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IL-15 and IFN-γ signal through the ERK pathway to inhibit HCV replication, independent of type I IFN signaling

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ABSTRACT

Despite effective new treatments for Hepatitis C virus (HCV) infection, development of drug resistance, safety concerns and cost are remaining challenges. More importantly, there is no vaccine available against hepatitis C infection. Recent data suggest that there is a strong correlation between spontaneous HCV clearance and human NK cell function, particularly IFN-γ production. Further, IL-15 has innate antiviral activity and is also one of the main factors that activates NK cells to produce IFN-γ. To examine whether IL-15 and IFN-γ have direct antiviral activity against HCV, Huh7.5 cells were treated with either IFN-γ or IL-15 prior to HCV infection. Our data demonstrate that IFN-γ and IL-15 block HCV replication *in vitro*. Additionally, we show that IL-15 and IFN-γ do not induce anti-HCV effects through the type I interferon signaling pathway or nitric oxide (NO) production. Instead, IL-15 and IFN-γ provide protection against HCV via the ERK pathway. Treatment of Huh7.5 cells with a MEK/ERK inhibitor abrogated the anti-HCV effects of IL-15 and IFN-γ and overexpression of ERK1 prevented HCV replication compared to control transfection. Our *in vitro* data support the hypothesis that early production of IL-15 and activation of NK cells in the liver lead to control of HCV replication.

1. Introduction

Hepatitis C virus infection presents a global health problem with approximately 3% of the world population currently infected [1]. Approximately 70–80% of those infected develop chronic hepatitis with a risk of developing end-stage cirrhosis and hepatocellular carcinoma (HCC) [2]. It is not well understood why 20–30% of infected individuals are able to spontaneously clear HCV upon HCV recognition [3–6]. This early virus clearance highlights the contribution of innate immunological events [7].

Upon HCV infection, hepatocytes, the primary targets of HCV [8], sense HCV viral components through various pattern-recognition receptors which leads to the subsequent production of type I and III interferons (IFNs) [9–12], Indeed, binding of type I IFN to its specific receptor (IFNAR) on the surface of target cells activates an intracellular signaling cascade resulting in the induction of IFN-stimulated genes (ISGs), which triggers a variety of actions to degrade viral RNA and/or inhibit translation of viral and host RNA [13–18].

In particular, IFN-α is a potent inhibitor of HCV replication and is currently used as a treatment for HCV infection. Though the use of IFN-α plays a major role in the clearance of HCV in some individuals, its potency varies significantly depending on the genotype of the virus, many unknown factors and is not 100 percent effective [19].

The liver is particularly enriched in macrophages (Kupffer cells) and NK cells, which are key components of the innate immune system [20]. As NK cells constitute a large proportion of the immune cell population of the liver, they are placed in a key position to combat HCV at the site of the infection [21,22]. There is a robust correlation between spontaneous HCV clearance and human NK cell function [23,24]. Further, IFN-γ production in the liver has been associated with HCV viral clearance in chimpanzees [25]. The modulatory effect of IFN-γ on IFN-α and association of IFN-γ gene polymorphisms in HCV clearance have been shown, previously [26–28].

Indeed, immune cells in the surrounding hepatic environment can produce IL-15, which can activate NK cell antiviral function. Upon detection of viral components, macrophages and dendritic cells (DCs)

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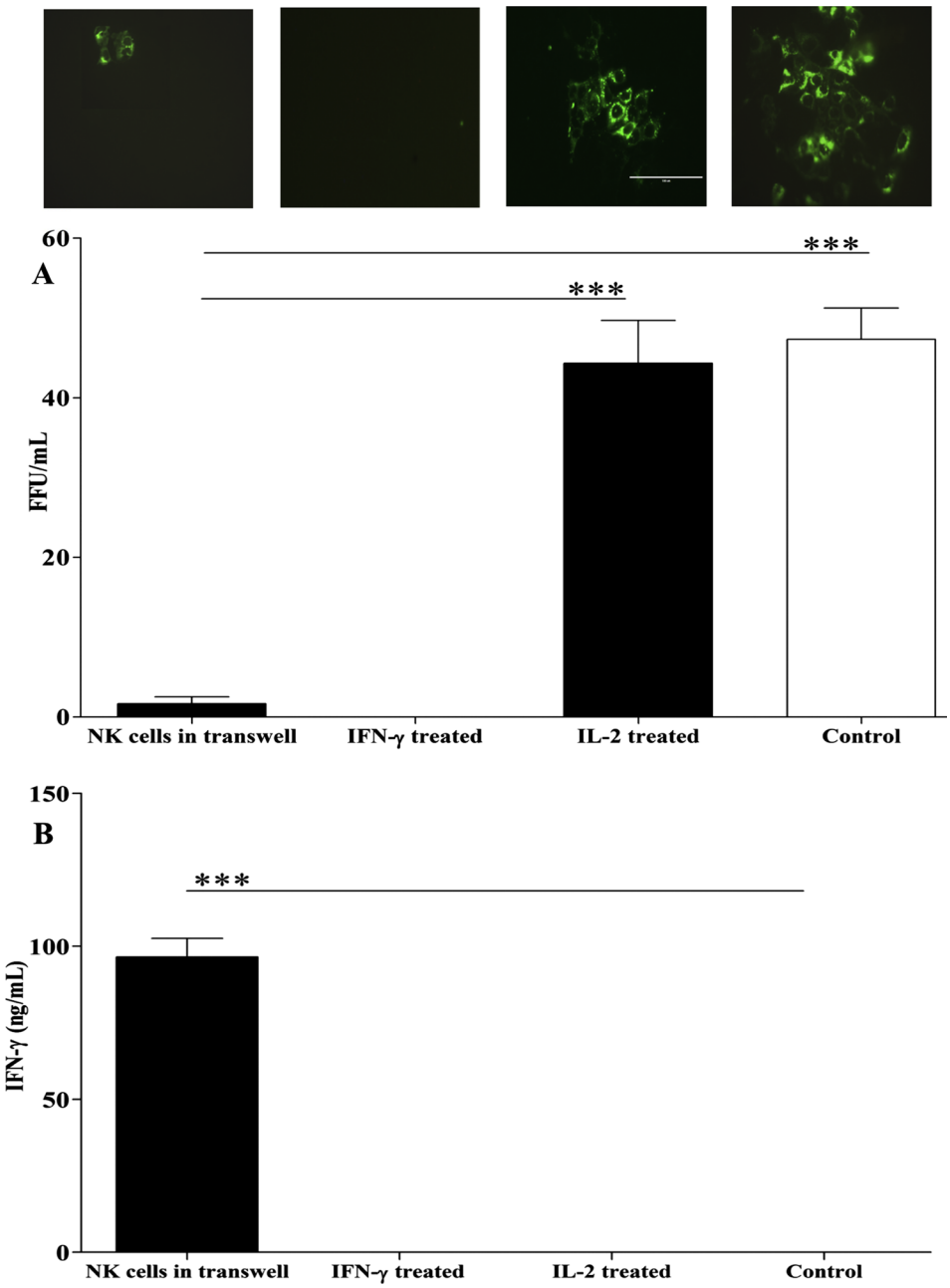

Fig. 1. Stimulated NK cells produce IFN-γ and inhibit HCV replication in Huh7.5 cells. Stimulated NK cells isolated from healthy volunteers were added to 6-well transwell plates with Huh7.5 cells grown on a coverslip. The transwell system made the exposure of soluble factors released by NK cells available to Huh7.5 cells. In second and third wells, IFN-γ and IL-2 were added, respectively, and as a control, Huh7.5 cells were cultured in another well without any treatment. The cells were incubated overnight in the CO_2 incubator and after washing with the medium, 100 µl of diluted HCV virus (10^{-2}) in complete medium was added to each well.

can produce IL-15, which can induce IFN-γ production from NK cells. IFN-γ is a potent antiviral cytokine and plays a key role in activating the adaptive immune response, which ultimately clears the virus infection.

In this study, we examined the direct anti-HCV effects of IL-15 and IFN-γ on an HCV-infected, cultured hepatic cell line, Huh7.5.

2. Materials and methods

2.1. Ethics statement

All work with viruses was completed with approval from the McMaster Presidential Biosafety Advisory Committee under BSL II+ conditions. Blood was taken from healthy adult volunteers with written informed consent and approval from the Human Integrated Research Ethics Board (HIREB) at McMaster University. All methods were performed in accordance with the relevant guidelines and regulations of McMaster University.

2.2. Cells and viruses

Human hepatoma cells, Huh7.5, were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), (Life Technologies Inc.), 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin (Sigma-Aldrich, Oakville, ON, Canada), 10 µM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (McMaster University) and 2 µM L-glutamine (L-glu) (Life Technologies Inc.). Experiments were performed with cells at passages 2–6. All cells were incubated at 37 °C in a

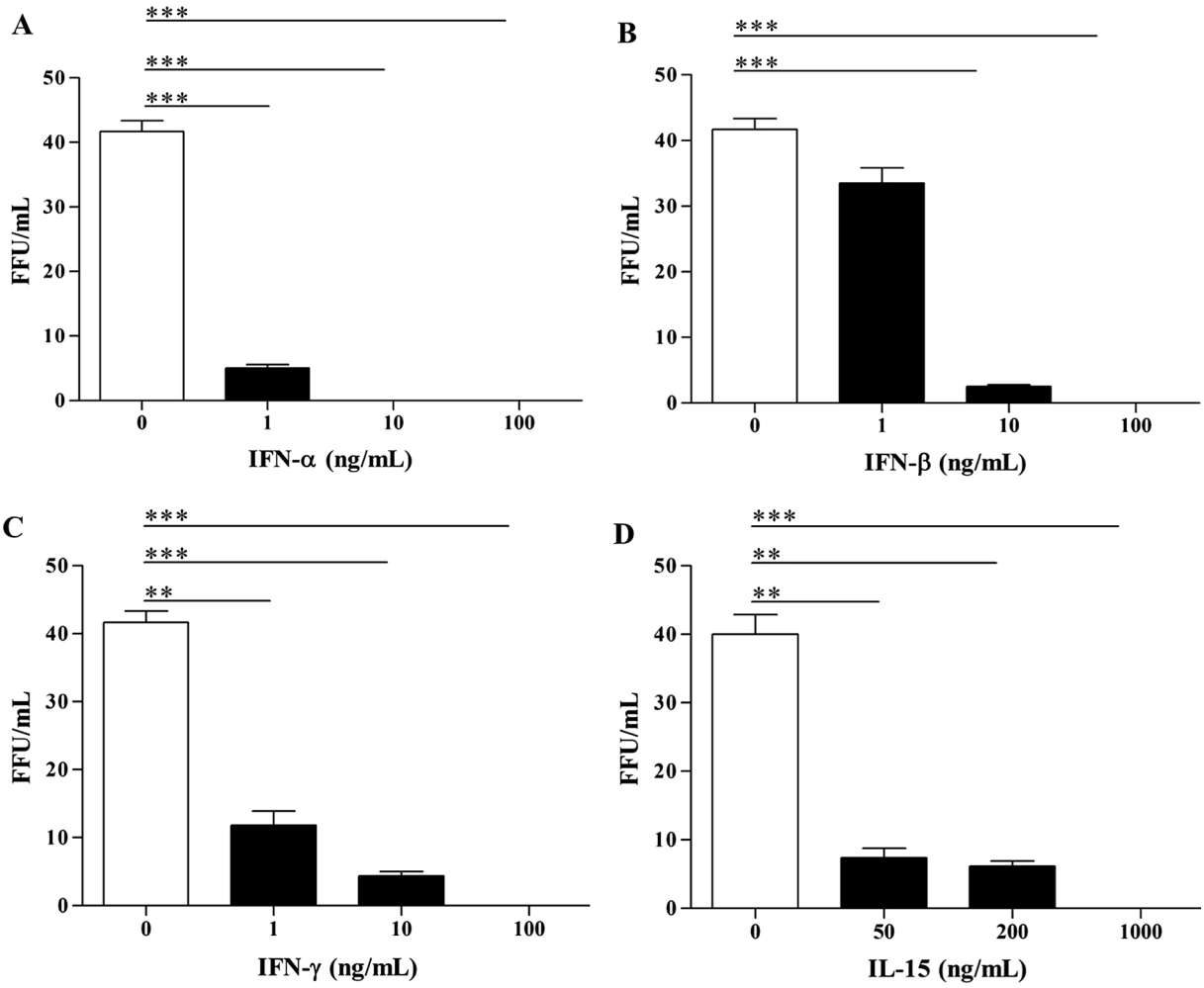

Fig. 2. IFN-α, IFN-β, IL-15 and IFN-γ induce an inhibitory effect on HCV replication in Huh7.5 cells in a dose-dependent manner. Huh7.5 cells in DMEM containing 10% FBS were trypsinized and 5×10^4 cells/well (1.2×10^5 cell mL^{-1}) in 400 µl medium were seeded in 8-well chamber slides. For treatment of cells, the cytokines were added in the following concentrations to designated wells (IFN-α: 100 ng mL^{-1}, IFN-β: 100 ng mL^{-1}, IFN-γ: 100 ng mL^{-1}, IL-15: 0.2 and 1 µg mL^{-1}). The cells were incubated overnight in a CO_2 incubator. The medium from each well was removed, and cells were washed with medium gently at least two times. 100 µl of diluted HCV virus (10^{-2}) in complete medium was added to each well (medium only was added for virus negative control) then cells were placed in an incubator for 4 h. The medium was then removed, and cells were washed twice before cytokines were replenished at their initial concentrations. The chamber slide was incubated for 72 h in a CO_2 incubator and the cells were stained using anti-HCV antibody. Slides were read under fluorescence microscopy and FFU numbers were recorded. When Huh7.5 cells were seeded and treated with IFN-α, IFN-β, IL-15 and IFN-γ at different doses, stronger inhibition was found at higher doses. IFN-α was used at 1, 10 and 100 ng mL^{-1}, IFN-β and IFN-γ were used at 1, 10 and 100 ng mL^{-1} and IL-15 was used at 50, 200 and 1000 ng mL^{-1}. HCV was used at a 10^{-2} dilution. (A), (B), (C), and (D) show the dose-dependent effect of IFN-α, IFN-β, IFN-γ and IL-15, respectively (***$P \leq 0.001$ and **$P \leq 0.01$).

humidified 5% CO_2 incubator. Vero cells (ATCC) were cultured in Minimum Essential Medium Eagle- Alpha Modification (α-MEM) media supplemented with 5% FBS in addition to all the additives mentioned above for DMEM. Hepatitis C Virus (HCV), the Japanese Fulminant Hepatitis 1 (JFH1) strain stocks were prepared in Dr. Jordan Feld's lab (Toronto Centre for Liver Disease, Toronto, ON, Canada). Vesicular Stomatitis Virus (VSV) expressing green fluorescent protein (GFP) was generated by Dr. Brian Lichty (McMaster University, Hamilton, ON, Canada). Bacteria (DH5α) containing GFP-ERK-1 plasmid (GFP-ERK1) was a gift from Rony Seger (Addgene) [29].

2.3. HCV infection of Huh7.5 cells

Briefly, Huh7.5 cells were seeded and grown in 8 or 16-well chamber slides as a monolayer (BD Biosciences, San Jose, CA) and then infected with HCV at 10^3–10^5 FFU mL^{-1}. Infection, immunofluorescence staining and microscopy were performed using the protocol explained by Yi [30].

2.4. Effect of natural IFN-γ released by human NK cells on HCV infection

Natural killer (NK) cells were cultured from peripheral blood mononuclear cells (PBMCs) of healthy volunteers [31]. Peripheral blood mononuclear cells were separated from whole blood using the Ficoll-Paque gradient density centrifugation method and were co-cultured at a 1:2 ratio with irradiated antigen presenting K562 cells which were genetically engineered to express membrane bound IL-21 (K562-mb-IL-21) in RPMI-1640 media supplemented with 10% FBS, 1% Penicillin and Streptomycin, 1% L-glutamine, 1% sodium pyruvate, 50 mM 2-ME and 50 U/mL of IL-2. Half of the co-culture media was replaced every two days along with the addition of IL-2 at a concentration of 50 U/mL. Every 7 days irradiated K562-mbIL-21 cells were replenished with the expanding NK cells in order to maintain the 1:2 ratio. Stimulated cells were treated with IL-12 at 10 ng/mL, IL-15 at 20 ng/ml, and IL-18 at 100 ng/mL for 18 h prior to their addition to the Huh7.5 cells. Huh7.5 cells were grown on coverslips in a 6-well plate. In one well, stimulated NK cells were added into the apical side of a transwell (Falcon) along with IL-2 (50 U/ml). In additional wells, IFN-γ

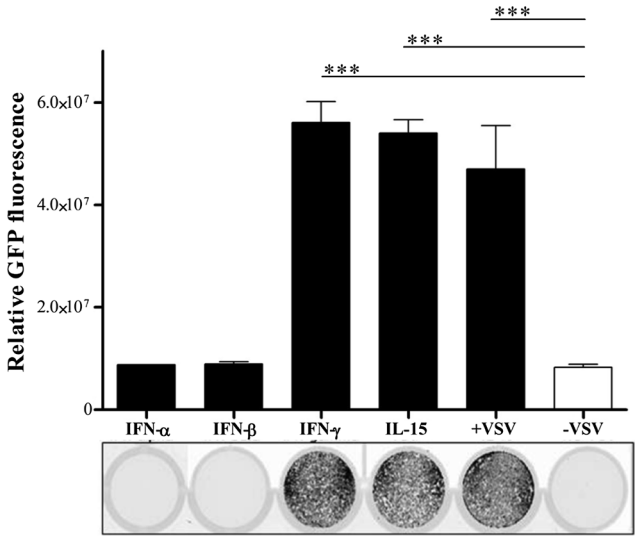

Fig. 3. IL-15 and IFN-γ do not induce type I IFN signaling. In a VSV-GFP assay, the supernatants of Huh7.5 cells treated with cytokine were added to Vero cell culture and the cells were incubated for one day. Media was then removed and VSV-GFP virus (200 µl in each well of 24-well-plate) was added. The plate was incubated for 40 min, washed, then 500 µl of Methylcellulose overlay was added. The plate was incubated for 24 h in the CO_2 incubator and then fluorescence intensity was recorded by Typhoon imager. The induced antiviral effect was detected only for IFN-α and, IFN-β.

(100 U/mL) or IL-2 (100 U/mL) were used to treat the cells. In the control wells, Huh7.5 cells were left untreated. After 24 h of incubation, the supernatants were collected and used for the measurement of IFN-γ. The cells were then infected with HCV as previously described. After infection, fresh stimulated NK cells were added in transwell and the cells were incubated for an additional 72 h. IFN-γ concentration was assayed using a DuoSet ELISA kit (R&D Systems).

2.5. Treatment of Huh7.5 cells with IL-15, IFN-γ, type I IFNs, cell supernatants, or nitric oxide (NO)

Huh7.5 cells were seeded to obtain a confluency of ~80% (10^5 cell mL^{-1}) on 8-well chamber plates (Lab-Tek, Rochester, NY, USA) one day prior to infection. Different concentrations of IFN-α, -β, -γ and IL-15 (R&D Systems Inc., MN, USA) were added in triplicate and the cells were treated prior to infection and again after infection. For the cell supernatant transfer, cells were treated with IL-15, IFN-γ, and type I IFNs for 24 h. The cells were washed three times and incubated in media without treatment for 24 h. The collected supernatants were transferred to new cultures of cells seeded in an 8-well slide chamber followed by HCV infection. For the NO treatment, the cells were treated with 0–200 µM of diethylenetriamine NONOate (DETA-NO) (Sigma, USA) which acts as a NO donor or with 0–200 µM of the control DETA (diethylenetriamine; Sigma, USA) diluted in complete DMEM media before infection with HCV. Following DETA-NO/DETA treatment, cells were infected with HCV. Fresh DETA or DETA-NO was added after each medium change.

Nitrite Oxide (NO) was measured in the supernatant of HCV infected and/or cytokine treated Huh7.5 cells using a commercial Griess assay kit (R&D Systems).

To investigate the role of iNOS in the antiviral response, cells were seeded the day prior to infection and treated with (L-nil), a selective inhibitor of iNOS and were subsequently infected with HCV.

2.6. VSV-GFP assay

Vero and Huh7.5 cells were seeded in 24-well plates. Huh7.5 cells were treated with IL-15, IFN-γ, and type I IFNs for 24 h. Supernatants were then collected and added to the Vero cells and incubated for another 24 h. The supernatants were removed and VSV-GFP assay was performed as described before [32].

2.7. Evaluation of the type I interferon response using PCR array and cytokine array

RNA was isolated from cells treated with IFNs (α, β, γ) and IL-15. cDNA was synthesized and subjected to QPCR as per manufacturer's instructions (SABiosciences). Raw Ct values were analyzed using the RT2 Profiler PCR Array Data Analysis (SABiosciences).

Overnight supernatants collected from the cultures of Huh7.5 cells treated with type I IFNs, IL-15 and IFN-γ were analyzed using the Human Cytokine 64-plex Discovery Assay (Eve Technologies Corp, Calgary, AB, Canada).

2.8. Inhibition of ERK/MEK1/2, p38, Jak/STAT, NF-κB, JNK and PI3K signaling

Following infection, Huh7.5 cells were cultured and treated with or without individual inhibitors or with IL-15 or IFN-γ as described in the previous section and the numbers of FFUs were measured. The inhibitors included BAY 11–7082 (NF-κB inhibitor; Calbiochem), SP600125 (JNK inhibitor; Sigma-Aldrich Canada, Oakville, ON, Canada), Jak Inhibitor I (Calbiochem, Merck, Whitehouse Station, NJ), PD98059 (ERK/MEK1/2 inhibitor; Calbiochem), SB203580 (p38 inhibitor; Calbiochem) and PI-103 (multi-targeted PI3K inhibitor for p110α/β/δ/γ; Cayman Chemical). All inhibitors were dissolved in water or dimethyl sulfoxide (DMSO) according to the manufacturer's protocol and used at concentrations ranging from 1 to 10 µM. DMSO alone was used as a control.

2.9. Evaluation of ERK expression in IFN and IL-15 treated Huh7.5 cells by protein immunoblotting

Huh7.5 cells were cultured and incubated overnight and were then stimulated with recombinant IL-15 and IFN-γ for 30 min, 2 h, 6 h, and 24 h. The cells were washed with cold PBS twice, lysed and immunoblotting was performed as described previously [33].

2.10. Transfection of Huh7.5 cells with ERK encoding plasmid

Bacteria (DH5α) containing GFP-ERK-1 plasmid (GFP-ERK1) [29] were grown in Luria-Bertani (LB) media with Kanamycin (50 µg mL^{-1}) at 37 °C. The plasmid was purified using a Maxiprep plasmid purification kit (Qiagen) and its concentration and purity were assessed by NanoVue. Huh7.5 cells were cultured in a 24-well plate and incubated for 24 h. Cells were then incubated with Opti-MEM® I Reduced Serum Medium (Thermo Fisher Scientific Inc.-Life Technologies™-Gibco®, Massachusetts, USA), containing 5 µg of purified plasmid DNA (GFP-ERK-1 plasmid and GFP control (empty) vector) and Lipofectamine® 2000 (Thermo Fisher Scientific Inc.-Life Technologies™-Invitrogen™, Massachusetts, USA). The cells were washed three times with DMEM and 1 mL of complete DMEM, supplemented with 10% FBS medium, was added to the wells. To the assigned wells, IL-15 (1 µg mL^{-1}) or IFN-γ (100 U mL^{-1}) was added. After 8 h, the cells were washed and infected with HCV (10^5 FFU mL^{-1}). At 4 h post-infection, the cells were washed and complete DMEM was added and incubated for three days. An anti-HCV core 1b secondary antibody (Texas Red-conjugated Goat Anti Mouse IgG antibody, Life Technologies) was used to detect presence of HCV in the Huh7.5 cells. The fluorescence was detected using a confocal microscope (EVOS cell imaging system, Thermo Fisher Scientific). Separate images were taken with green and red filters. Overlay images were created by the overlay tool in EVOS.

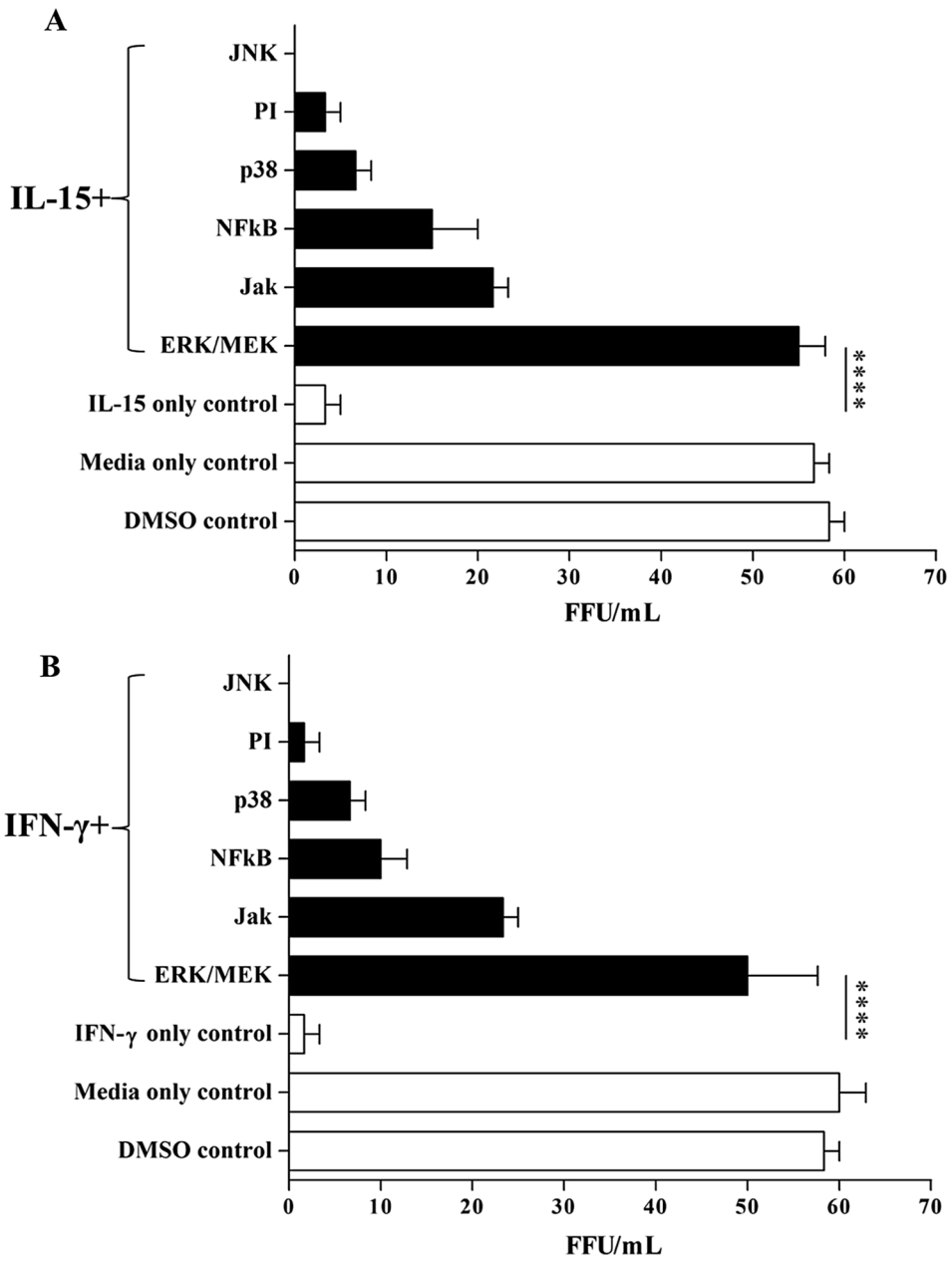

Fig. 4. Inhibitors of MEK/ERK/1/2 and Jak signaling significantly impact the anti-HCV effect of IL-15 and IFN-γ. To identify the signal transduction pathways involved in the anti-HCV effects induced by IL-15 and IFN-γ, cells were cultured in 16-well chamber slides and treated with/without inhibitors individually and with IL-15 or IFN-γ. The cells were infected with HCV and the FFUs were measured. The inhibitors included BAY 11–7082 (NF-κB inhibitor), SP600125 (JNK inhibitor), Jak Inhibitor I, PD98059 (MEK/ERK1/2 inhibitor), SB203580 (p38 inhibitor) and PI-103 (multi-targeted PI3K inhibitor. Inhibitors were dissolved in water or DMSO according to the manufacturer's protocol and used at a concentration of 15 μM. DMSO was used as a mock control. These results show that the inhibitors of MEK and JAK showed the most HCV-inhibitory effect induced by IL-15 (A) and IFN-γ (B) (****$P \leq 0.0001$).

2.11. Statistical analysis

GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA) was used for generating graphs and conducting statistical analyses. Figures represent mean values ± standard deviation (SD). The student's one-tailed *t*-test and one- or two-way analysis of variance (ANOVA) with Tukey or Bonferroni post-tests were used to assess statistical significance.

3. Results

3.1. IL-15 and IFN-γ inhibit HCV replication in Huh7.5 cells

First, we examined the effect of soluble factors released by activated human NK cells on HCV infection. Expanded NK cells from PBMCs of healthy volunteers were co-cultured with irradiated K562-mbIL-21 cells to induce expansion and proliferation of NK cells. These NK cells were then stimulated with a combination of IL-12, IL-15, and IL-18 for 18 h were added in transwell with Huh7.5 cells. The Huh7.5 cells were then infected with HCV and incubated for 3 days. In the wells with stimulated NK cells very few FFUs were detected or not observed, compared to control cells or cells treated with IL-2 (Fig. 1A). Similarly, we failed to observe FFU in IFN-γ-treated cells. Since NK cells are potent producers of IFN-γ as part of the innate immune response and have long been known to exert antiviral effects through IFN-γ, the concentration of IFN-γ produced by the stimulated NK cells was examined. A significantly high concentration of IFN-γ in the supernatant of stimulated cultured NK cells was found (Fig. 1B). Overall, our results demonstrate that soluble factors released from stimulated NK cells can potently inhibit HCV replication.

Next, to examine whether recombinant IL-15 or IFN-γ can decrease HCV replication in hepatocytes, Huh7.5 cells were treated with increasing doses of these cytokines. The antiviral properties of IL-15 and IFN-γ, as well as IFN-α and IFN-β, followed a dose-dependent effect, with IFN-α showing the strongest antiviral effect (Fig. 2).

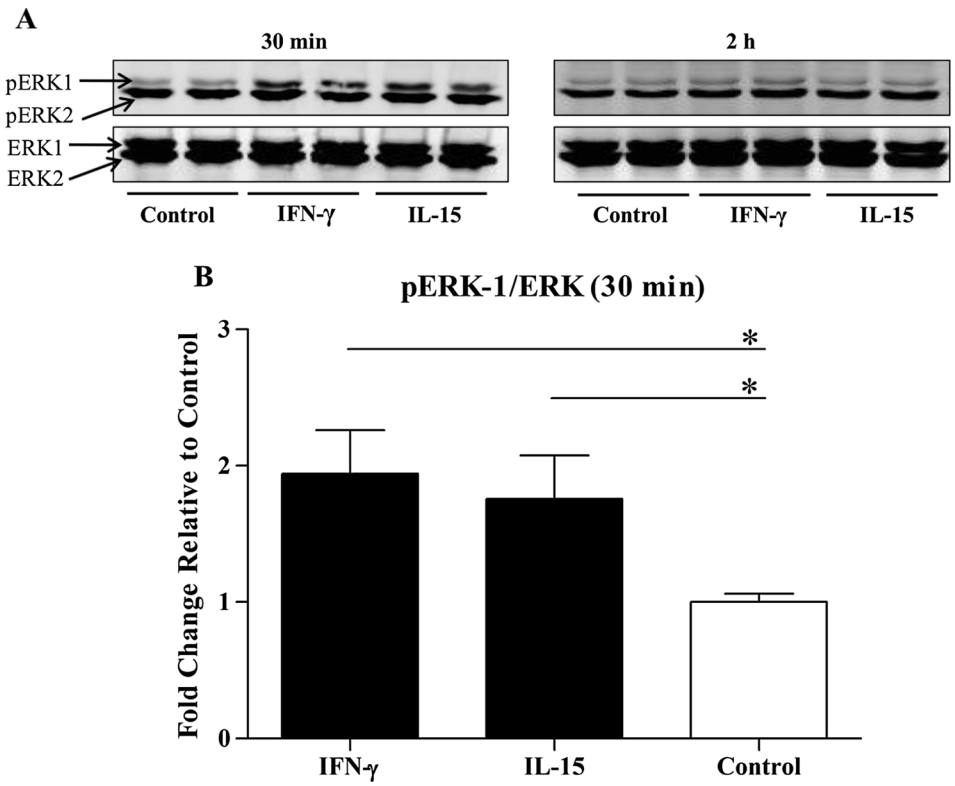

Fig. 5. IL-15 and IFN-γ lead to increased pERK1 in Huh7.5 cells. The cultured Huh7.5 cells were treated with IL-15 and IFN-γ for 30 min, 2 h, 6 h, and 24 h. The cells were lysed and p-ERK1/2 was measured using Western blotting. (A) The blots show that using inhibitor (PD98059) and DMSO alone do not have any effect on ERK or pERK expression. The increase of p-ERK1/2 was found only at the 30 min time point. (B) pERK2 concentration was corrected relative to total ERK1/2 and fold-changes relative to the control at each time point were calculated. IL-15 and IFN-γ resulted in a significant increase of pERK1 in Huh7.5 cells at 30 min (*$P \leq 0.05$).

3.2. The antiviral effects of IL-15 and IFN-γ are independent of NO production and type I IFN signaling

After finding that both IL-5 and IFN-γ were able to decrease HCV replication in Huh7.5 cells, we next wanted to determine whether their antiviral properties related to their ability to induce NO production or induce an antiviral state. To examine the possible involvement of NO production after IL-15 and IFN-γ treatment, NO was examined 24 h post-treatment. The levels of NO in the supernatants post-treatment did not significantly differ compared to control Huh7.5 cells. The results suggest that Huh7.5 cells do not produce soluble NO when treated with IFN-α, IFN-β, IL-15 or IFN-γ (Supplementary Fig. 1). However, addition of DETA-NO to Huh7.5 cells led to a direct dose-dependent inhibition of HCV replication, suggesting that NO has anti-HCV activity in this cell type.

We then wanted to assess whether IL-15 or IFN-γ were able to induce a protective antiviral stat in Huh7.5 cells. VSV-GFP showed that the supernatants from IL-15 or IFN-γ treated Huh7.5 cells were not able to induce an antiviral state in Vero cells as we found treated replication of VSV-GFP in Vero cells overlayed with supernatants from IFN-γ or IL-15-treated Huh7.5 cells (Fig. 3). Further, upon investigation of the type I IFN signaling expression profile in Huh7.5 cells post-IL-15 and -IFN-γ treatment, no significant upregulation of genes related to type I IFN signaling pathway was detected. As a control, stimulation of Huh7.5 cells with IFN-α or IFN-β induced upregulation of genes downstream of the type I IFN signaling pathway (Supplementary Fig. 2). Further, IL-15 and IFN-γ did not induce production of the type I IFN-induced cytokines MIP1-α, TRAIL, IP-10, and MIP-1β (Supplementary Fig. 3). These results demonstrate that the type I IFN signaling pathway and ISG expression are not involved in the antiviral effects of IL-15 and IFN-γ during HCV infection.

We then examined whether soluble-mediated factors released by IL-15 and IFN-γ treated Huh7.5 cells were involved in protection against HCV infection. The obtained data demonstrate that the inhibition of HCV replication induced by IFN-β, IL-15 or IFN-γ in Huh7.5 cells is not dependent on the production of soluble mediators produced by the Huh7.5 cells. Instead, these data indicate that IL-15 and IFN-γ affect the hepatocytes directly (Supplementary Fig. 4).

3.3. Inhibition of ERK/MEK1/2 signaling pathway diminishes the anti-HCV effects of IL-15 and IFN-γ

After determining that IL-15 and IFN-y prevent HCV infection by stimulating hepatocytes directly, we examined possible antiviral mechanisms occurring intracellularly. During IFN-γ or IL-15 stimulation, inhibition of the ERK/MEK1/2 pathway resulted in a significant increase in the number of HCV FFU compared to Huh7.5 cells treated with IL-15 or IFN-γ without inhibitors (Fig. 4A and 4B, respectively). We then examined the expression and phosphorylation of ERK1 and ERK2 at different times post-IL-15 or IFN-γ treatment was investigated. Western blot analysis using specific antibodies against ERK1 and ERK2 proteins, revealed that IL-15 and IFN-γ induce phosphorylation of ERK1 in Huh7.5 cells after 30 min of stimulation, which ultimately resolved within 2 h (Fig. 5A and B).

3.4. ERK overexpression confers resistance to HCV replication

The contribution of ERK/MEK1/2 signaling to the anti-HCV effect was examined by transfecting Huh7.5 cells with a plasmid expressing both GFP and ERK proteins. As a control, Huh7.5 cells were transfected with a GFP-expressing plasmid. The images taken from two different conditions, transfection with ERK-GFP and GFP alone, illustrate that there was little colocalization of ERK protein expression (GFP) with HCV-infected cells (PE-Texas Red; Fig. 6A). Analysis of the images revealed that less than 2% of the Huh7.5 cells had co-localization of both ERK protein expression and HCV infection (Fig. 6B), as compared to 45% co-localization in the in the control GFP expressing cultures. Further, there was significantly fewer numbers of HCV FFU in wells with ERK overexpression compared to cells transfected with GFP alone (Fig. 6C). The measurement of intensity of red light showed that GFP does not interfere with HCV infection (Fig. 6D). Overall, our data suggests that ERK expression is sufficient to confer resistance to HCV

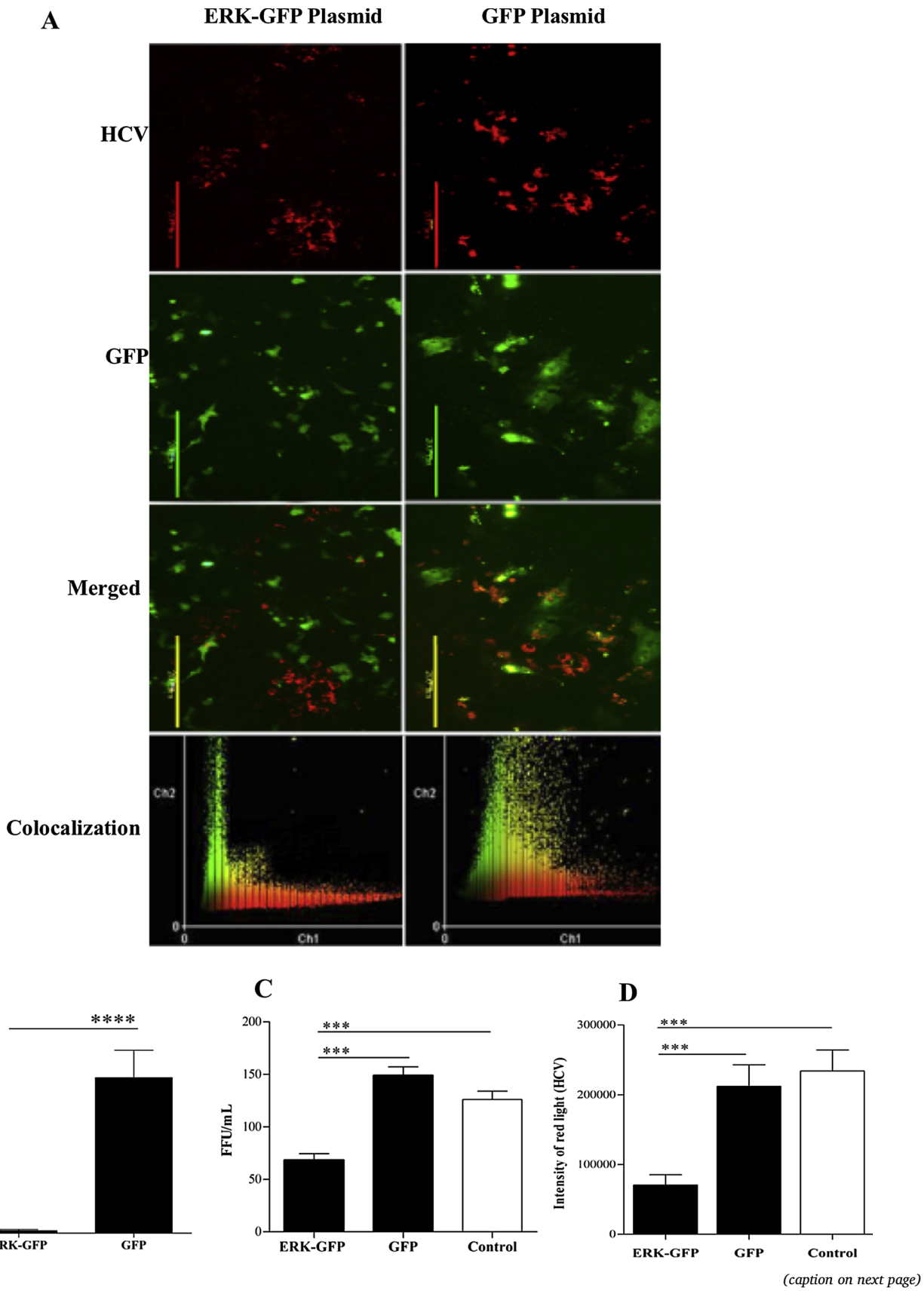

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Fig. 6. Huh7.5 cells transfected with ERK1 are resistant to HCV replication. The Huh7.5 cells were cultured in a 24-well plate and then transfected with the GFP-ERK-1 plasmid. After 8 h, the cells were infected with HCV (10^5 FFU mL^{-1}). Four hours post-infection cells were washed, complete DMEM was added and cells were incubated for 72 h. Immunofluorescence staining was then completed using secondary antibody (Texas Red-conjugated Goat Anti Mouse IgG antibody, Life Technologies). Fluorescence was detected with a confocal microscope (EVOS cell imaging system, Thermo Fisher Scientific). Separate photographs were taken with green (GFP) and red (HCV infection) filters and merged images were made by the overlay tool in EVOS. The likely effect of GFP expression on HCV infection was evaluated using Huh7.5 cells transfected only with GFP expressing plasmid as a control. (A) The pictures were analyzed using ImageJ. Co-localized spots were detected when the cells expressed ERK1 (Manders' coefficient = 0.459), showing that ERK expression confers resistance to HCV infection in Huh7.5 cells. The co-localization was found to be much higher in the cells that were transfected with GFP plasmid (Manders' coefficient = 0.958). Rectangular dashed line in scatter plots shows the number of cells that were positive for HCV and GFP staining (yellow dots). (B) The comparison of % co-localization between two groups was done using ImageJ and shows a significant difference (**$P \leq 0.01$). (C) Quantification of FFUs from the different treatment conditions showed a significant decrease in HCV infection after ERK expression. (D) Measurement of the intensity of green light showed that control plasmid (expressing GFP only) d did not interfere with HCV replication (*$P \leq 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

infection in Huh7.5 cells.

4. Discussion

HCV infection and transmission continues to persist worldwide and leads to liver damage and hepatocellular carcinoma in chronically infected individuals. The first generation of medicine for the treatment of HCV was an IFN-α monotherapy. To improve upon this therapy, the combination of ribavirin and IFN-α or pegylated interferon (PEG-IFN) was approved for use. More recently, the newest generation of HCV therapy includes interferon-free treatment, such as HCV protease inhibitors and an NS5B polymerase inhibitor [34–37]. Even with these treatments, HCV remains a worldwide public health problem as a significant proportion of infected individuals have a suboptimal response to treatment. Indeed, a number of factors, including HCV genotype, viral load, past treatment experience, degree of liver damage, and individual ethnicity, have all been shown to have an effect on an individual's response to treatment and their ability to clear the virus [19,38–40]. Therefore, understanding alternative methods of modulating the immune response against HCV can open new windows for finding more efficacious treatments as well as the development of a much needed preventative vaccine.

NK cells, as part of the innate immune system, are important players in the response against HCV infection and the subsequent outcome of HCV infection [41,42]. NK cells are potent antiviral innate immune effector cells that upon activation can limit virus replication through both inflammatory cytokine production and cytotoxicity. Furthermore, the high proportion of NK cells within the liver leaves them perfectly poised to rapidly respond to HCV infection. In particular, IFN-γ from NK cells can induce macrophages to produce NO, a potent antiviral molecule, as well as initiate the adaptive immune response against virus infection.

IL-15, which can be released from macrophages and DCs during infection, has been shown to stimulate NK cell proliferation as well as their production of IFN-γ. In this paper, the effects of both IL-15 and IFN-γ on HCV infection in a hepatic cell line was examined. We found that IFN-γ production from stimulated NK cells were able to potently inhibit HCV infection in Huh7.5 cells. Further, the direct addition of IFNs (α, β, γ) and IL-15 to Huh7.5 cells prior to HCV infection, results in these cells becoming resistant to HCV entrance and/or proliferation. These antiviral effects behaved in a dose-dependent manner, which resulted in a decrease in the size of FFUs after infection (unpublished data). This suggests that an antiviral state is induced in neighboring cells and/or cell-cell transmission of HCV is prevented with treatment [43,44]. Sene et al. found that HCV infection downregulates NKG2D expression on NK cells, reducing their cytotoxic function and IFN-γ secretion. Interestingly, IL-15 was able to reverse the downregulation of NK cell NKG2D expression [45].

In this study, to understand the underlying mechanism by which IL-15 and IFN-γ inhibit HCV replication in Huh7.5 cells, the potential involvement of the type I IFN pathway was examined. We and others have shown that type I IFNs are effective HCV inhibitors. IFN-α, -β and -γ induce the transcription of interferon-stimulated genes that can effectively inhibit virus replication [46]. After IL-15 and IFN-γ treatment, we found no significant increase of ISGs in the Huh7.5 cell line was not found, which suggests that IL-15 and IFN-γ, in particular, do not impart their antiviral activities through the type I IFN signaling cascade or through the activation of ISGs. However, it has been found in previous studies that Huh7.5 cells have a dominant negative mutation in the RIG-I gene and are susceptible to an absence of type I interferon production in response to viral infection [47]. As a result, we analyzed the change in protein levels of selected type I IFN-induced cytokines in the supernatant of Huh7.5 cells after treatment by IFN-α, IFN-β, IL-15, and IFN-γ. The significant increases for TRAIL, MIP-1α, and MIP-1β in the supernatants of cells treated with IFN-α and IFN-β were detected. We found elevation of IP-10 was only in cells treated with IFN-α. An increase in IFN-induced cytokines upon IL-15 or IFN-γ treatment was not found. Altogether, our data showed that IL-15 and IFN-γ do not inhibit HCV replication through the type I signaling pathway or through the activation of ISGs.

IL-15 and IFN-γ have previously been shown to stimulate NO production, a potent antiviral molecule that can inhibit virus replication. Indeed, it has been shown that IL-15 stimulation of murine macrophage RAW 264.7 cells results in the release of NO, which subsequently inhibited vaccinia virus (VV) replication in a bystander human 293 cell line in a dose-dependent manner [48]. However, our data suggest that IL-15 and IFN-γ do not inhibit virus replication through NO production. However, when NO was added to the Huh7.5 cells, there was a significant reduction in HCV replication. Though IL-15 and IFN-γ do not significantly induce NO production from Huh7.5 cells, these cytokines can induce NO production from other immune cells in the surrounding liver environment.

The transfer of soluble factors from Huh7.5 cells treated with IFNs (α, β, γ) and IL-15 to new Huh7.5 cells prior to infection did not inhibit HCV replication, suggesting that IL-15 and IFN-γ directly inhibit HCV replication within the hepatocytes themselves. To test this postulate, cells stimulated with IL-15 and IFN-γ were treated with inhibitors of the main signaling pathways prior to HCV infection. It has been shown that the IFN-γ- ERK axis plays a major in the antiviral activity of the NK cell supernatant. Yu et al. showed MKK1/2/ERK pathway is involved in IL-2 activation of NK cells [49]. Further, Jiang confirmed (MAPK-ERK) plays a critical role in the regulation of NK cell cytotoxicity [50].

Our data suggest that the ERK/MEK signaling pathway play a major role in the antiviral response induced by these two cytokines. Further, cells treated with IL-15 and IFN-γ displayed a significant increase of pERK1 at 30 min post-stimulation. This highlighted the role of the ERK/MEK pathway in inhibiting HCV replication in hepatocytes.

ERK 1 and 2 are the two main protein products in ERK pathway. Both proteins are activated by dual phosphorylation on their regulatory Tyr and Thr residues located within the Thr-Xaa-Tyr motif [51]. In the hepatocyte cell line, IL-15 and IFN-γ induce phosphorylation of ERK1, which is decreased after 2 h of stimulation. Murphy *et al* showed that signal duration is likely to influence the transcription and activity of immediate early genes, such as c-Fos, c-Jun, c-Myc and Egr1 [52]. We found that the over-expression of ERK1 in hepatocytes was sufficient to prevent hepatocyte infection. The majority of cells that were induced to

express ERK1 were negative for HCV infection, which suggests that expression of ERK confers resistance to HCV infection in hepatic cells. This is in agreement with other studies that have found that the MEK/ERK pathway is important in determining the outcome of HCV infection and HCC development [53]. Indeed, Ndjomou *et al* found that the MEK/ERK signaling pathway had a negative regulatory role on HCV infection [54]. Though the interaction between MEK/ERK signaling and HCV outcome has been studied, the role of the MEK/ERK pathway in HCV infection remains controversial [53–63]. Activation of the Ras/Raf/MEK/ERK pathway by HCV can augment HCV replication by enhancing hepatoma cell proliferation and subsequently its own propagation [53,55]. In contrast, a negative regulatory role of the MEK/ERK signaling pathway in HCV replication in NIH3T3 cells has been illustrated, where inhibition of the MEK/ERK pathway during HCV infection increased HCV replication [54,57]. In another study, exposing hepatic cells to HCV E2 protein upregulated the ERK signaling pathway [63]. Additionally, it has been shown that ERK inhibitors are able to partially modulate the expression of interferon-stimulated genes [61]. It has been suggested using ERK inhibitors with the current IFN-α-based regimen may improve overall treatment efficacy by blocking the bile acid-mediated promotion of HCV replication [62].

Though this *in vitro* model is able to mimic the interactions between HCV, hepatic cells and NK cells, there are limitations to this *in vitro* technique as it is not a reflection of the liver microenvironment that hosts a variety of cell types that can interact with each other. In the future, an investigation of IL-15, NK cells, and IFN-γ should be investigated in a double-humanized mouse model containing both a human immune system and human liver cells in order to understand the antiviral response of NK cells in an *in vivo* HCV infection.

This study provides direct evidence that ERK signaling inhibits HCV replication and show that IFN-γ released from human NK cells is able to inhibit HCV replication in a hepatocyte cell line. Interestingly, Huh7.5 cells express more IL-15Ra during HCV infection which makes them more responsive to IL-15 (Supplementary Fig. 5). In understanding the mechanism by which IL-15, NK cells, and IFN-γ are able to prevent HCV replication, this information can be used to develop effective therapeutics as well as open up future avenues of research to investigate the spontaneous clearance of HCV in particular individuals.

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Conflict of interest

The authors declare no conflict of interest.

Author contribution

Designed and conceived the experiments: AAA, KLM, CDR, FV. Contributed reagents, materials and analysis tools: JJF, RSR. Performed experiments: FV, SEC, MVC, EL, BC, AD. Wrote the manuscript: FV, AJL, AAA.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.06.006.

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