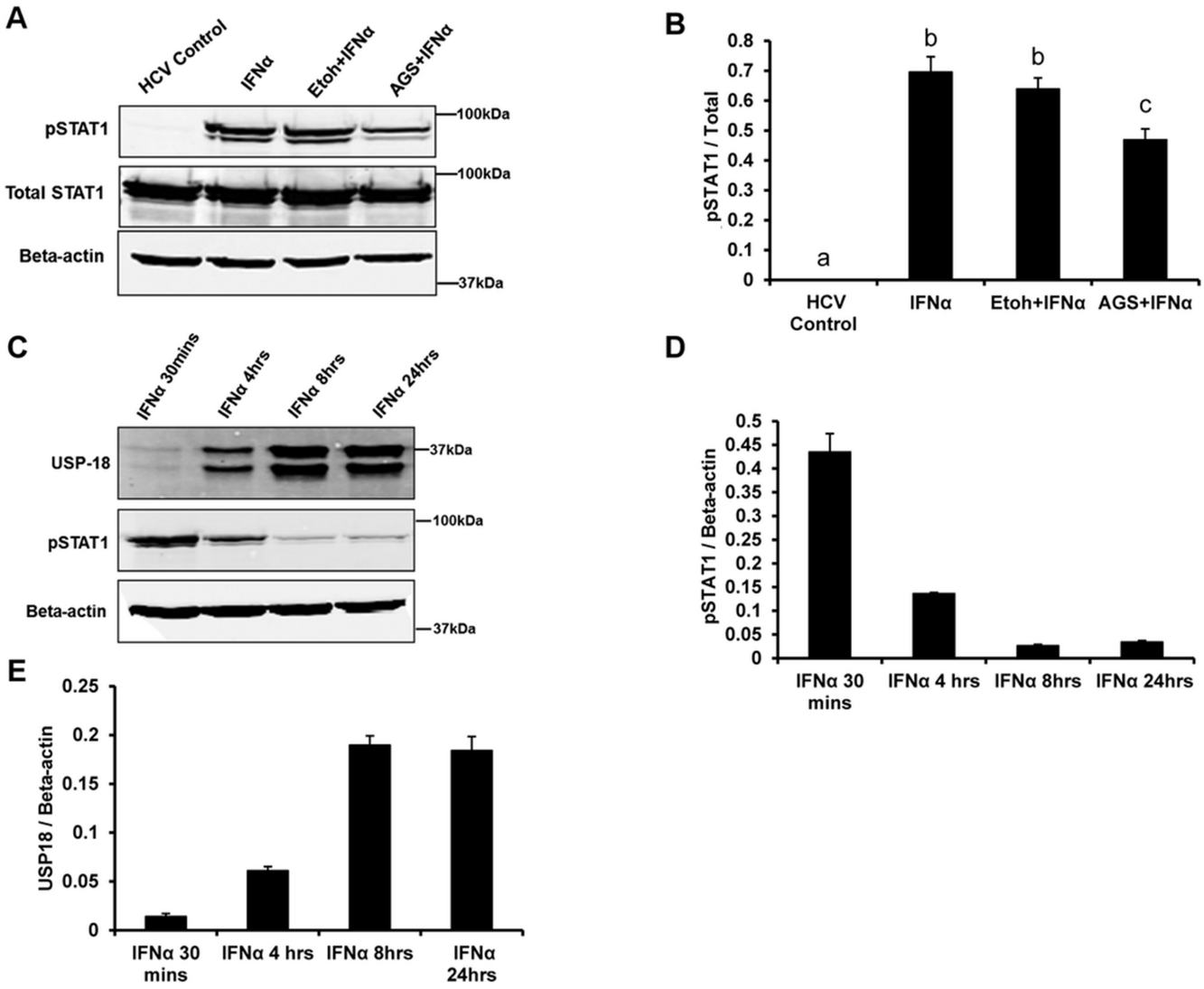

Fig 1. Acetaldehyde decreases STAT-1 phosphorylation.

HCV-infected RLW cells were treated with 50 mM ethanol or AGS for 48 hrs, then IFNα signaling was induced by 1000 units of IFNα for 0.5 hr. (A) Immunoblot analysis of cell lysates from control (without IFNα), IFNα, ethanol(Etoh)+IFNα and AGS+IFNα were probed with anti-pSTAT-1, anti-total STAT-1 and anti-β-actin antibody as a loading control (25 μg of protein loaded per lane), immunoblots were developed using an Odyssey® infrared imaging system. (B) Quantification of Immunoblot bands using Li-Cor software.(C) **Inverse kinetics of STAT-1 activation and USP18 treated with IFNα.** Kinetics of STAT-1 activation and USP18 were measured by immunoblot analysis from cell lysates of HCV-infected RLW cells treated with 1000 units of IFNα for 0.5, 4, 8, 24 hrs were probed for anti-USP18, anti-pSTAT-1 and anti-β-actin as a loading control (25 μg of protein loaded per lane). Blots were developed using the Odyssey® infrared imaging system. (D, E) Quantification of immunoblot bands using Li-Cor software. All data (representative results and quantification) were generated from 3 independent experiments and presented as Mean ± SEM. Bars with different letters are significantly different at $p \leq 0.05$.

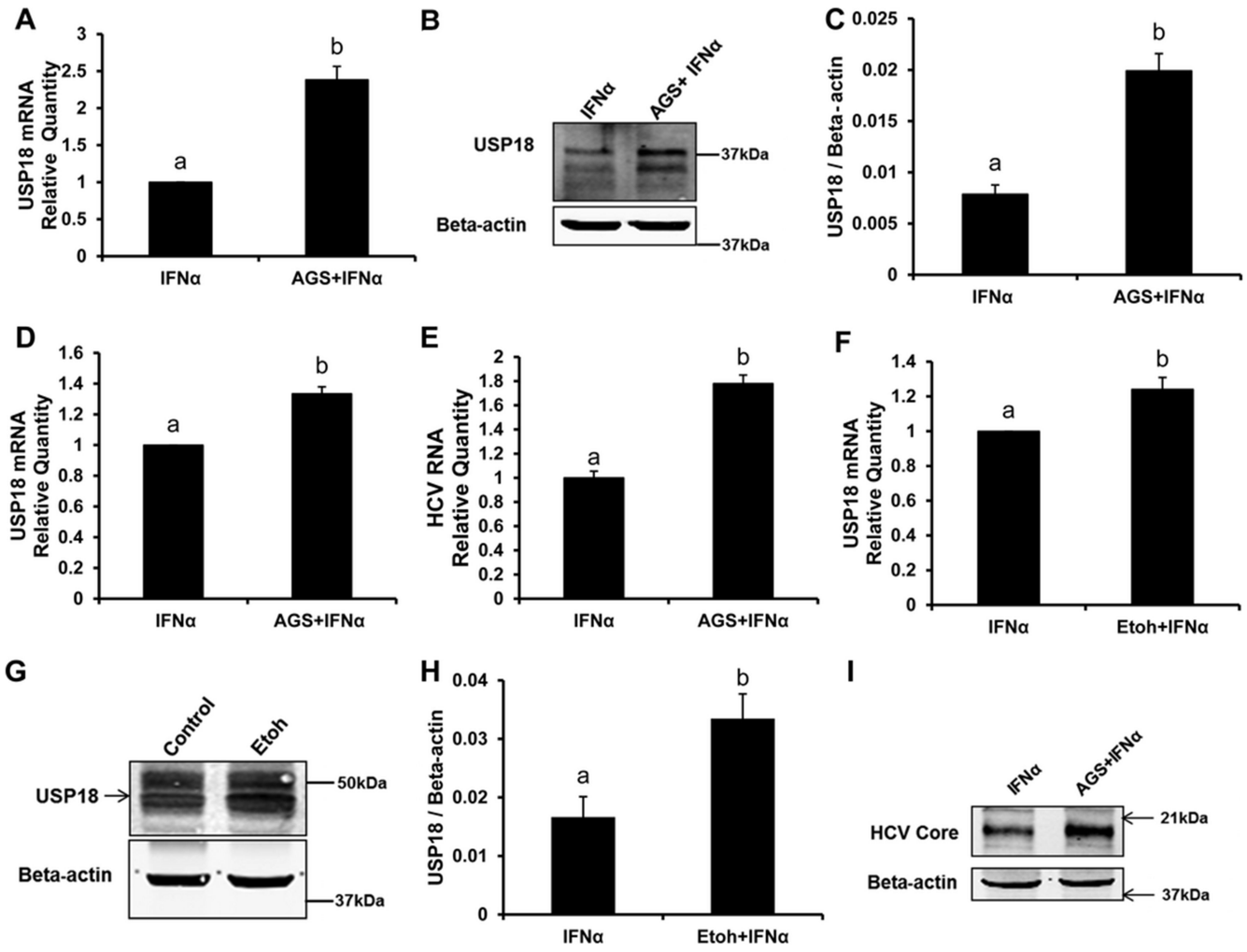

Fig 2. Acetaldehyde increases both mRNA and protein expression of USP18 in liver cells.

(A) HCV-infected RLW cells were exposed to AGS for 48 hrs, then cells were treated with 1000 units of IFNα for 1 hr. Real-time PCR analysis was performed for USP18 mRNA; GAPDH was used to normalize the gene of interest. Data presented as the relative quantity in relation to IFNα. (B) Immunoblot analysis of cell lysates from HCV-infected RLW cells exposed to AGS for 48 hrs and treated with 1000 units of IFNα for 1 hr were probed with anti-USP18 and anti-β-actin antibody as a loading control (25 μg of protein loaded per lane), blots were developed using an Odyssey® infrared imaging system. (C) Quantification of immunoblot bands using Li-Cor software. (D, E) Human hepatocytes were attached to collagen-coated 6-well plates and then infected with patient serum. Infected cells were cultured for 3 days and then exposed to AGS for 48 hrs. After incubation, cells were stimulated with human IFNα 1000 IU, for 1 hr, and processed for real-time PCR for the mRNA expression of USP18 and the levels of HCV RNA. (F, G) C57Bl/6J mice transgenically expressing HCV structural proteins (6-8 weeks old) were pair-fed control and ethanol as described in Materials and Methods. Four hrs prior to sacrifice, each mouse was injected intraperitoneally with mouse IFNα, (1000 IU). USP18 mRNA and protein expression were detected by real-time PCR analysis and western blot, respectively. GAPDH

was used to normalize the gene of interest and β-actin as a loading control for the protein of interest (25 μg of protein loaded per lane); immunoblots were developed using Odyssey® infrared imaging system. (G) Quantification of immunoblot bands using Li-Cor software. (I) HCV core protein levels were measured in HCV Tg mice by western blot fed control or ethanol diets, and β-actin was used as a loading control for the protein of interest (25 μg of protein loaded per lane). All data (representative results and quantification) were generated from 3 independent experiments and presented as Mean ± SEM. Bars with different letters are significantly different at $p \leq 0.05$.

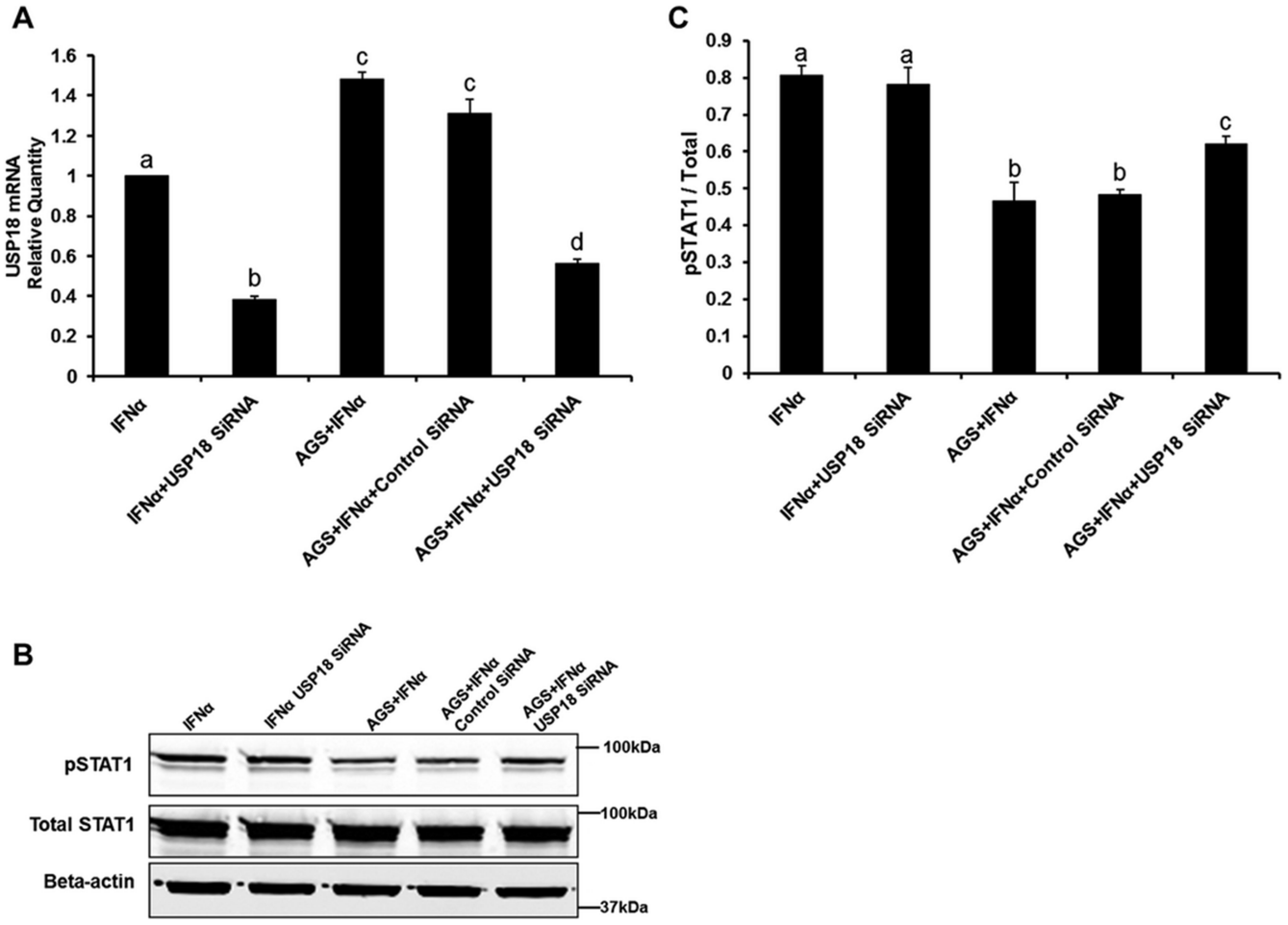

Fig 3. Silencing of USP18 attenuates the effects of AGS on STAT-1 activation.

(A) Knock-down efficiency of USP18 using siRNA (USP18 siRNA) was analysed by real-time PCR; non-silencing siRNA (scrambled siRNA) was used as a control. GAPDH was used to normalize the gene of interest. HCV-infected RLW cells were transfected with USP18 siRNA. After 48 hrs of transfection, cells were exposed to AGS for 48 hrs, then treated with 1000 units of IFNα for 1 hr. Real-time PCR analysis was performed for the mRNA expression of USP18; GAPDH was used to normalize the gene of interest. (B) HCV-infected RLW cells were transfected with USP18 siRNA. After 48 hrs of transfection, cells were exposed to AGS for 48 hrs, then treated with 1000 units of IFNα for 1 hr. Cell lysates (25 μg of protein loaded per lane) were used for immunoblot analysis of STAT-1 phosphorylation, total STAT-1 and β-actin. Immunoblots were developed using Odyssey® infrared imaging system. (C) Quantification of immunoblot band using Li-Cor software. All data (representative results and quantification) were generated from 3 independent experiments and presented as Mean ± SEM. Bars with different letters are significantly different at $p \leq 0.05$.

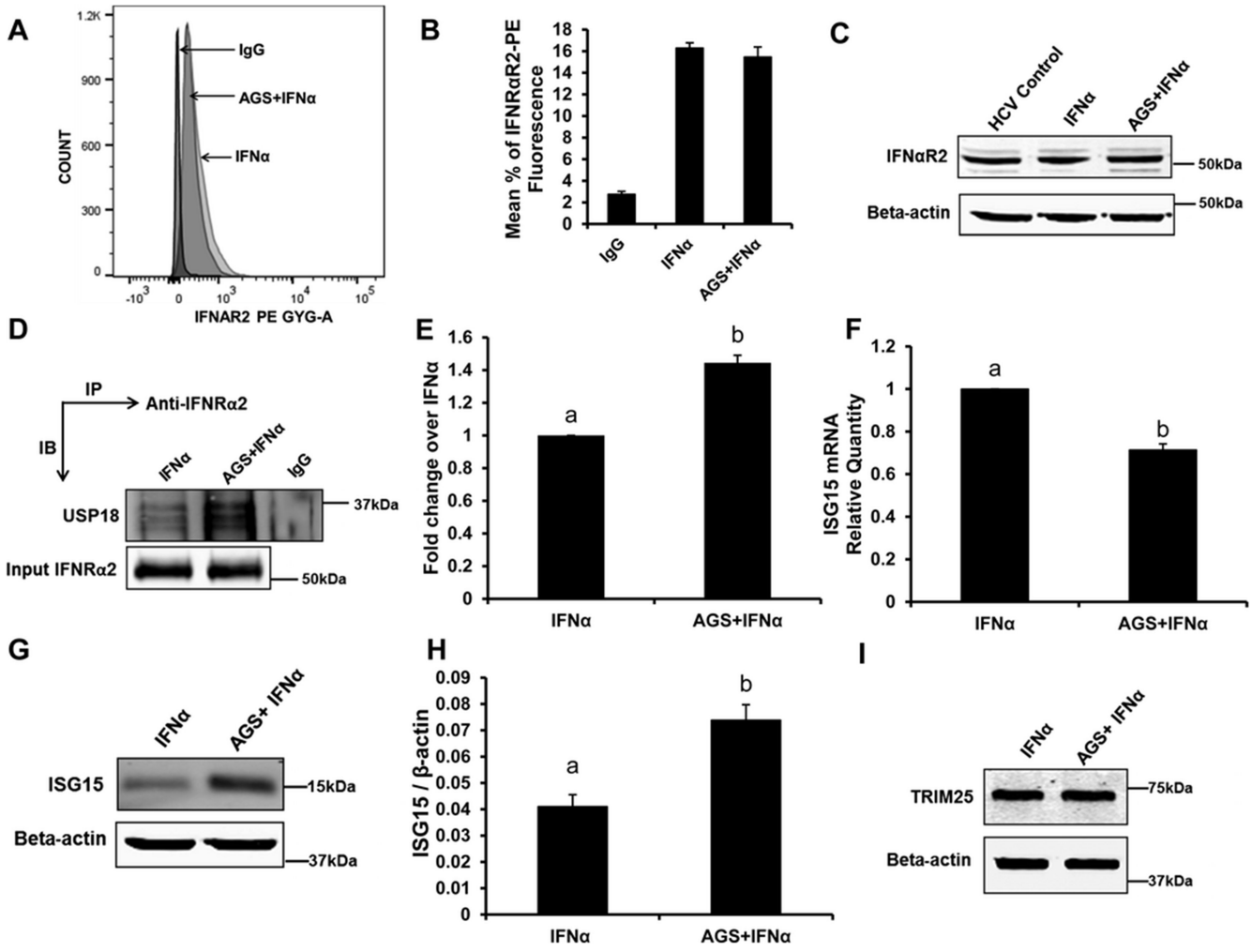

Fig 4. Acetaldehyde does not affect IFNαR2 levels in HCV-infected RLW cells.

HCV-infected RLW cells were exposed to AGS for 48 hrs, then treated with 1000 units of IFNα for 1 hr. (A) Non-permeabilized cells were used for flow cytometry analysis of cell surface IFNαR2 levels using fluorescent dye phycoerythrin (PE)-labelled anti-IFNαR2 antibody or mouse IgG as an isotype control (B) Quantification of IFNαR2-PE staining expressed as mean percentage of fluorescence (C) Cell lysates were used for immunoblot analysis of IFNαR2. (D) IFNαR2 and USP18 interaction detected by immunoprecipitation. HCV-infected RLW cells were exposed to AGS for 48 hrs then treated with 1000 units of IFNα for 1 hr. Cell lysates were immunoprecipitated with anti-IFNαR2 antibody and then were immunoblotted using anti-USP18 antibody. As a negative control, cell lysates were immunoprecipitated with isotype specific IgG antibody. Immunoblots were developed using Odyssey® infrared imaging system. (E) Quantification of immunoblot bands using Li-Cor software. .Data presented as the fold change in relation to IFNα. **(F, G, H, I) Effect of acetaldehyde on unconjugated ISG15 and TRIM 25.** HCV-infected RLW cells were exposed to AGS for 48 hrs, then treated with 200 units of IFNα for 4hrs. (E) Real-time PCR analysis was performed for the mRNA expression of ISG15; GAPDH was used to normalize the gene of interest. (F, H) Cell lysates were used for immunoblot analysis of unconjugated

ISG15 and TRIM25. β-actin was used as a loading control. Immunoblots were developed using Odyssey® infrared imaging system. (G) Quantification of immunoblot bands using Li-Cor software. All data (representative results and quantification) were generated from 3 independent experiments and presented as Mean ± SEM. Bars with different letters are significantly different at $p \leq 0.05$.

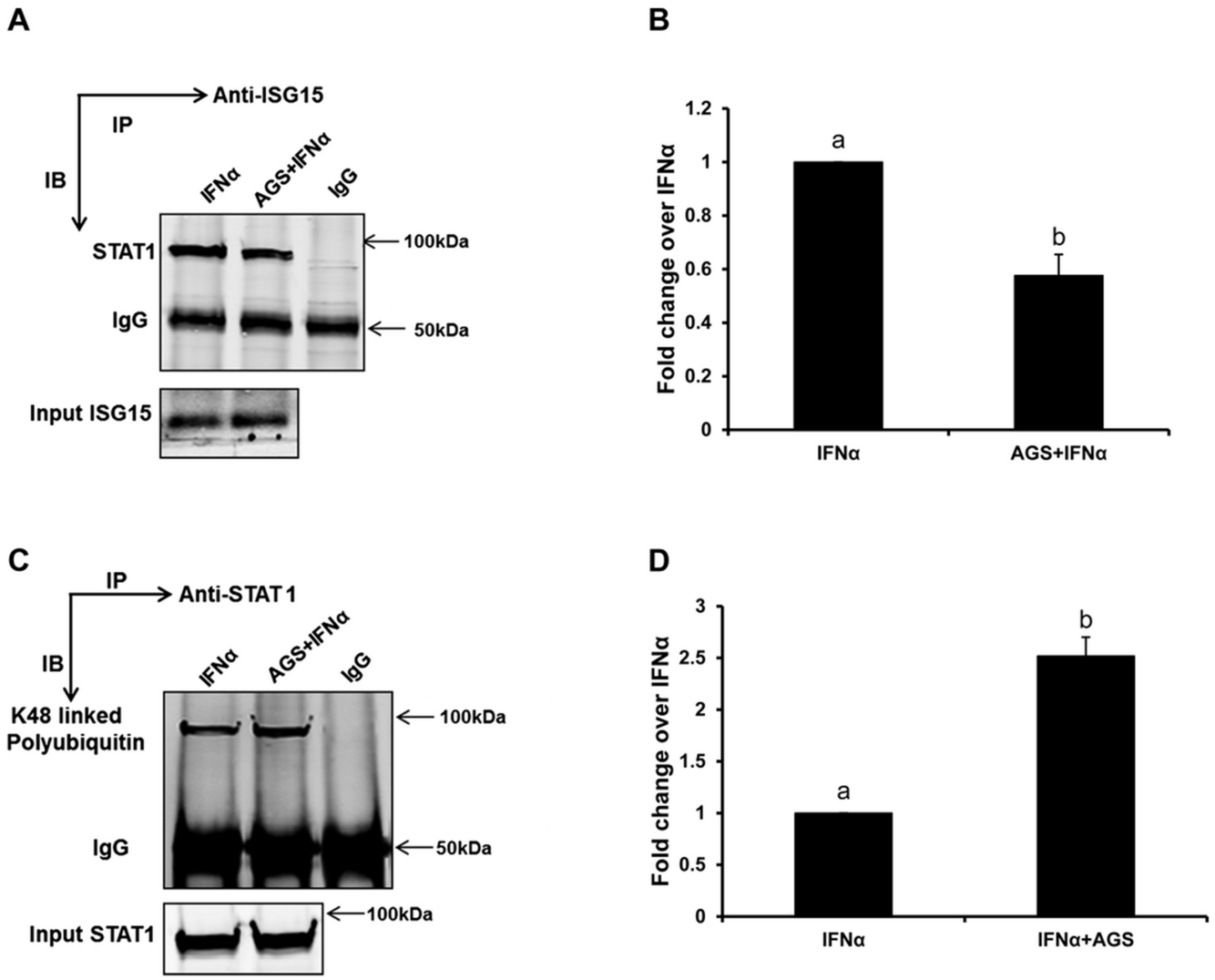

Fig 5. Acetaldehyde differentially affects STAT-1 ISGylation and STAT-1-Ubiquitination.

HCV-infected RLW cells were exposed to AGS for 48 hrs, then treated with 1000 units of IFNα for 1 hr. A. Cell lysates were immunoprecipited with anti-ISG15 (or isotype-specific antibody) followed by immunoblotting with anti-STAT-1. C. Cell lysates were immunoprecipitated with anti-STAT1 or isotype-specific antibody followed by immunoblotting with anti-K48 linked polyubiquitin antibody. Immunoblots were developed using the Odyssey® infrared imaging system. B and D, Quantification of immunoblot bands using Li-Cor software. Data presented as the fold change in relation to IFNα. All data (representative results and quantification) were generated from 3 independent experiments and presented as Mean ± SEM. Bars with different letters are significantly different at $p \leq 0.05$.

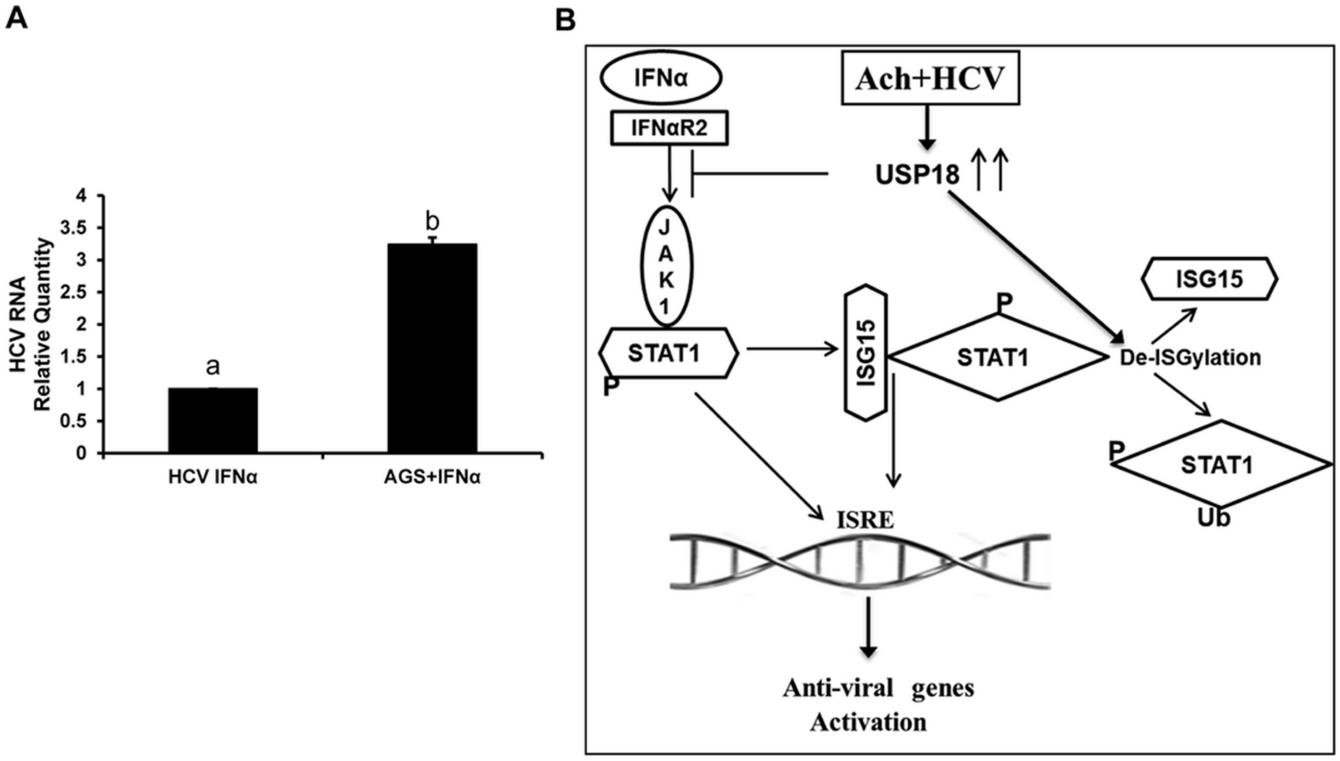

Fig 6.

(A) Effect of AGS on HCV infection. HCV-infected RLW cells were exposed to AGS, then treated with 1000 units of IFNα for 1 hr. Real-time PCR analysis was performed for the mRNA expression of HCV RNA; GAPDH was used to normalize the gene of interest. All data were generated from 3 independent experiments and presented as Mean ± SEM. Bars with different letters are significantly different at $p \leq 0.05$. **(B) Ethanol metabolism-induced suppression of STAT1 activation by IFNα in HCV-infected liver cells: involvement of USP18 (schematic diagram).** Acetaldehyde increases USP18 levels. This (i) dysregulates IFNαR2-JAK1 cross-talk, thereby reducing downstream STAT1 phosphorylation; (ii) increases delSGylation of STAT1 leading to destabilization of pSTAT1; (iii) de-ISGylation of STAT1 increases STAT1-K48 polyubiquitination, promoting its degradation by 26S proteasome. Suppression of STAT1 phosphorylation by acetaldehyde will ultimately decrease activation of anti-viral genes in HCV-infected liver cells.