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Regulating Action of in Vitro Hepatitis C Virus Infection on Interferon-Induced Interferon Stimulating Genes in Murine Macrophages

Dolgorsuren Sandagdorj[1], Batkhishig Munkhjargal[1], Baasansuren Enkhjargal[1], Baljinnyam Tuvdenjamts[1,3], Enkhjin Zorigt[1], Budjav Jadamba[1], Altanshagai Chinbat[1], Khongorzul Samdankhuu[4], Ulziisaikhan Jambalganii[1], Batsuren Boldbaatar[1], Tsogtsaikhan Sandag[1], Bilegtsaikhan Tsolmon[2], Enkhsaikhan Lkhagvasuren[1]

[1]Department of Microbiology and Immunology, School of Pharmacy and Bio-Medicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia; [2]Core Laboratory, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia; [3]Institute of Medical Sciences, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia; [4]School of Nursing, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia

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Corresponding Author
Enkhsaikhan Lkhagvasuren, PhD
Department of Microbiology and Immunology, School of Pharmacy and Bio-Medicine, Mongolian National University of Medical Sciences, Ulaanbaatar 14210, Mongolia
Tel: +976-89981001
E-mail: enkhsaikhan@mnums.edu.mn

Objectives: The objective of this study was to determine the in vitro regulating effect of Hepatitis C virus (HCV) infection on interferon stimulating genes (ISGs) stimulated by IFN-γ and Imiquimod (TLR7 ligand) on murine macrophages. iNOS and STAT1 were measured by RT-PCR, and immunoblotting (IB). Nitric oxide production was measured by Griess Reagent Assay. **Results:** HCV inhibits IFN induced iNOS mRNA and also protein expression. HCV significantly reduced IFN-γ induced ISGs (iNOS mRNA, iNOS protein, s727-STAT1, tyr701-STAT1). **Conclusion:** These results indicate that in vitro hepatitis C virus infection is involved in the regulation of IFN-γ induced ISGs in the levels of gene and protein expression of iNOS and STAT1 transcription factors.

Key words: HCV, Nitric Oxide, IFN-γ, RAW264.7

Introduction

HCV mainly replicates within hepatocytes in the liver, although there is a clear evidence for replication in lymphocytes or monocytes [1]. In 50-80% of all patients, the infection becomes chronic and results in liver fibrosis, cirrhosis, primary liver cancer, and eventually death [2, 3]. For these reasons, it is the primary indication for liver transplantation. Since the virus

was discovered in 1989, significant public health and research advances have led to great progress in curbing new infections and developing life-saving treatment options [4].

However, globally 130-150 million people have been infected with HCV (World health organization strategy 2016) and worldwide 1.4 million people die each year from HCV-related acute inflammation, primary liver cancer and cirrhosis. The prevalence of HCV infection in Mongolia is estimated about 14% of Mongolian population of which 95% are 1b genotypes [5]. The high prevalence of HCV infection is because single-use hypodermic needles were not available in Mongolia prior to 1990 during the Soviet era [6].

Although the hepatocyte is the main target of HCV infection, there are some difficulties in studying HCV-infected hepatocyte culture in vitro. Consequently, macrophage, dendritic cell, and fibroblast cultures are often used to study HCV infection. What are the clinical implications of the proposed role of macrophages and monocytes in HCV infection? Macrophages are fairly long-lived cells, and generally survive for weeks [7]. Macrophages in patients with HCV infection may act as a carrier of the infection to other cells, infecting microglial/macrophage cells, and may allow HCV to pass through the blood-brain barrier to infect brain astroglial cells [8-10]. But little else is known about HCV infection of macrophages.

Sometimes an adaptive immune response to HCV infection is not determined in vitro. During HCV infection, the immune system is inadequate to eradicate the virus. After HCV infection, IFN is secreted from macrophage and other cells, then IFN-stimulating gene (ISG) expression is activated [11]. After infecting hepatocytes, Natural killer T-cells may directly limit HCV replication by secreting IFN-γ locally. IFN-γ directly inhibits HCV replication in vitro. It has been shown that IFN-γ secreted from immune cells of chimpanzee liver plays a main role in eliminating the HCV from the body [11, 12]. But little is known about these mechanisms in HCV infected macrophages.

The IFN-γ receptor (IFNγR) is expressed in hepatocyte and other cells. When IFN-γ binds to its receptor, it induces STAT1 (Signal transducer and activator of transcription 1) protein phosphorylation by Jak1 (Januse kinase 1) and Jak2 (Januse kinase 2). Phosphorylated STAT1 forms a homodimer, moves to the nucleus, and interacts with GAS (Gamma Interferon-activated site) to initiate ISG gene transcription. An example of a ISG is NOS2 also known as iNOS (Nitric oxide synthase) gene [13]. iNOS synthesizes nitric oxide (NO) which becomes an important mediator of chronic liver cell inflammation. Because of the many sources and pleiotropic effect of NO on the liver and other organs, its effect on the regulatory function of organs is unclear and controversial.

Tissue nitric oxide levels increase due to cytokines and endotoxins that depend on iNOS enzyme activity. Even though nitric oxide's role in chronic hepatitis pathogenesis is not fully understood, overproduction of NO is related to liver tissue damage during inflammation. Liver inflammation, fatty liver disease and cirrhosis are the direct result of HCV infection and indirect result of inflammation and oxidative stress damage. Many study results show that after HCV infection, expression of iNOS increases [14-16]. However, depending on the situation, NO has either a protective or cell damaging effect; over synthesis of NO may promote fibrosis and inflammation [11]. So we examined the signalling pathway of IFN-γ through STAT1 phosphorylation, iNOS and its mRNA expression and NO production.

Even though, there are large numbers of HCV and ISG gene cross-talking studies, the relationship between innate immunity, IFN-γ stimulated antiviral pathway, and adaptive immune response is not fully understood in macrophages and further studies about their interaction are needed. The objective of this study was to determine the in vitro regulating effect of HCV infection on interferon stimulating genes (ISGs) stimulated by IFN-γ and TLR7 ligand in HCV infected macrophages.

Materials and Methods

1. Cell culture

The murine macrophage cell line RAW 264.7 was used in this study. RAW 264.7 cells were cultured in RPMI-1640 or DMEM supplemented with 10% inactivated fetal calf serum (FCS), an antibiotic mixture (penicillin G, streptomycin, amphotericin B), and incubated at 37°C and humidified in 5% CO2 until cell growth reached 85% of surface area of the culture plates. The cell culture suspension was prepared with trypsin and ethylene-amin tetra acid (EDTA).

Sample collection:

HCV positive and negative serums were obtained from 10 patients who volunteered to participate to the study and viral

RNA loads of their sera were confirmed by Bona-Vita Laboratory and the laboratory of the Liver Center, in Mongolia. We used patients sera whose viral RNA loads were 1000000 U/ml and 3700000 U/ml respectively. For the HCV negative group, we used serum from a person who tested negative for HCV, HBV and HIV.

2. Infection of cell culture with patient-derived serum:
Serum from the patients who were infected with HCV genotype 1b were incubated with PEG8000 and PBS at 4°C for 12 hours (Serum I: 10^6u/ml, serum II: 3,7 x 10^6u/ml, in 1,2 cell: 1 virus RNA), and then centrifuged at 1600g. After centrifugation, the virus containing supernatant was added to the cell cultures in a 6 well plate.

3. qRT-PCR determination of HCV RNA:
RAW264.7 cells were lysed with Nucleospin RNA isolation kit (Machery Nagel). The extracted RNA was run with OneStep qRT-PCR Hepatitis C Virus® genes Advanced Kit (Primer Design, UK) in accordance with the manufacturer's protocol.

One-step qRT-PCR combines the reverse transcription and real-time PCR reaction in a simple closed tube protocol. This saved significant bench time and also reduced errors. The sensitivity of a one-step protocol was also greater than a two-step because the entire biological sample is available to the PCR without dilution. Amplification was performed for 50 cycles.

4. Determination of Nitrite using the Griess Reaction (Nitrite determinations analysis)
The Griess Reagent System used sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This system detected NO production in a variety of cell and tissue liquid such as plasma, serum, urine and tissue culture medium. A nitrite standard reference curve was established for each assay for accurate quantitation of NO production NO_2 levels in experimental samples.

5. qRT-PCR determination of iNOS mRNA
Cells were lysed with Macherey Nagel NucleoSpin RNA plus kit and, RNA concentration was measured by Flurometer method. Unlike identification of HCV RNA, we performed a 2-step qRT-PCR for a detection of the RNA.

6. Immunoblotting:
Protease and phosphatase inhibitor containing lysate solution was used for cell lysation, and protein concentration was measured by Pierce BCA protein assay kit. Proteins were resolved by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes. Protein expression was quantified using Image J densitometry analysis and normalized to b-actin expression.

7. Statistical analysis
Statistical analysis was performed with the Student t-test using Microsoft Excel software. A p-value <0.05 was considered significant.

8. Ethical statement
The study was approved by the Ethical Review Committee of Mongolian National University of Medical Sciences. Informed consent was obtained from the participants who donated their serum.

Results

Determination of Serum Derived HCV's Effect on IFN Stimulated NO Production

After detecting HCV RNA in RAW264.7 cell line, we measured the NO production in supernatant of the culture. We determined activation ISG by NO production and, the effect of HCV on it.

NO production increased with time and dose independently when treated with IFN-γ alone, but on the other hand IFN-γ and HCV positive serum when combined together had lowering effect (Figure 1A, B). This effect was more clearly evident at 36 and 48 hours ($p<0.01$).

Identitification of HCV effect on IFN-γ stimulated expression of iNOS mRNA

In the control group there was no iNOS production. Whereas IFN-γ alone increased iNOS mRNA expression by 9.6 fold in the infected celled compared to the control group. HCV alone slightly induced iNOS mRNA expression. But when HCV is combined with IFN-γ the induced iNOS mRNA expression

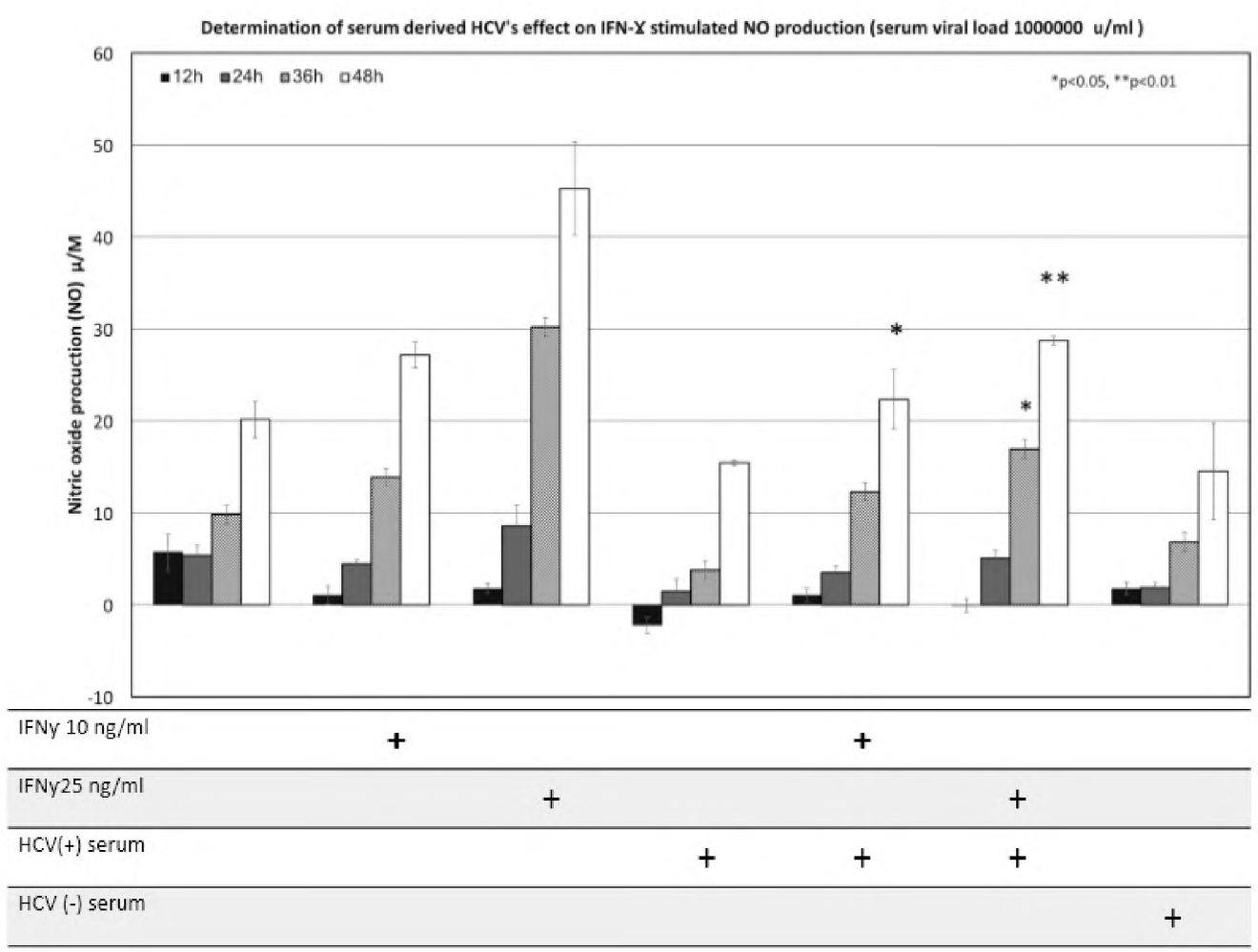

Figure 1A. Effect of HCV(+) serum on IFN-γ induced NO production in RAW264.7 cells. RAW264.7 cells were pretreated with HCV(+) serum (1000000 u/ml) and then stimulated with IFN gamma. The levels of NO production in the culture supernatant were determined by Griess reagent assay on 12, 24, 36 and 48 hours. **p<0.01

decreased (Figure 2). These results show that HCV infection inhibits IFN-γ induced iNOS mRNA expression in murine macrophage RAW264.7 cells.

Identitification of HCV effect on IFN-γ stimulated protein expression of iNOS

According to our immunoblot results, when IFN-γ or imiquimod were used alone, there was increased protein expression of iNOS. When IFN-γ and imiquimod were combined the protein expression increased but but less than the sum of the effects of IFN-γ and imiquimod alone. But when treated with HCV positive serum alone, the protein expession decreased. Furthermore, HCV inhibited the protein activation in the group treated with both IFN-γ and imiquimod (Figure 3). From these results HCV inhibits IFN-γ induced protein expression of iNOS in RAW264.7 cell.

Determination of HCV effect on Type II interferon stimulated Y701 STAT1 protein phosphorylation

As an immunoblotting assay result, IFN-γ alone increased the p-Y701STAT1 protein synthesis. But the Y701STAT1 protein phosphorylation increased even more when imiquimod was combined with IFN-γ. However, in HCV-infected macrophage cells, the IFN-γ +imiquimod-stimulated Y701STAT1 protein phosphorylation was inhibited (Figure 4). We used β-actin as an internal control. According to our immunoblotting results,

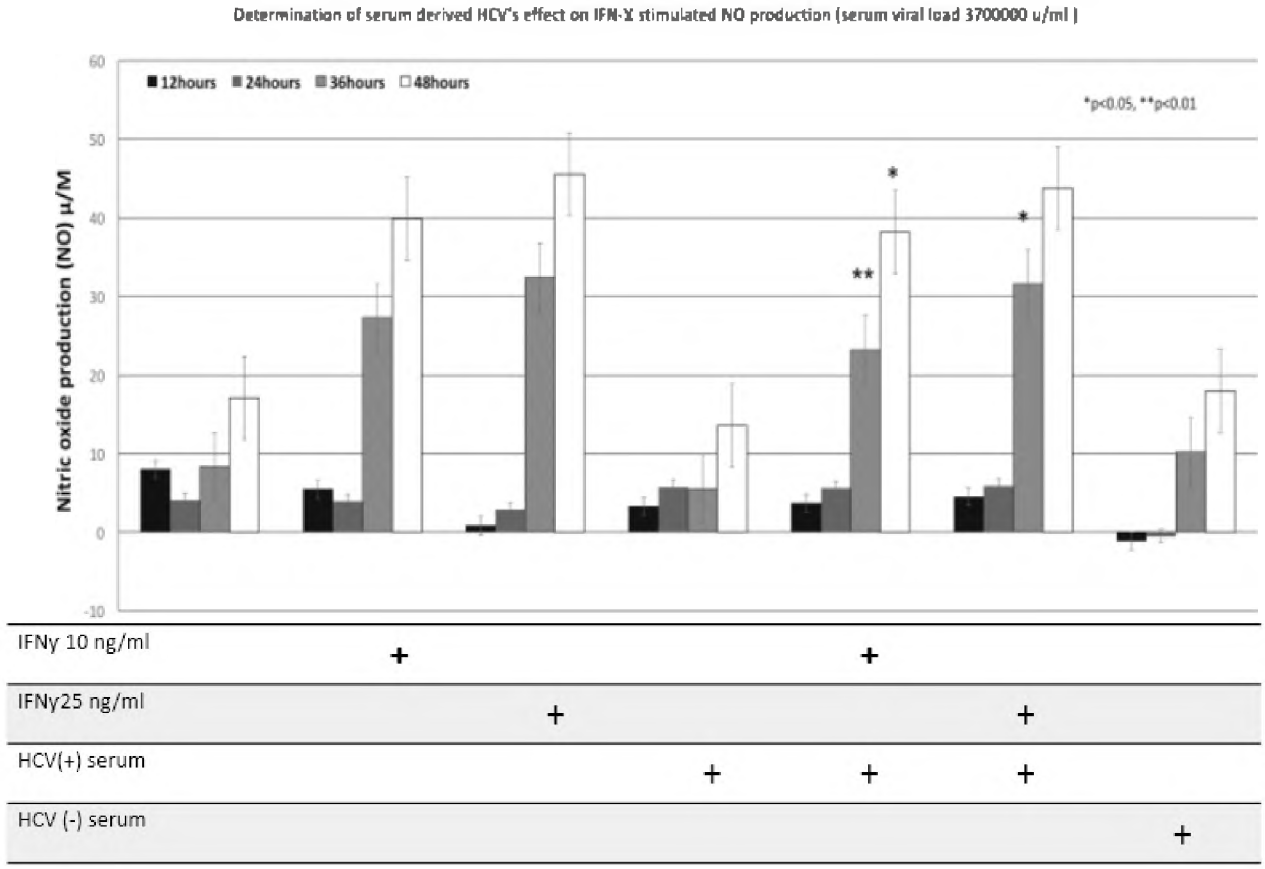

Figure 1B. Effect of HCV(+) serum on IFN-γ induced NO production in RAW264.7 cells. RAW264.7 cells were pretreated with HCV(+) serum (3700000 u/ml) (B), and then stimulated with IFN gamma. The levels of NO production in the culture supernatant were determined by Griess reagent assay on 12, 24, 36 and 48 hours. **p<0.01

HCV infection decreases IFN-γ and imiquimod stimulated p-Y701 STAT1 protein phosphorylation.

Determination of HCV effect on IFN-γ and imiquimod stimulated S727 STAT1 protein phosphorylation.

IFN-γ and imiquimod alone increased the protein expression of p-S727-STAT1. Whereas, in HCV positive serum treated group they inhibited protein phosphorylation (Figure 5). From these results we determined that HCV infection decreases effects on IFN-γ and imiquimod induced phosphorylation of S727-STAT1. HCV infection interferes IFN-γ signalling pathways and limits both phosphorylation of serine and tyrosine residues which is super activation of STAT1.

Discussion

The purpose of this study was to determine NO production, mRNA and protein expression of iNOS and activation of STAT1 in HCV infected murine macrophage linear cell model.

George et al. noted result that viral RNA was detectable in 7 types of cells in vitro after 48 hours of incubation with HCV positive serum [17]. Despite that, according to the studies of HCV infection of human linear cells by Nicola et al. (2012), Jason et al. (2013), Moetaz et al. (2012), HCV infection can damage many kinds of human cells of, so more study is needed on this field [18-21]. Giuseppe et al. showed that HCV enters the cell by entering the bloodstream, being transported by high

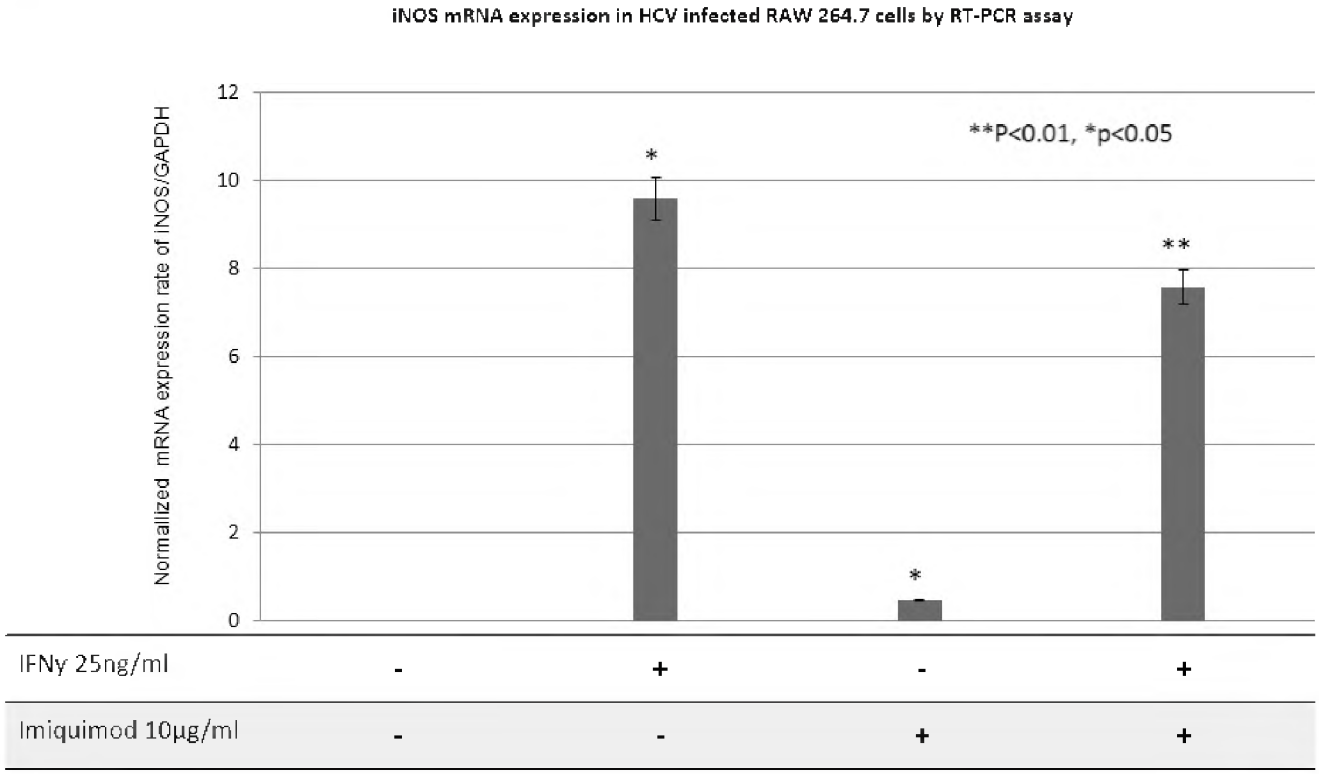

Figure 2. iNOS mRNA expression in HCV infected RAW 264.7 cells. HCV infected RAW264.7 cells were stimulated with IFN gamma and Imiquimod. The iNOS mRNA expression in the HCV infected RAW 264.7 cells was determined by RT-PCR assay after HCV infection in 2 days. **p<0.01

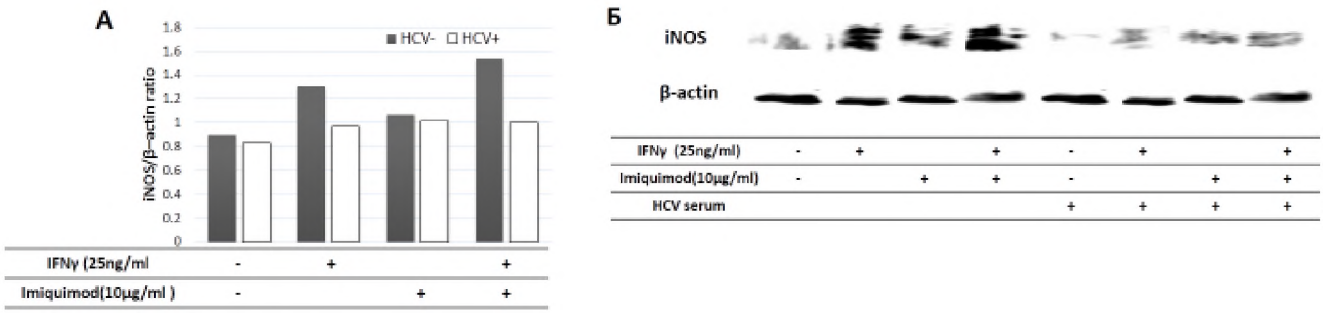

Figure 3. Determination of HCV effect on iNOS expression stimulated by Type II interferon. (A) Compared result of quantitative analysis of immunoblotting results done with the help of ImageJ program. (B) Immunoblotting result. After 48 hours of treating imiquimod with HCV(+) serum, it was treated by imiquimod (10 m/ml) for 1 hour and by IFN-γ (25 ng/ml) for 24 hours. Then iNOS expression was determined by immunoblotting assay. β-actin was used as an internal control.

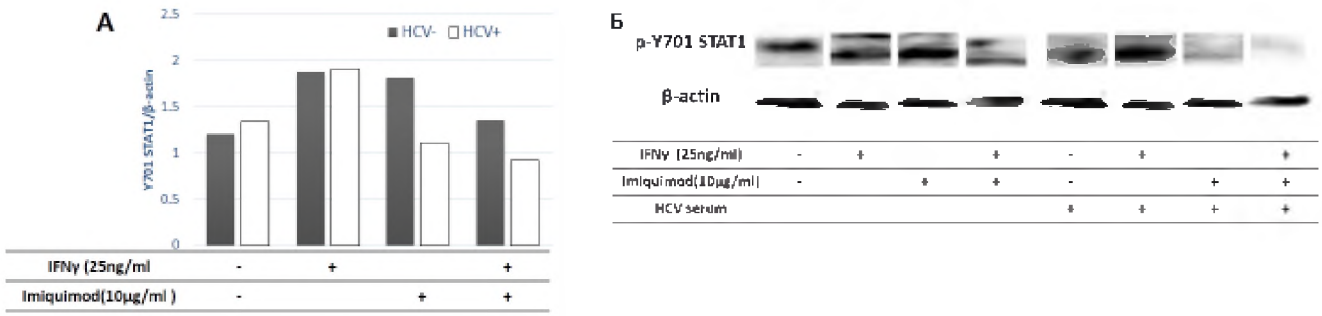

Figure 4. Determination of HCV effect on IFN-γ stimulated p-Y701STAT1 protein synthesis. (A) Compared result of quantitative analysis of immunoblotting results done with the help of ImageJ program. (B) Immunoblotting result. After 48 hours of treating RAW264.7 cell with HCV(+) serum, it was treated by imiquimod (10 mg/ml) for 1 hour and by IFN-γ (25 ng/ml) for 2 hours. Then Y701 STAT1 protein phosphorylation was determined by immunoblotting assay. β-actin was used as an internal control.

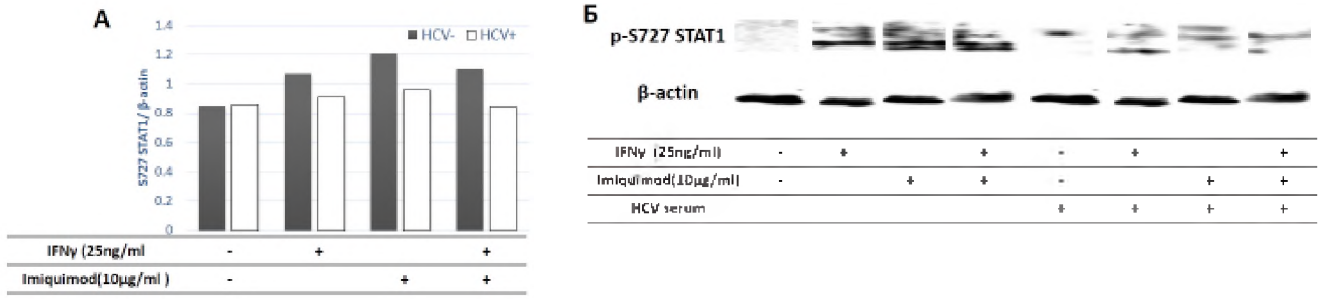

Figure 5. Determination of HCV effect on IFN-γ stimulated p-S727STAT1 protein synthesis. (A) Compared result of quantitative analysis of immunoblotting results done with the help of image J program. (B) Immunoblotting result. After 48 hours of treating RAW264.7 cell with HCV(+) serum, it was treated by imiquimod (10 mg/ml) for 1 hour and by IFN-γ (25 ng/ml) for 2 hours. Then S727 STAT1 protein phosphorylation was determined by immunoblotting assay. β-actin was used as an internal control.

density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL) to the liver and binding to hepatocyte surface receptor called CD81, then disrupting the claudin, occludin tight junctions [21, 22]. HCV infection can damage not only liver but also other organs because CD81 receptor is expressed on many cell surfaces such as macrophages, thyrocytes and retinal cells [23, 24]. HCV infection of mouse macrophage in our study can be explained by "quasispecies" feature of this virus. In the study of Ramirez et al. the ability of HCV to create a mutation and shift is explained by the term "quasispecies" [25-27].

We determined IFN-γ induced gene activation by the level of IFN-γ stimulated NO production and showed that HCV evades from host immune response. This may be the indirect mechanism by which the hepatitis C virus escapes host immune response. IFN-γ, which is a type II IFN, participates in IFN induced activation of a group of genes. One of the IFN induced genes is a gene which codes iNOS protein and iNOS is an enzyme that directly participates in NO synthesis [27]. From this result we see that HCV infection inhibits IFN-γ induced gene activation and decreases IFN-γ induced NO production. In other words, HCV works against host cell primary immune response. However, IFN-γ has a strong antiviral effect, resulting in a decrease of NO production in HCV positive serum groups. This supports the hypothesis of Ibrahim and Gomaa's (2010) that when the cell is treated with IFN-γ, signal transduction of IFN-γ decreases due to HCV infection, making it possible for the virus to evade host immune mechanism [25].

In the study about the relationship between HCV infection and NO production, Keigo et al. (2014) determined that non-

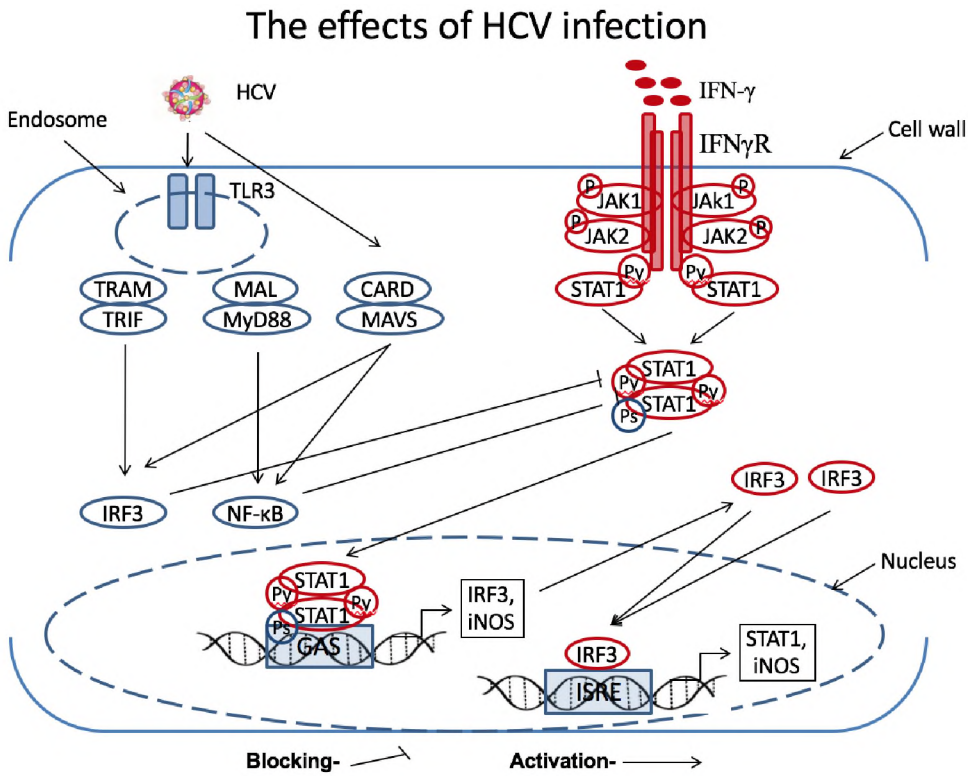

Figure 6. A possible molecular mechanism of HCV infection on IFN-γ stimulated signal transduction on RAW264.7 macrophage cells.

structural proteins of HCV had an effect on NO production. The noted that non-structural protein-3 (NS3) of HCV, plays the main role in NO production [28]. Therefore, using these recombinant proteins, we can induce NO production and know how IFN-γ inhibits NO production and the signalling pathway of IFN-γ. Scott et al. (2015) and other researchers studied STAT1 activation in several types of cell lines including macrophages. Unlike those studies, we determined that IFN-γ induced STAT1 activation in a HCV infected murine macrophage cell line.

According to our study, HCV inhibits IFN-γ induced phosphorylation of Y701 STAT1 and iNOS protein and mRNA expression in murine macrophage cells. In addition, HCV down-regulates TLR7 ligand dependent S727 STAT1 activation in response to IFN-γ treatment. Those results indicate that HCV could be a negative regulator on the super-activation of STAT1 transcription factor at the both sites of Serine and Tyrosine phosphorylation.

A possible molecular mechanism of HCV infection on IFN-γ stimulated signal transduction on RAW264.7 macrophage cells in shown in Figure 6.

Hepatitis C virus (HCV) triggers several transcription factors such as nuclear factor kappa B (NF-kB) and interferon regulatory factor-3 (IRF3) through TRIF (TIR-domain-containing adapter-inducing interferon-β), TRIF-related adaptor molecule (TRAM), MyD88 adapter-like (Mal) and Myeloid differentiation primary response 88 (MYD88) via toll-like receptor 3 (TLR3) signalling. In addition, HCV also stimulates IRF3 and NF-kB through caspase-recruitment domain/Mitochondrial antiviral-signalling protein (CARD/MAVS) pathways [29]. On the other hand, IFN-γ induces gene activation of interferon stimulating genes (ISG) by JAK/STAT1 signaling pathways. This leads to the production of iNOS, a key enzyme on the synthesis of nitric oxide production. HCV infection might downregulate IFN-γ signalling via inhibition of STAT1 super activation at serine and tyrosine phosphorylation sites.

Serena et al. (2009) determined that HCV expression leads to increased baseline and impaired TLR7 ligand-induced IRF-7 expression in HCV-infected human hepatoma cell line HUH.7 and Japanese fulminant hepatitis JFH-1 cells. They found a significant decrease of TLR7 expression in the presence of

HCV infection in both in vitro in hepatoma cells and in vivo in human HCV-infected livers [30]. Many researchers have studied type I IFN and TLR7 ligand-induced activation of STAT1 in HCV infected cells, but there are few studies about IFN-γ - induced activation of STAT1 [30-32].

Our results showed that HCV infected serum significantly reduced IFN-γ induced ISGs (iNOS mRNA, iNOS protein, s727-STAT1, tyr701-STAT1) and its final product, nitrous oxide.

But our study has some limitations. First, our cells were infected with HCV for a relatively short period of time, making our results difficult to compare to other studies. Second, we did not use human primary liver cell cultures from HCV infected patients. Instead, we used mouse macrophage cell lines. In future studies, we will do similar experiments on different cell lines other than macrophage cells to test the hypothesis that HCV infects cell types other than hepatocytes in in vitro.

Lastly, we still have many unanswered questions regarding NO production during HCV infection, the mechanism of HCV evasion from IFN and the immune system, HCV heterogeneity, the distribution of quasispecies, how IFN stimulates other genes and how these impact potential therapies.

In conclusion, our results indicate that in vitro hepatitis C virus infection does involve the regulation of IFN-γ induced ISGs level of gene and protein expression of iNOS and STAT1 transcription factors. Further studies are needed regarding HCV viral RNA replication and how IFN-γ affects HCV replication in different cell types using TLR signalling [32, 33].

Conflict of Interest

The author states no conflict of interest.

Acknowledgements

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