# Hepatitis C Virus Impairs Natural Killer Cell Activity via Viral Serine Protease NS3

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# Hepatitis C Virus Impairs Natural Killer Cell Activity via Viral Serine Protease NS3

Directed by Professor Jae Myun Lee

The Doctoral Dissertation
submitted to the Department of Medical Science,
the Graduated School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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June 2017

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June 2017

# ACKNOWLEDGEMENTS

7 년여의 학위 과정 기간을 거쳐 이렇게 박사 학위를 받게 된 지금, 그 어떤 말로도 기쁨을 다 표현할 수 없을 것 같습니다. 대학을 졸업하면서 질병의 근원을 연구하고 해결하는 의과학자의 길을 걷겠다 생각하고 훌륭한 연구 업적을 이루겠다 다짐했지만, 지난 시간들을 돌이켜 보면 언제나 실패들의 연속을 겪어야만 했습니다. 어려운 공부와 연구를 하기에는 제 자신이 부족하다는 생각에 괴로웠고, 때로는 연구 과정에서 오는 큰 좌절과 상실감으로 이 길을 택한 스스로에 대한 원망으로 모든 것을 포기하고 도망치고 싶은 생각도 많았습니다. 그렇지만 주변의 많은 분들께서 격려와 도움을 주셔서 극복할 수 있었습니다.

먼저 부족한 저에게 공부할 기회를 주시고 좋은 환경에서 연구할 수 있도록 지원을 아끼지 않으셨던 이재면 교수님께 진심으로 감사합니다. 교수님께서 해주신 격려와 가르침 덕분에 어려웠지만 이렇게 논문을 완성할 수 있었습니다. 그리고 과학자의 기본 소양과 연구에 대한 접근 방법을 깨우쳐 주신 박전한 교수님께 깊은 감사를 드립니다. 교수님의 가르침이 앞으로 의과학자로 살아 가는 동안 흔들림 없이 연구를 대하는 기준이 될 것입니다. 또한 제가 실험실에 들어와 자연살해세포와 C 형 간염바이러스 연구를 할 수 있도록 기틀을 잡아주시고, 논문의 부족한 부분을 채울 수 있도록 도와 주신 이화여자대학교 윤주천 교수님께 감사 드립니다. 바쁜 시간에도 논문 심사를 해주신 윤호근 교수님과 정재호 교수님께도 감사 드립니다. 해외 학회를 갈 수 있도록 도와주시고 관심

갖고 봐주신 최인홍 교수님, 실험에 필요한 재료들을 제공해주신 카이스트 신의철 교수님, 포항공과대학 장승기 교수님께도 감사의 말씀 드립니다.

학위 과정 시작부터 끝까지 실험실에서 함께 생활 하면서 여러모로 도와주신 분들께도 감사의 인사를 드립니다. 전체적인 실험의 기본기들을 가르쳐주신 권승현 선생님 항상 감사합니다. 제 실험에 관심 많이 가져주신 이현규 선생님, 늘 진심 어린 조언을 해주시고 가르쳐주신 박광환 선생님, 실험실에서 가장 오랜 시간을 함께 하며 흑역사부터 보고 도와주신 심두희 선생님, 학위 과정 마지막에 실험이 잘 풀리지 않아 좌절할 때 함께 고민해 주시고 조언도 많이 해주신 차혜란 선생님, 논문 마무리를 위해 조언 많이 해주신 박필구 선생님, 언제나 즐거운 대화로 큰 고민도 작은 고민거리처럼 느끼게 만들어 주신 김민정 선생님 정말로 감사합니다. 실험적으로 별로 도와준 것 없이 여러 가지로 도움만 받아 미안한 용근이, 작은 것부터 실험까지 늘 친절하게 도와준 혜미와 경주, 바쁘게 실험 하느라 정신 없는 국영 쌤 그리고 함께 졸업하는 수지와 신입생 수진이까지 모두에게 깊은 감사를 드립니다.

미생물학교실의 여러 선생님들과 선·후배님들에게도 감사를 전합니다. 물심양면으로 지원해주신 고시환 선생님, 실험 때문에 채혈 많이 도와주신 김영미 선생님, 만날 때 마다 걱정해 주시고 조언해 주신 곽만섭 선생님과 이강무 선생님, 지금은 질병관리본부에 계시는 양은정 선생님 감사합니다. 졸업 때문에 오랜 시간 함께 마음 고생 했던 진원이형, 미국에서 멋진 인생을 살고 있을 샛별이, 열심히 연구중인 지애 쌤과

태강이, 먼저 졸업하고 회사로 떠난 승재 쌤과 상준이에게 감사의 말씀 전합니다. 실험 얘기와 시시콜콜한 얘기도 많이 했던 후배 수민이와 많이 친해지지 못해 아쉬운 후배들 (희욱, 유진, 한구, 원태) 모두 감사합니다. 학위 과정 동안 만나면 우울한 얘기만 했었는데 그때마다 잘 들어주고 격려해준 고마운 친구들 (민욱, 재혁, 봉근, 수진, 성연, 원석, 류미, 제선, 지섭, 현아) 감사합니다. 실험의 기본기를 가르쳐 주신 서울의대 미생물학교실 (박정규 교수님, 김봉긔 선생님, 윤일희 선생님, 김조민 선배님, 신진영 선생님, 양승하 선생님, 종형이 형, 수영이 누나) 감사드립니다.

끝으로 오랜 시간 저보다 더 마음 고생 하시면서도 곁에서 묵묵히 지켜봐 주시고 응원만 해주신 사랑하고 존경하는 아버지, 어머니, 그리고 언제나 자랑스러운 동생 감사합니다. 남들은 일찍 취업하고 결혼해서 가정 꾸리고 사는데, 저는 이제야 학업이 끝났네요. 오랫동안 부족한 아들·오빠 뒷바라지 하느라 고생만 하셨는데 앞으로 보답할 수 있도록 하겠습니다. 손자 잘되라고 걱정 많이 해주신 할머니, 외할아버지, 외할머니께도 감사를 드립니다. 4 년의 시간 동안 한결 같은 마음으로 옆에서 응원해주고 사랑해준 여자친구 민정에게도 진심으로 감사하고 사랑한다고 전합니다.

2017 년 06 월
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### Hepatitis C Virus Impairs Natural Killer Cell Activity via Viral Serine Protease NS3

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(Directed by Professor Jae Myun Lee)

Hepatitis C virus (HCV) infection is characterized by a high frequency of chronic cases owing to the impairment of innate and adaptive immune responses. The modulation of natural killer (NK) cell functions by HCV leads to an impaired innate immune response. However, the underlying mechanisms and roles of HCV proteins in this immune evasion are controversial, especially in the early phase of HCV infection. To investigate the role of HCV non-structural proteins especially NS3 in the impairment of NK functions, NK cells were isolated from the peripheral blood mononuclear cell (PBMC) by negative selection. To assess the direct cytotoxicity and IFN-γ production capability of NK cells, NK cells were co-cultured with uninfected, HCV-

infected, HCV-NS3 expressing construct-transfected Huh-7.5 cells, or HCV-NS replicon cells. To determine the effect of an NS3 serine protease inhibitor, HCV-infected Huh-7.5 cells were treated with BILN-2061. Then, NK cells were harvested and further co-cultured with K-562 target cells. NK cell functions were analyzed by flow cytometry (FACS) and enzyme-linked immunosorbent assay (ELISA). When co-cultured with HCV-infected Huh-7.5 cells, the natural cytotoxicity and IFN-γ production capability of NK cells were significantly reduced. NK cell functions were inhibited to similar levels upon co-culture with HCV-NS replicon cells, HCV-NS3 expressing construct-transfected Huh-7.5 cells, and HCV-infected Huh-7.5 cells. These reductions were restored by BILN-2061-treatment. Furthermore, BILN-2061 treatment significantly increased degranulation against K-562 target cells and IFN-γ productivity in NK cells. Consistent with these findings, the expression levels of activating NK cell receptors, such as NKp46 and NKp30, were also increased by BILN-2061 treatment. Together, in HCV-infected Huh-7.5 cells, the serine protease NS3 may play a role in the abrogation of NK cell functions in the early phase of infection through downregulation of NKp46 and NKp30 receptors on NK cells. These results suggest that NS3 represents a novel drug target for the treatment of HCV infections.

Key words: hepatitis C virus, non-structural protein NS3, serine protease, early phase of infection, natural killer cell, activating receptor, NKp46, NKp30

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## I. INTRODUCTION

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is an enveloped, positive-sense RNA virus.[1] Seven major genotypes and more than 100 subtypes have been identified.[2] HCV is parenterally transmitted, mainly from injection drug abuse and unsafe transfusions and therapeutic injections.[3,4] Approximately 170 million people in the world are infected by HCV. HCV infection is characterized by its chronicity.[5] Up to 80% of infected patients develop chronic hepatitis as a consequence of impairment of innate and adaptive immune response.[2,3,5] Spontaneous clearance of HCV is rare in the chronic phase of infection. Chronic hepatitis progresses to liver fibrosis,

cirrhosis, and hepatocellular carcinoma (HCC).[6,7] Although impairment of adaptive immune responses against HCV has been revealed, that of innate immune responses, especially natural killer (NK) cell responses, remains unclear and still has a lot of controversy.[3,5]

NK cells constitute a major component of the intrahepatic lymphocyte and provide innate immune responses against many pathogens.[2,3,8] They serve as the front line of defense against virus infections because they recognize and rapidly kill virus-infected cells in the early phase of infection.[2,9,10] The outcome of engagement between receptors of the NK cells and ligands of the target cells is determined through the balance of signals from inhibitory and activating pathways. The main classes of NK cell receptors include the predominantly inhibitory killer immunoglobulin-like receptors (KIR), C-type lectin-like receptors of the CD94/natural killer group 2 (NKG2) family comprising inhibitory (NKG2A) and activating (NKG2C/D) isoforms, as well as the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46 that deliver activating signals.[2,11-15] NK cell inhibitory receptors such as NKG2A/CD94 and killer cell Ig-like receptors (KIR) recognize self or normal cells through target cells' expression of class I major histocompatibility complex (MHC) molecules to prevent cytolysis. On the other hand, activating receptors such as NKp46, NKp30, NKp44, and NKG2D transduces activating signals upon binding to ligands on the target cells whose class I MHC

molecules are downregulated. NK cells directly lyse target cells through the secretion of cytotoxic granules, granzyme, and perforin.[2,16] In addition, NK cells produce proinflammatory cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α.[9,17] These cytokines exert a regulatory function on the adaptive immune system such as T cells, dendritic cells (DC), and macrophages.[9,18] These immune-regulatory functions of NK cells played a critical role in the crosstalk between innate and adaptive immunity.

Recent studies have implicated NK cells as important players in host defense in all stages of HCV infection and suggest that NK cells may even protect from HCV acquisition.[2] The role of NK cells in the early phase of HCV infection displayed increased multifunctional responses with enhanced NCR (NKp46, NKp44) and NKG2A expression, cytotoxicity, and IFN-γ production.[19] Another study demonstrated clear race and gender related differences in expression of NKp46, which correlates with differential HCV natural history, supporting the biological relevance of NKp46 in innate immune protection.[20] NKp46 is also considered the major human NCR involved in NK cell-mediated killing.[21] These studies supported that NK cell activity contributes to anti-HCV defense in the earliest stages of infection.

It has been thought that NK cells are activated early in acute HCV infection, although their precise role is unclear.[22-24] NK cell IFN-γ production and cytotoxicity or degranulation were higher in patients with acute HCV

infection than in healthy individual controls.[22,23] In another study, circulating NK cells were significantly elevated in the acute phase of HCV infection compared to HCV-negative controls. Furthermore, NK cells from acutely infected patients showed increased degranulation in response to K-562 cells.[24] Phenotypic alterations of NK cells in acute HCV infection have been reported, but are difficult to interpret.[2] A previous study showed increased expression of NKG2D on NK cells, irrespective of the outcome, as compared with healthy controls which is consistent with activation.[22] Another study also showed that NK cells from acutely infected patients demonstrated lower frequencies of NKp46 and NKp30 expressing NK cells, and these lower levels correlated with HCV clearance.[24] This finding is controversial, as high levels of NKp46[19] and NKp30[25] expression have recently been associated with protection against HCV infection in exposed, uninfected individuals, and NKp46 expression correlates with anti-HCV activity *in vitro*.[2,20,26]

In chronic HCV infection, NK cells are activated, but may display alterations in phenotypes and functions.[27] Previous studies showed that NK cells from chronically HCV infected patients express higher levels of several activating receptors, such as NKp46 and NKp30.[8,28] Chronic exposure of NK cells to endogenous IFN-α can result in increased STAT expression, and preferentially STAT1 over STAT4 phosphorylation.[29] Several studies have shown that NK cells, obtained from chronically infected patients, are impaired in anti-viral

effector functions.[30,31] NK cells also seem to be impaired especially in their ability to secrete IFN-γ.[3]

It has been suggested that HCV alters innate immune responses at multiple levels. HCV-infected cells evade NK-mediated lysis in the early phase of infection. HCV activates regulatory T (Treg) cells that secrete transforming growth factor (TGF)-β and interleukin (IL)-10. High levels of Treg cells are also associated with impaired NK cell functions.[32] A previous study reported that cell-to-cell contact with HCV-infected cells reduces the functional capacity of NK cells. Moreover, the inhibition of NK cell functions is associated with downregulation of activating NK receptors,[33] suggesting that a viral protein(s) may affect infected cells, which negatively signal NK cell functions. However, HCV protein(s) that may contribute to the evasion of HCV-infected cells from NK cytotoxicity through impairment of NK cell functions is still elusive.

HCV circulates in the blood and replicates on the surface of hepatocytes. HCV is internalized via clathrin-mediated endocytosis.[1] This process requires the tetraspanin CD81,[34] the scavenger receptor class B member I (SRB1),[35] and the tight junction proteins claudin 1 (CLDN1)[36] and occludin (OCLN).[37,38] HCV translation and replication start in the cytosol after endocytosis and pH-dependent release from early endosomes.[1,5]

HCV translation is initiated through an internal ribosomal entry site (IRES) in the 5' untranslated region (UTR) and generates a single polyprotein of approximately 3,000 amino acids.[5] The translation product of the HCV genome is a form of polyprotein that is cleaved by viral enzymes and host proteases to yield structural (S) proteins comprising Core, E1, E2, and non-structural (NS) proteins including NS2, NS3, NS4A, NS4B, NS5A, and NS5B.[2,5] HCV-NS3 displays two types of roles; first, it has a serine protease function in the N-terminal domain that cleaves the polyprotein into individual non-structural proteins. Second, it acts as an RNA dependent helicase and displays ATPase activity with its C-terminal domain.[39] As a cofactor, HCV-NS4A forms a complex with HCV-NS3 and is essential for the enhancement of proteolytic activity of the HCV-NS3 serine protease.[5,40,41]

HCV viral proteins also have evasion mechanisms for immune responses. For examples, in previous studies, HCV-Core protein impaired NK cell cytotoxicity after upregulating the MHC class I molecule on liver cells via p53 and TAP1.[42] HCV-E2 protein, an envelope protein of HCV, may cross-link the CD81 on NK cells and, decrease the release of IFN-γ and cytotoxic granules.[32,43] Furthermore, HCV-NS3/4A cleaves adaptor molecules IPS-1 and TRIF[44] and HCV-NS5A protein downregulates expression of NKG2D on NK cells via TLR4, thus impairing the NK cell functions.[45]

The current standard of care for HCV patients is pegylated IFN (PEG-IFN) and ribavirin (RBV), which leads to sustained virologic response (SVR) in genotype 1 of HCV and genotype 2 of HCV patients.[41] The failure of many patients to achieve SVR, as well as the significant side-effect profile of PEG-IFN and RBV, highlight the need for new drugs with better efficacy and tolerability. Therefore, HCV-NS3 protease and HCV-NS5B polymerase are considered to be prime targets. Inhibitors of each enzyme, such as simeprevir for HCV-NS3 and sofosbuvir for HCV-NS5B, have shown promising anti-viral activity in clinical trials.[40,41]

In this study, HCV protein(s) that modulates NK cell functions through cell-to-cell interaction of NK cells with HCV-infected Huh-7.5 cells was investigated. It was found that co-cultivation of NK cells with HCV-infected Huh-7.5 cells significantly reduced NK cell degranulation and IFN-γ secretion. Also, the effects of HCV non-structural (NS) protein on NK cell functions were assessed using replicon cells, containing HCV-NS proteins, and the HCV-NS3 overexpression system. Cell-to-cell contact with HCV-NS replicon cells or HCV-NS3 expressing construct-transfected Huh-7.5 cells reduced NK cell functions as much as HCV-infected Huh-7.5 cells. These reductions were restored by treatment of HCV-infected Huh-7.5 cells with an HCV-NS3 serine protease inhibitor, BILN-2061. Additionally, this restoration was correlated with increased expression of NK cell activation receptors, NKp46 and NKp30.

These findings suggest that HCV serine protease, HCV-NS3, might play an important role in the impairment of NK cell functions in the early phase of infection.

## II. MATERIALS AND METHODS

### 1. Cell lines

Human hepatoma Huh-7.5 cells (provided by C. Rice, The Rockefeller University, New York, NY, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (complete DMEM; all from HyClone, South Logan, UT, USA). Human myelogenous leukemia K-562 cells (ATCC Number: CCL-243) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin, and 2.05 mM $_{L}$-glutamine (complete RPMI 1640; all from HyClone). HCV-NS replicon cells (provided by S. K. Jang, Pohang University of Science and Technology, Pohang, South Korea) were maintained in complete DMEM containing 500 μg/ml G418 (Duchefa Biochemie, Haarlem, Nederland).[46] All cells were cultured at 37℃ in a 5% $CO_2$ incubator.

### 2. Generation of HCVcc

Cell-culture-derived infectious HCV (HCVcc) (genotype 2a, JFH-1 strain) was produced as described by previous studies.[33,47] The JFH-1 expression construct (provided by T. Wakita, National Institute of Infectious Diseases and

Toray Industries, Tokyo, Japan) was linearized, and full-length JFH-1 RNA was transcribed using a MEGAscript T7 *in vitro* transcription kit (Ambion, Austin, TX, USA). Huh-7.5 cells were transfected with the transcribed RNA using DMRIE-C reagent (Invitrogen, Carlsbad, CA, USA). The supernatant collected from HCV RNA-transfected Huh-7.5 cells was used to infect naïve Huh-7.5 cells to make HCV stocks. The supernatant of HCV-infected Huh-7.5 cells was filtered through 0.45-μm syringe filters (Millipore, Billerica, CA, USA) and concentrated using Centricon Plus-70 (Millipore) according to the manufacturer's instructions.

To determine the infectious HCV titer, $1 \times 10^4$ Huh-7.5 cells were seeded in the wells of 96-well flat-bottom culture plates (Nunc, Roskilde, Denmark). On the next day, the infectious supernatant was 10-fold serially diluted in complete DMEM and 100 μl each dilution in triplicate was added to Huh-7.5 cells. The infectious supernatant was removed 4 hr after incubation and replaced with complete DMEM. Three days after infection, Huh-7.5 cells were washed with Phosphate-buffered saline (PBS) (Hyclone) and fixed for 20 min with 50 μl 100% Methanol at -20℃. Fixed cells were washed with PBS and blocked with IF buffer, containing 1% bovine serum albumin (BSA) (Affymetrix, Cleveland, OH, USA) and 0.25 mM EDTA in PBS for 1 hr at room temperature (RT). The cells were stained with mouse monoclonal anti-HCV-Core antibody (Thermo Scientific, Grand Island, NY, USA) diluted in IF

buffer (1:300) for 1 hr at RT, washed with PBS, and incubated with secondary FITC-conjugated goat anti-mouse IgG antibody (Jackson Immuno Research, West Grove, PA, USA) diluted in IF buffer (1:50) for 1 hr at RT. Then, the cells were washed and determined focus forming units (FFU). Images were visualized on Carl Zeiss Axio Imager.M2 immunofluorescence microscope (Carl Zeiss, Jena, Germany).

**3. Peripheral blood mononuclear cell (PBMC) isolation and NK cell purification**

Peripheral blood mononuclear cell (PBMC) was freshly isolated from the whole blood of healthy donors by Ficoll-Paque (GE Healthcare Life Science, Piscataway, NJ, USA) density gradient centrifugation as described by a previous study.[32] Separated PBMC was frozen and stored in a liquid nitrogen tank until use. Donors gave written informed consent under protocols approved by the Institutional Review Boards (IRB) of Severance Hospital Yonsei University Health System. Frozen PBMC was thawed and suspended in complete RPMI 1640 in the presence of 50 U/ml Benzonase (Novagen, Madison, MI, USA), which was added to remove DNA released from dead cells and to prevent clumping of PBMC. Cells were centrifuged and the supernatant was removed. After washing with complete RPMI 1640, cells were rested without any cytokines for 18 hr at 37℃ in a $CO_2$ incubator.

NK cells were isolated from the rested PBMC by negative selection using a human NK cell isolation kit (Miltenyi Biotec, Bergisch, Gladbach, Germany) according to the manufacturer's instruction. The purity of isolated NK cells was measured by LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) after staining the cells with anti-CD3-allophycocyanin (APC)-H7 and anti-CD56-PerCP-Cy5.5 or anti-CD56-APC antibodies (all antibodies were diluted 1:100 in FACS buffer) (BD Bioscience). When the frequency of $CD3^-$ $CD56^+$ cells was above 90%, isolated NK cells were used for the following experiments.

**4. Degranulation of NK cells**

NK cell degranulation was assessed as described by a previous study.[33] Huh-7.5 cells were suspended in complete DMEM and seeded in 96-well flat-bottom culture plates (Nunc, Roskilde, Denmark) at $1 \times 10^4$ cells per well. On the next day, Huh-7.5 cells were infected with HCVcc at a multiplicity of infection (MOI) of 1. Three days after infection, when NK cell responses peaked in general virus infections *in vivo*,[33] the supernatant was removed from the culture wells, and NK cells in complete RPMI 1640 were added to the seeded Huh-7.5 cells at a 1:1 ratio for 18 hr.

To determine the effects of HCV-NS3, NK cells were also co-cultured with HCV-NS3-DNA (provided by Eui-Cheol Shin, Korea Advanced Institute of

Science and Technology, Daejeon, South Korea)-transfected or HCV-NS replicon cells.

To evaluate the effect of an HCV-NS3 serine protease inhibitor, NK cells were co-cultured with BILN-2061-treated HCV-infected Huh-7.5 cells. The direct cytotoxicity of NK cells was determined as described by a previous study.[33] After co-culture for 18 hr, NK cells were harvested and further co-cultured with K-562 target cells in the presence of 20 μl/ml anti-CD107a-FITC antibody (BD Biosciences) in 96-well round-bottom culture plates (Corning Inc., Corning, NY, USA) for 4 hr. After the co-culture with K-562 target cells, NK cells were stained with anti-CD56-PerCP-Cy5.5 or anti-CD56-APC antibodies. Cells were fixed with 1% formaldehyde and analyzed with the LSR II flow cytometer (BD Biosciences) and FlowJo_V10 software (Tree Star, Ashland, OR, USA).

**5. IFN-γ production of NK cells**

NK cells were co-cultured with uninfected, HCV-infected, HCV-NS3 expressing construct-transfected Huh-7.5 cells, BINL-2061 treated HCV-infected Huh-7.5 cells or HCV-NS replicon cells for 18 hr, and then were harvested on 96-well round-bottom culture plates (Corning Inc.). Then measured intracellular IFN-γ as described by a previous study,[33] NK cells were added to the same number of K-562 cells, and treated with 10 ng/ml

recombinant human interleukin (IL)-12, and 100 ng/ml IL-15 in the presence of brefeldin A (BD Biosciences) for 6 hr. After the stimulation, supernatant was collected from the cultures and immediately stored at -20℃ for later cytokine analysis. Cells were fixed and permeabilized with cytofix/cytoperm solution (BD Biosciences). Intracellular IFN-γ was detected by staining with the Alexa Fluor 488-conjugated anti-IFN-γ antibody (BD Biosciences) and analyzed with the LSR II flow cytometer and FlowJo_V10 software. Secreted IFN-γ in the collected supernatant was measured by a human IFN-γ enzyme-linked immunosorbent assay kit (Enzo Life Sciences, Farmingdale, NY, USA or ATGen, Seongnam, South Korea) according to the manufacturer's instructions.

**6. Receptor expression of NK cells**

To determine changes in the expression levels of NK cell receptors induced by cells described above, NK cells, isolated from PBMC of healthy donors were co-cultured with uninfected, HCV-infected, BILN-2061-treated HCV-infected Huh-7.5, HCV-NS3 expressing construct-transfected Huh-7.5 cells or HCV-NS replicon cells for 18 hr. Then the NK cells were harvested and stained with anti-NKp46-PE, anti-NKp30-PE, anti-NKG2D-APC (BD Biosciences), anti-2B4-APC and anti-NKp44-PE (BioLegend, San Diego, CA,

USA). Cells were fixed with 1% formaldehyde and analyzed with the LSR II flow cytometer and FlowJo_V10 software.

**7. Confocal microscopy**

For HCV-NS3 immunostaining, cells were seeded in four-well chamber slides (Nunc), washed with PBS, and fixed with 3.7% formaldehyde for 10 min at room temperature (RT). Cells were permeabilized with 0.1% Triton X-100 in PBS buffer for 5 min at RT, and then blocked with 1% BSA in PBS buffer for 20 min at RT. After washing with PBS, cells were incubated with mouse monoclonal anti-HCV-Core antibody diluted 1:300 in PBS or anti-HCV-NS3 antibody diluted 1:50 in PBS for 1 hr at RT. Slides were washed with PBS and mounted using VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were visualized on the Carl Zeiss LSM-700 confocal microscope.

**8. Western blot analysis**

Cells were lysed with RIPA buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Then, bicinchoninic acid assay was performed to determine the protein concentration. Cell lysates were separated by 10% glycine/sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to a 0.45 μm

nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked for 1 hr with 5% skim milk in PBST buffer, and then incubated at 4℃ overnight with anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA; 1:8,000), anti-HCV-NS3 (1:50), and anti-HCV-Core (1:1,000) in 3% BSA in PBST buffer. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was used as the secondary antibody (1:5,000 or 1:8,000), and bands were visualized by enhanced chemiluminescence (ECL) (Advansta, Menlo Park, CA, USA) according to the manufacturer's instructions.

**9. Real-time PCR analysis**

Total RNA was extracted with TRIzol reagent (Thermo Scientific), and cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR for HCV-Core mRNA transcription was performed using Kappa SYBR master system (Applied Biosystems, Grand Island, NY, USA). Primer sequences for real-time PCR were the following: forward primer R6-130-S17 (nucleotides 130-146), 5'-CGGGAGAGCCATA -GTGG-3'; the reverse primer R6-290-R19 (nucleotides 290-272), 5'-AGTACCACAAGGCCTTTCG-3'.[48]

**10. Statistical analysis**

Student's *t* tests and repeated-measures one-way analysis of variance (ANOVA) were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA), with *p*-values of $<0.05$ were considered being significant.

## III. Results

### 1. HCV-infected Huh-7.5 cells reduced the functional capacity of NK cells

NK cells were purified from PBMC of 14 healthy donors and the frequency of the isolated NK cells was determined (**Fig. 1**).

To investigate the modulatory effect of HCV-infected Huh-7.5 cells on NK cell functions, NK cells were co-cultured for 18 hr with HCV-infected Huh-7.5 cells or uninfected Huh-7.5 cells. Then, NK cell cytotoxicity against K-562 cells was assessed by staining the expression of CD107a, a marker of NK cell cytotoxic granules. IFN-γ productivity was measured by intracellular flow cytometry. Co-cultivation of NK cells with HCV-infected Huh-7.5 cells significantly reduced CD107a expression against K-562 cells and IFN-γ production compared with NK cells alone or NK cells co-cultured with uninfected Huh-7.5 cells (**Fig. 2**). These observations are in accordance with previous data, where direct cell-to-cell contact NK cells and HCV-infected Huh-7.5 cells reduced functions of NK cells.[33]

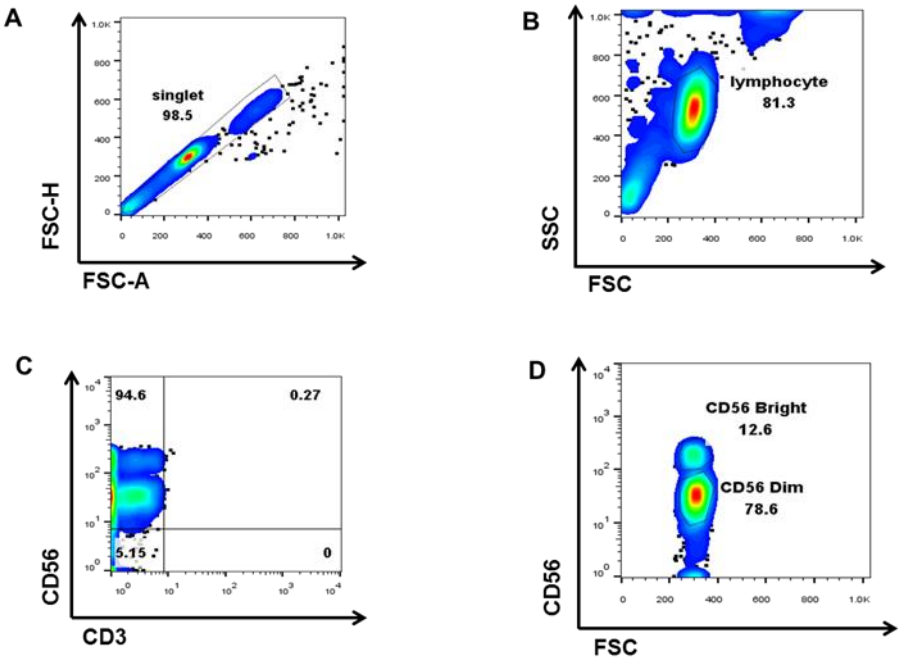


**Figure 1. Purification of NK cells from PBMC.** (A-C) Gating strategies for purified NK cells. NK cells with purity above 90% were used for subsequent experiments. (D) NK cells were also distinguished as two subsets different from their functional capacity.

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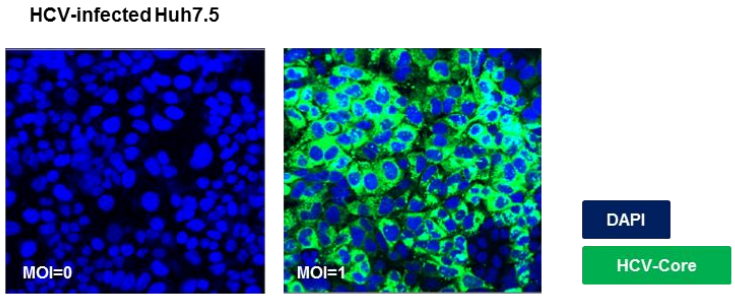


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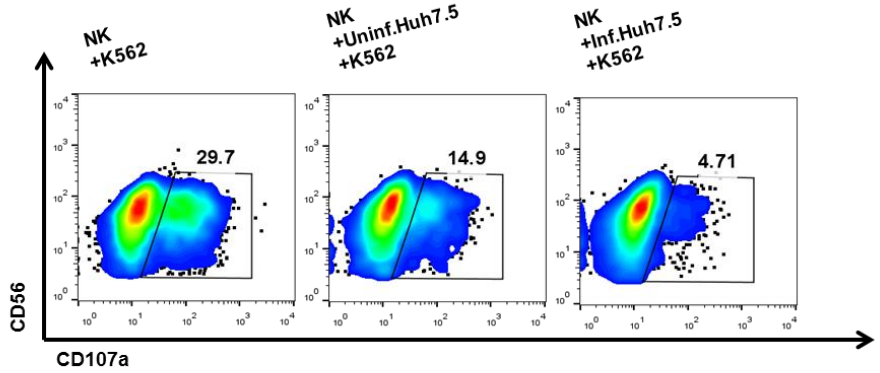


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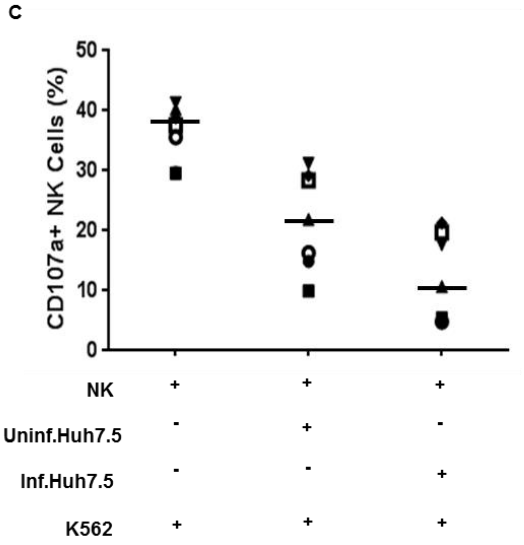


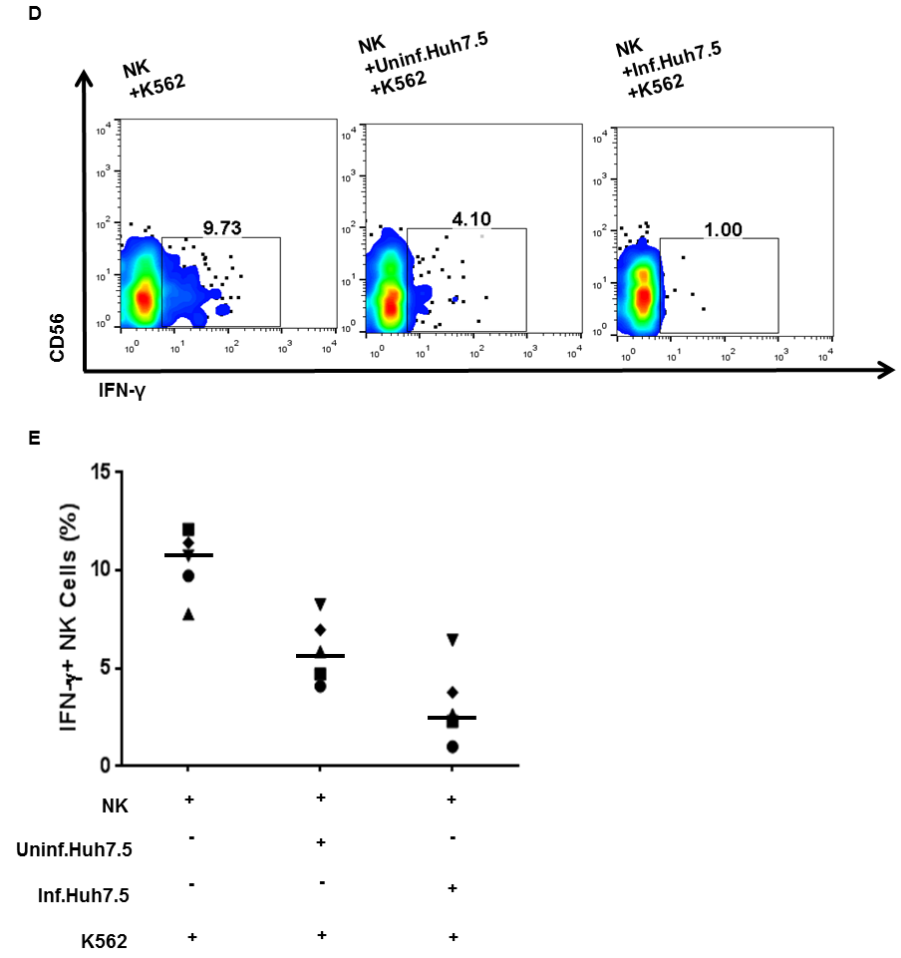
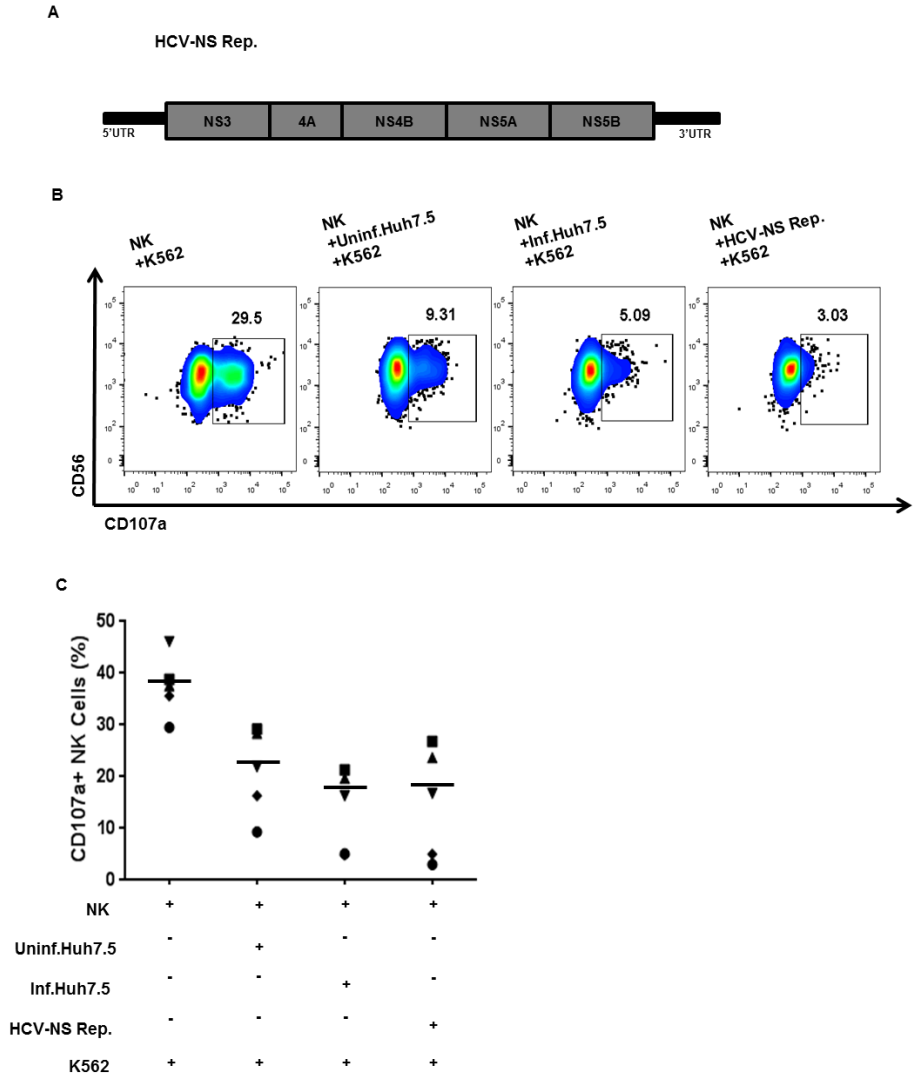



**Figure 2. Reduced NK cell cytotoxicity and IFN-γ production after direct contact with HCV-infected Huh-7.5 cells.** (A) Expression of HCV-Core protein in HCV-infected Huh-7.5 cells. Huh-7.5 cells were infected with HCV-JFH1 (MOI = 1), and three days later, cells were fixed and stained for HCV-Core protein (*green*) immunofluorescence with DAPI (*blue*) nuclear staining. (B, C) NK cell degranulation after co-cultivation with HCV-infected Huh-7.5 cells. Primary human NK cells were pre-incubated with uninfected or

HCV-infected Huh-7.5 cells for 18 hr, and harvested NK cells were co-cultured with K-562 cells at a 1:1 ratio for 4 hr. NK cell degranulation was measured by CD107a expression. (D, E) IFN-γ production by NK cells after co-cultivation with HCV-infected Huh-7.5 cells. Primary human NK cells were pre-incubated with uninfected or HCV-infected Huh-7.5 cells for 18 hr and harvested NK cells were then co-cultured with K-562 cells at a 1:1 ratio in the presence of 10 ng/ml recombinant human interleukin (IL)-12 and 100 ng/ml recombinant human IL-15 for 6 hr. IFN-γ producing cells were measured by intracellular staining of IFN-γ and flow cytometry analysis. (B) Representative pseudocolor plots obtained from seven independent individuals and (C) their linear progression plots. (D) Representative pseudocolor plots obtained from five independent individuals and (E) their linear progression plots. Bar represents the median value.

**2. HCV-NS replicon cells reduced functional capacity of NK cells**

Next, to investigate whether the non-structural proteins of HCV can reduce NK cell functions, NK cells were co-cultured with HCV-NS replicon cells (schematic diagrams shown in **Fig. 3A**). NK cytotoxicity and IFN-γ productivity were reduced by co-cultivation of NK cells with HCV-NS replicon cells, similar to those obtained from the co-cultivation of NK cells with HCV-infected Huh-7.5 cells (**Fig. 3B–3E**). Just as HCV-infected Huh-7.5 cells, NK cells co-cultured with HCV-NS replicon cells showed significantly reduced expression levels of CD107a against K-562 cells (**Fig. 3B and 3C**). Furthermore, it was also found that significantly reduced IFN-γ producing cells similar to those co-cultured with HCV-infected Huh-7.5 cells (**Fig. 3D and 3E**). The data demonstrate that HCV-infected cells regulate NK cell functions via cell-to-cell interaction and HCV-NS proteins may be involved in this modulation.



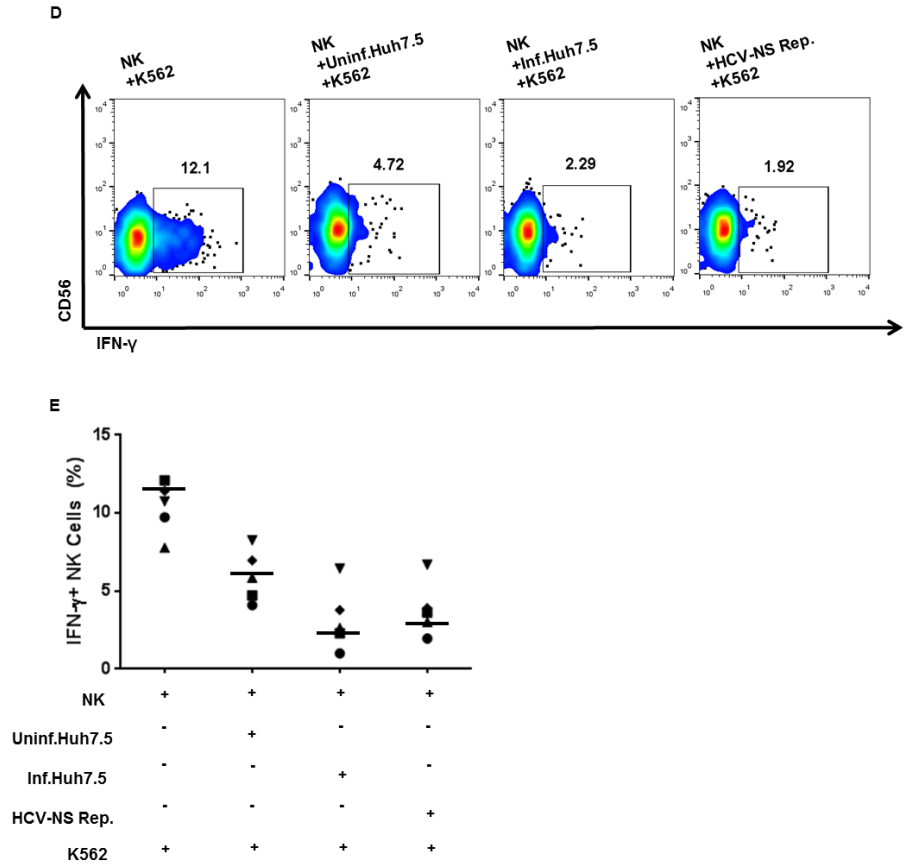



**Figure 3. HCV-NS protein expressing cells reduced NK cell cytotoxicity and IFN-γ production.** (A) Schematic diagrams of HCV-NS replicon constructs. (B, C) Degranulation of NK cells after co-cultivation with HCV-infected Huh-7.5 cells or HCV–NS replicon cells. NK cells were pre-incubated with HCV-infected Huh-7.5 cells or HCV-NS replicon cells for 18 hr, harvested, and co-cultured with K-562 cells at a 1:1 ratio for 4 hr. NK cell degranulation was measured by estimating CD107a expression. (D, E) IFN-γ production by NK cells after co-cultivation with HCV-infected Huh-7.5 cells

or HCV–NS replicon cells. NK cells were pre-incubated with HCV-infected Huh-7.5 cells or HCV-NS replicon cells for 18 hr and harvested then co-cultured with K-562 cells at a 1:1 ratio with treatment of 10 ng/ml recombinant human IL-12 and 100 ng/ml recombinant human IL-15 for 6 hr. IFN-γ production was assessed by intracellular staining of IFN-γ followed by flow cytometry. (B, D) Representative pseudocolor plots obtained from five independent individuals and (C, E) their linear progression plots. Bar presents the median value.

**3. HCV-NS3 reduced functional capacity of NK cells**

In order to identify the HCV-NS protein responsible for reduced NK cell cytotoxicity and IFN-γ production, HCV-NS3 was focused on because it acts as a serine protease and helicase, both of which are essential for viral replication.[5] The HCV-NS3 overexpression system were used to demonstrate the effect of HCV-NS3 on NK cell functions. In order to verify HCV-NS3 expression, western blot analysis and confocal microscopy were performed in HCV-NS replicon cells and HCV-NS3 expressing construct-transfected Huh-7.5 cells. The expression level of HCV-NS3 in these two cells was compatible to that in HCV-infected Huh-7.5 cells (**Fig. 4A and 4B**). The cytotoxicity and IFN-γ production capability of NK cells were also reduced by co-cultivation with HCV-NS3 expressing construct-transfected Huh-7.5 cells, as in the case of HCV-NS replicon cells (**Fig. 4C–4F**). Reduced IFN-γ production by HCV-NS replicon cells and HCV-NS3 expressing construct-transfected Huh-7.5 cells was confirmed using ELISA (**Fig. 4G**). These results indicate that HCV-NS3 expressed in the HCV-infected cells might play a role in the modulation of NK cell functions.

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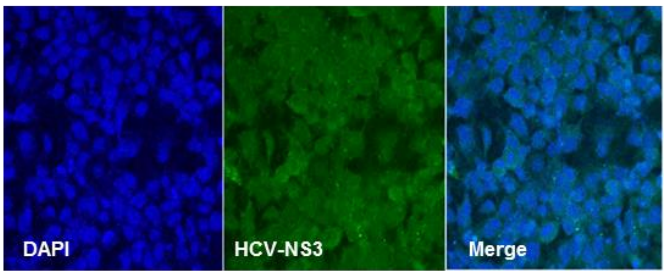


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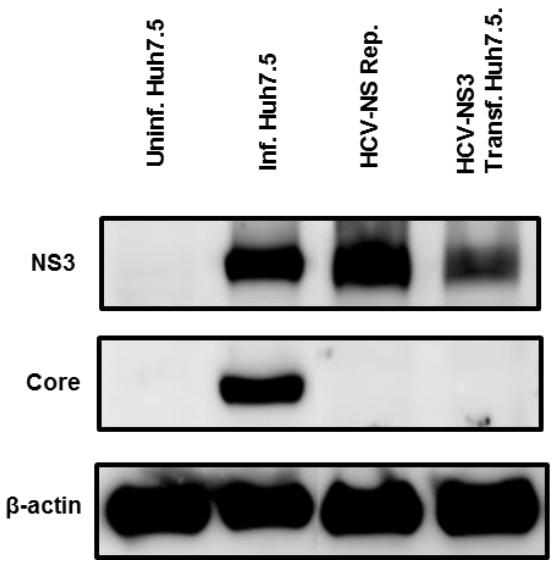

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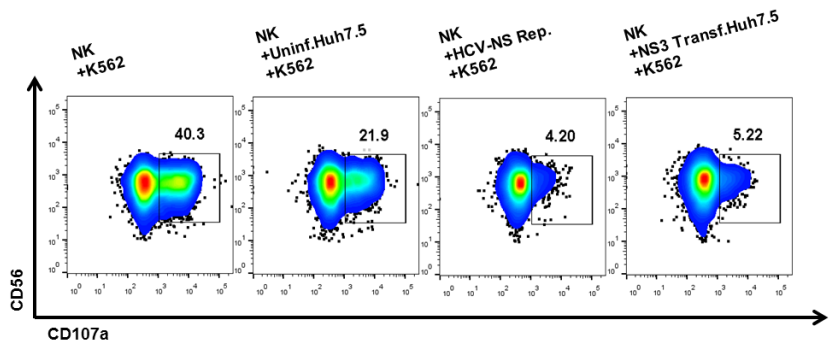


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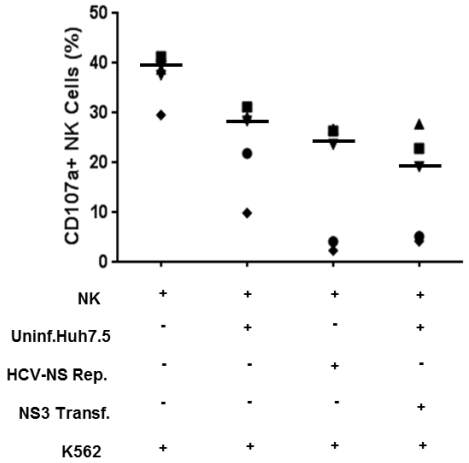
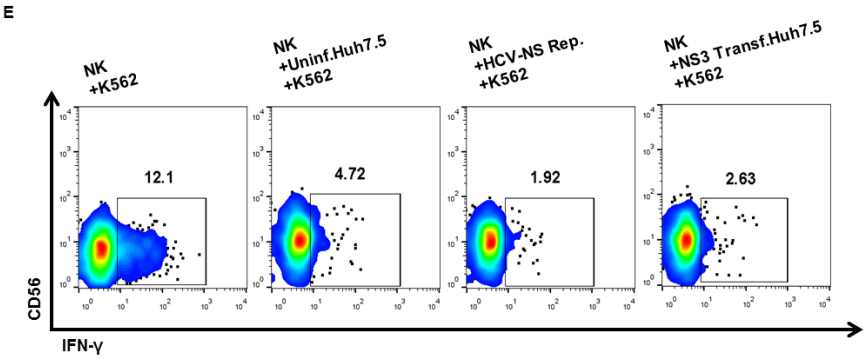
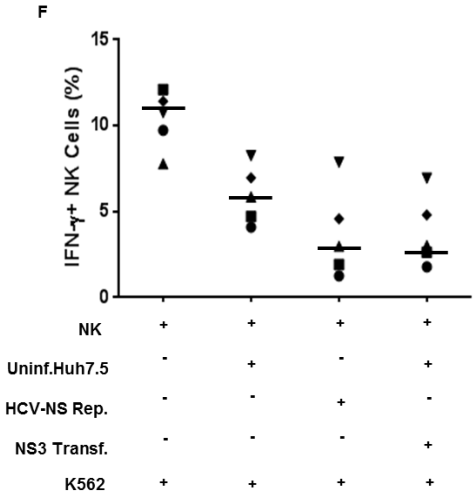




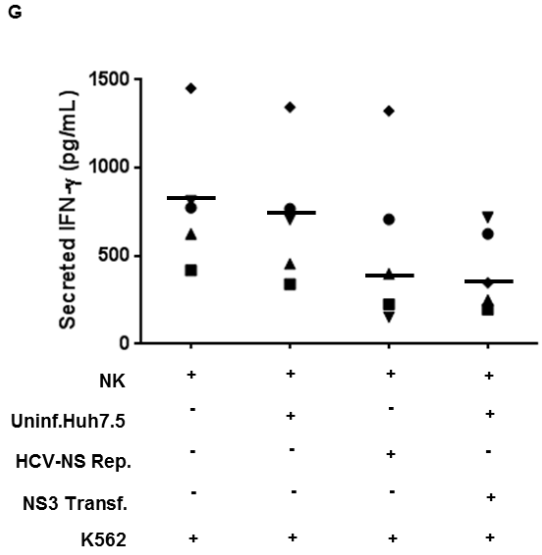
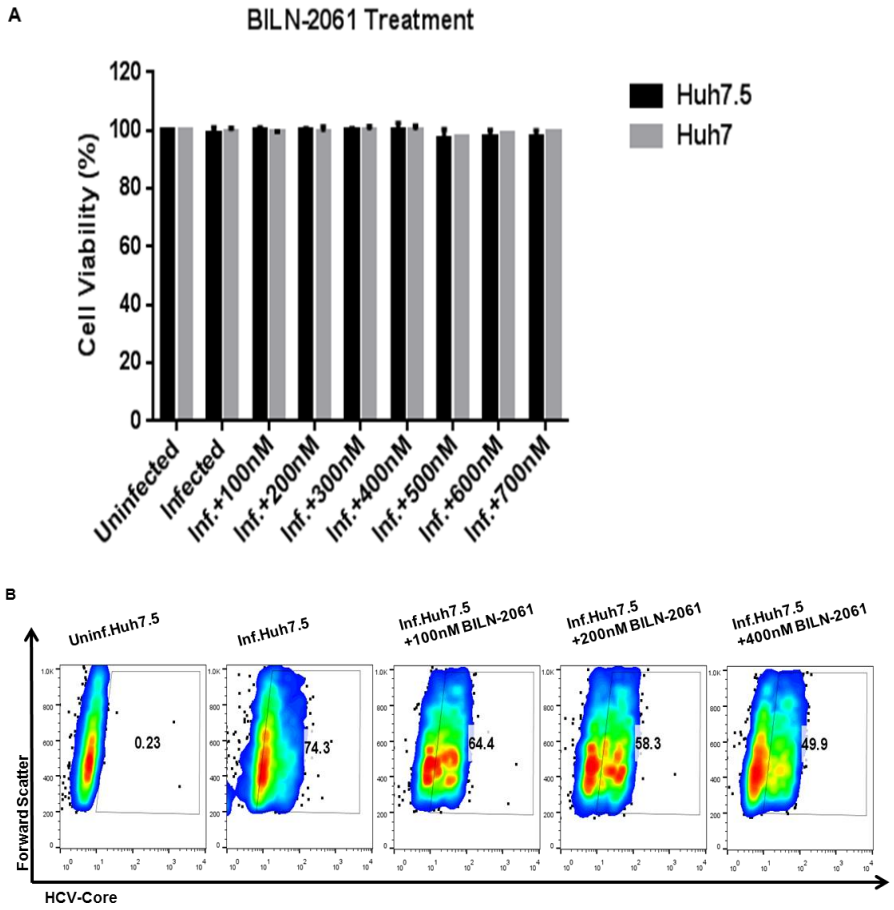



**Figure 4**. **HCV-NS replicon cells and HCV-NS3** expressing construct-**transfected Huh-7.5 cells attenuated NK cell functions.** (A) Expression levels of HCV-NS3 protein in HCV-NS replicon cells and HCV-NS3 expressing construct-transfected Huh-7.5 cells. Confocal microscopy was performed after 48 hr of HCV-NS3 expressing construct-transfection. Transfected Huh-7.5 cells were fixed and stained with HCV-NS3 (*green*) and 4',6-diamidino-2-phenylindole (DAPI; *blue*). Transient transfection was carried out using Lipofectamine 2000 (Invitrogen) (B) Western blotting was performed using anti-HCV-NS3 and anti-HCV-Core antibodies. As a loading control, human β-actin was used. (C, D) NK cell degranulation after co-cultivation with HCV-NS3 expressing construct-transfected Huh-7.5 cells. NK cells were pre-incubated with uninfected, HCV-NS replicon, or HCV-NS3 expressing construct-transfected Huh-7.5 cells for 18 hr, harvested, and co-

cultured with K-562 cells at a 1:1 ratio for 4 hr. NK cell degranulation was measured by estimating CD107a expression. (E, F) IFN-γ production by NK cells after co-cultivation with HCV-NS3 expressing construct-transfected Huh-7.5 cells. NK cells were pre-incubated with uninfected, HCV-NS replicon, or HCV-NS3 expressing construct-transfected Huh-7.5 cells for 18 hr, harvested, and co-cultured with K-562 cells at a 1:1 ratio with treatment of 10 ng/ml recombinant human IL-12 and 100 ng/ml recombinant human IL-15 for 6 hr. IFN-γ production was assessed by intracellular staining of IFN-γ. (G) IFN-γ secretion by NK cells after co-cultivation with HCV-NS3 expressing construct-transfected Huh-7.5 cells. NK cells were pre-incubated with HCV-NS replicon or HCV-NS3 expressing construct-transfected Huh-7.5 cells for 18 hr, harvested, and co-cultured with K-562 cells at a 1:1 ratio with treatment of 10 ng/ml recombinant human IL-12 and 100 ng/ml recombinant human IL-15 for 18 hr. Secreted IFN-γ in the culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). (C, E) Representative pseudocolor plots obtained from five independent individuals and (D, F) their linear progression plots. (G) Data from five independent individuals. Bar presents the median value.

**4. Treatment of HCV-infected cells with HCV-NS3 inhibitor, BILN-2061 restored NK cell functions**

In order to verify the role of HCV-NS3 in reduced NK cell degranulation and IFN-γ production, HCV-infected Huh-7.5 cells were treated with an HCV-NS3 inhibitor, BILN-2061. BILN-2061 concentrations of up to 700 nM did not affect the cell viability of Huh-7.5 and Huh-7 cells (**Fig. 5A**). BILN-2061 treatment decreased the expression levels of HCV-Core protein and HCV-NS3 protein in a dose-dependent manner (**Fig. 5B-5E**). First, the effect of BILN-2061 treatment on NK cells was determined. Treatment alone with 400 nM BILN-2061 did not have a direct effect on NK cell functions and activating receptors expression (**Fig. 6A-6C**). After 4 hr of HCV infection, the medium of Huh-7.5 cells was replaced with fresh medium containing 400 nM BILN-2061. Treatment of HCV-infected Huh-7.5 cells with BILN-2061 restored NK cell degranulation (from 4.86% of untreated group to 7.10% of treated group) (**Fig. 7A and 7B**). This restoration of function by BILN-2061 treatment was also observed in the case of IFN-γ production (**Fig. 7C and 7D**) and secretion (**Fig. 7E**). These observations corroborate the data above (**Fig. 4**), suggesting HCV-NS3 plays a role in the modulation of NK cell functions.



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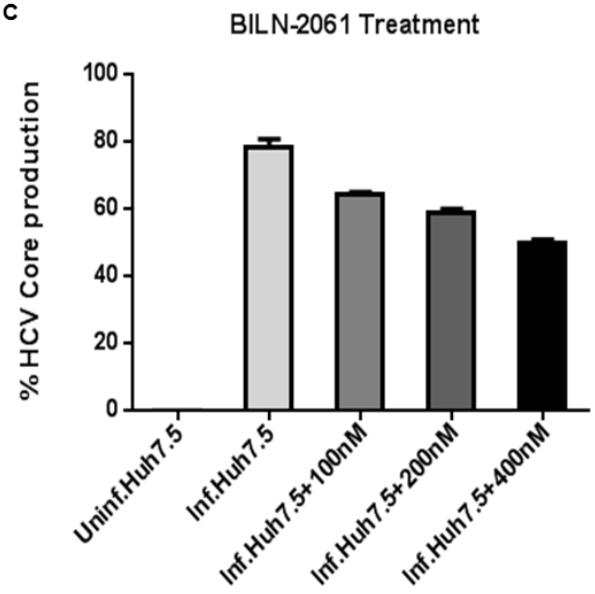


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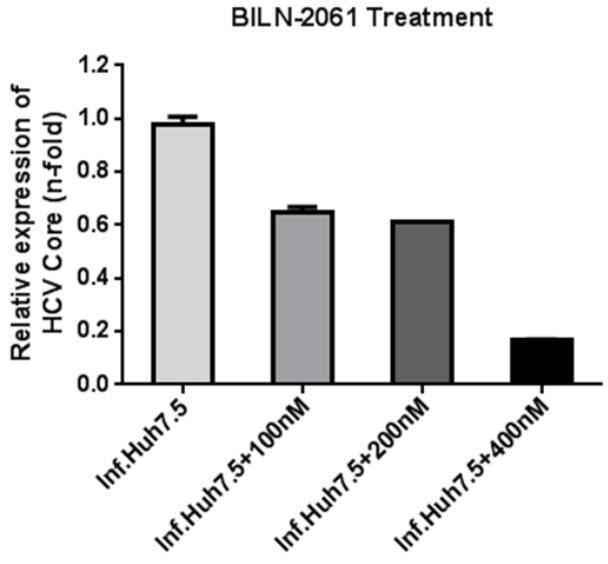


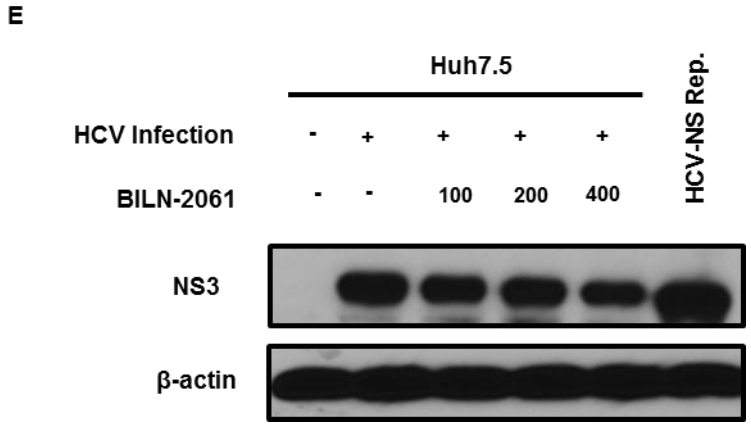



**Figure 5. Determination of the optimal concentration of BILN-2061, an HCV-NS3 inhibitor.** (A) Effect of BILN-2061 on cell viability. Huh-7.5 and Huh-7 cells were seeded in a 96-well flat bottom culture plate and infected with HCV at an MOI of 1 in Huh-7.5 cells and at an MOI of 10 in Huh-7 cells. After 4 hr, the supernatant was removed, the medium was replaced with complete DMEM, and the cells were treated with BILN-2061 (100-700 nM) for 48 hr. Cell viability assay was performed using CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan). BILN-2061 did not affect cell viability up to 700nM. (B) Effect of BILN-2061 on HCV replication. HCV-infected Huh-7.5 cells were treated with BILN-2061 (100-400 nM) for 48 hr. HCV replication was determined by estimating HCV-Core expression levels by using flow cytometry (stained with anti-HCV-Core antibody). HCV replication in HCV-infected Huh-7.5 cells was reduced by BILN-2061 treatment in a dose-dependent manner. Representative pseudocolor plots of

the results from three independent experiments. (C) Results from independent experiments of (B) repeated three times. (D) Effect of BILN-2061 on HCV-Core transcription. HCV-infected Huh-7.5 cells were treated with BILN-2061 from 100 nM to 400 nM for 48 hr. Real-time PCR was performed to detect HCV-Core mRNA. HCV-Core mRNA expression levels in BILN-2061 treated, HCV-infected Huh-7.5 cells decreased in a dose-dependent manner. (E) Effect of BILN-2061 on HCV-NS3 expression in HCV-infected Huh-7.5 cells. HCV-infected Huh-7.5 cells were treated with BILN-2061 (100-400 nM) for 48 hr. Western blot analysis was performed using anti-HCV-NS3 and anti-β-actin antibodies. BILN-2061-treated HCV-infected Huh-7.5 cells reduced HCV-NS3 expression in a dose-dependent manner. Thus, the optimal concentration was determined to be 400 nM BILN-2061 for further experiments.

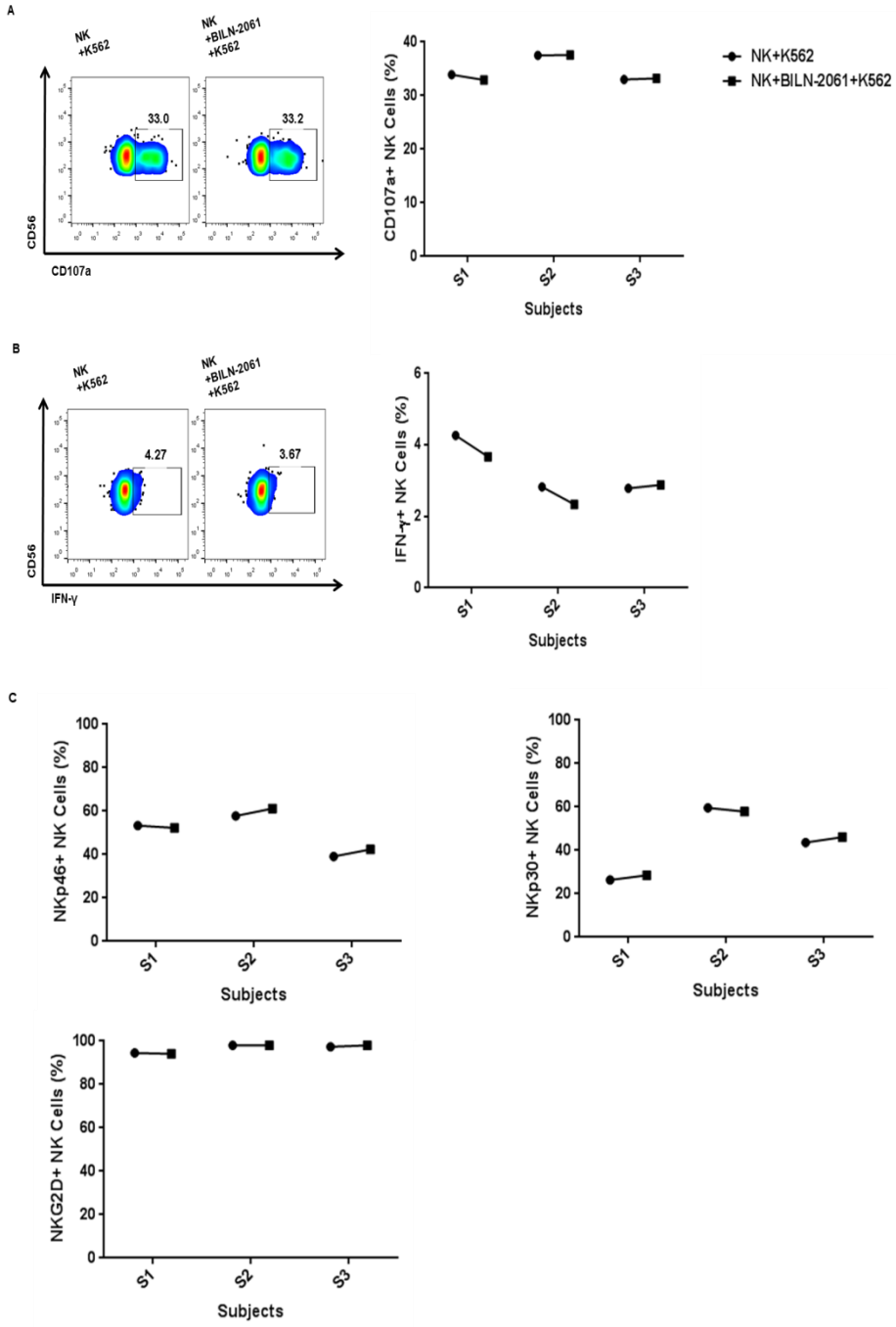
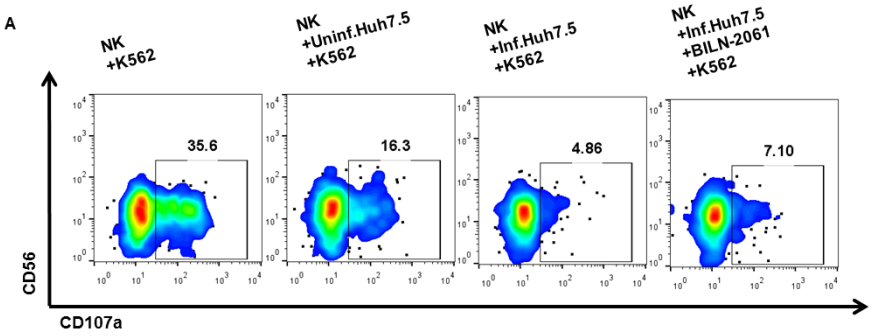
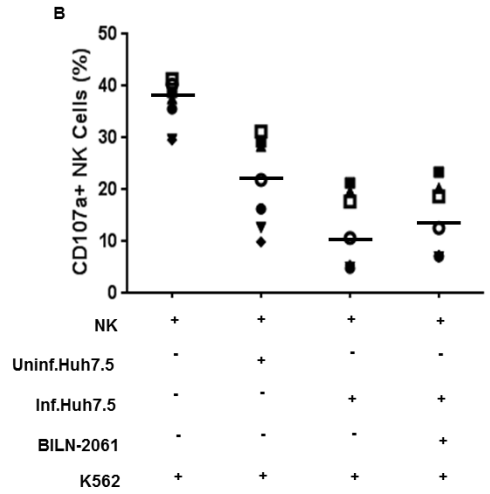
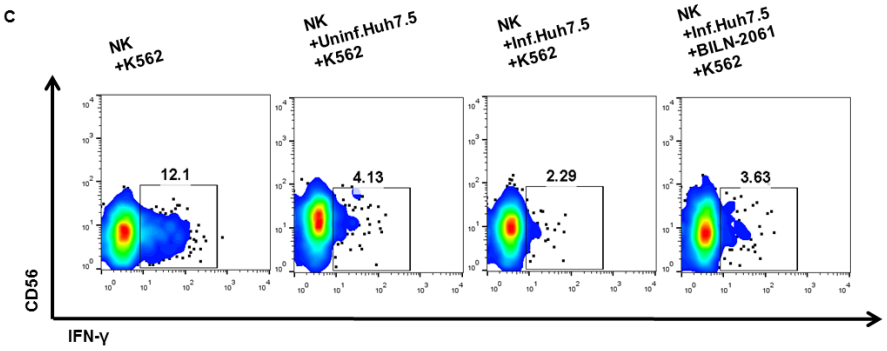
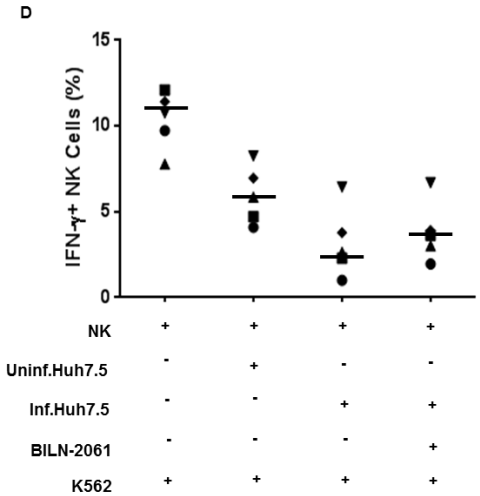


**Figure 6. Treatment with 400 nM of BILN-2061 did not affect NK cell functions.** (A) NK cell degranulation after treatment with BILN-2061. NK cells were seeded in a 96-well round bottom culture plate and treated with 400 nM of BILN-2061 for 18 hr, and then co-cultivated with K-562 cells at a 1:1 ratio for 4 hr. (B) IFN-γ production by NK cells treated with 400 nM BILN-2061 for 18 hr, then co-cultured with K-562 cells at a 1:1 ratio with treatment of 10 ng/ml IL-12 and 100 ng/ml IL-15 for 6 hr. IFN-γ production was assessed by intracellular staining of IFN-γ. (C) NK cells were treated with 400 nM BILN-2061 for 18 hr. The frequency of activating NK cell receptors expressing NK cells was measured by flow cytometry. Frequencies of $NKp46^{+}$, $NKp30^{+}$, and $NKG2D^{+}$ NK cells are shown. (A-C) Data from three independent individuals.





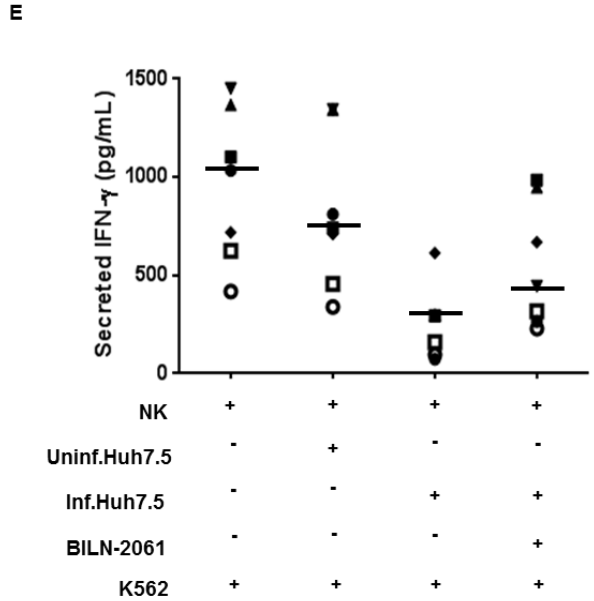



**Figure 7. NK cell functions were restored after interaction with BILN-2061-treated HCV-infected Huh-7.5 cells.** (A, B) NK cell degranulation after co-cultivation with BILN-2061-treated HCV-infected Huh-7.5 cells. NK cells were pre-incubated with uninfected, HCV-infected, or BILN-2061-treated HCV-infected Huh-7.5 cells for 18 hr, and harvested, and co-cultured with K-562 cells at a 1:1 ratio for 4 hr. NK cell degranulation was measured by estimating CD107a expression. (C, D) IFN-γ production by NK cells after co-cultivation with BILN-2061-treated HCV-infected Huh-7.5 cells. NK cells were pre-incubated with uninfected, HCV-infected, or BILN-2061-treated HCV-infected Huh-7.5 cells for 18 hr, harvested and co-cultured with K-562 cells at a 1:1 ratio with treatment of 10 ng/mL IL-12 and 100 ng/mL IL-15 for 6 hr. IFN-γ production was assessed by intracellular staining of IFN-γ. (E)

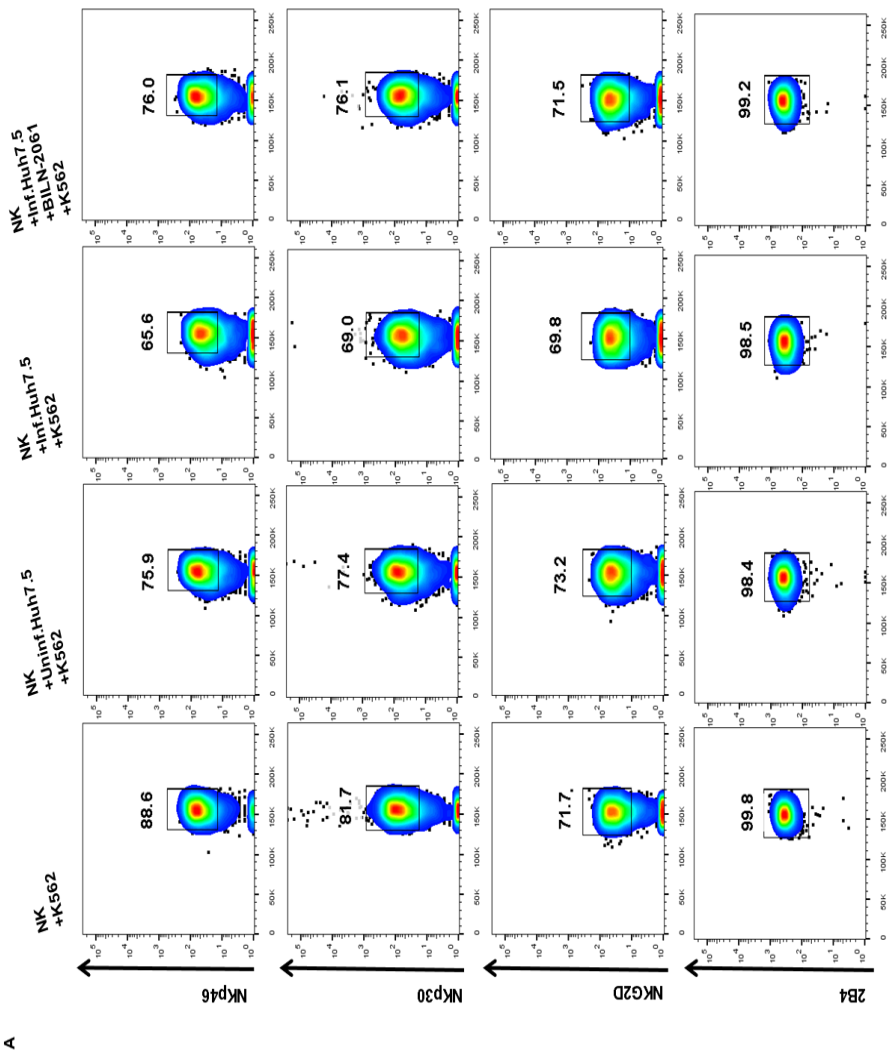
IFN-γ secretion by NK cells after co-cultivation with BILN-2061-treated HCV-infected Huh-7.5 cells. NK cells were pre-incubated with uninfected, HCV-infected, or BILN-2061-treated HCV-infected Huh-7.5 cells for 18 hr, harvested and co-cultured with K-562 cells at a 1:1 ratio with treatment of 10 ng/mL IL-12 and 100 ng/mL IL-15 for 18 hr. Secreted IFN-γ in the supernatant was measured by ELISA. (A, C) Representative pseudocolor plots obtained from seven and five independent individuals, respectively. (B, D) Their linear progression plots. (E) Data from seven independent individuals. Bar represents the median value.

**5. Restoration of NK cell functions upon BILN-2061 treatment was associated with increased NKp46 and NKp30 expression**

A previous study reported that activating receptors expressed on NK cell surface are downregulated after co-cultivation with HCV-infected cells, and that this downregulation is correlated with the functional impairment of NK cells.[33] To investigate the mechanism of this functional impairment by HCV-infected cells, the surface expression of various activating receptors was examined after co-culture of NK cells with HCV-infected Huh-7.5 cells. In accordance with the previous report,[33] co-culture of NK cells with HCV-infected cells decreased NK cell population expressing activating receptors such as NKp46 and NKp30 (**Fig. 8A**).

To investigate whether BILN-2061 affects the surface expression of NK cell activating receptors, surface expression of the activating receptors was evaluated after treatment of HCV-infected Huh-7.5 cells with BILN-2061. Treatment of these cells with BILN-2061 increased not only the NK cell population expressing activating receptors (**Fig. 8B**), but also the expression levels of the activating receptors (**Fig. 9**). Flow cytometric data showed that NK cell population expressing NKp46 and NKp30 decreased after being co-cultured with HCV-infected Huh-7.5 cells (**Fig. 8A and 8B**). In contrast, these effects were not observed for NKG2D and 2B4. A previous study indicated that NKp30 surface expression is downregulated (determined by assessing the mean fluorescence intensity [MFI]) following co-culture with HCV-infected Huh-7.5 cells.[49] It was observed that the NKp46 and NKp30 expression levels

of total NK cells were decreased after co-culture with HCV-infected Huh-7.5 cells. The surface expression levels were restored when NK cells were co-cultured with BILN-2061-treated HCV-infected Huh-7.5 cells. The fold change in MFI of NKp46 and NKp30 expression on NK cells increased upon co-culture with BILN-2061-treated HCV-infected Huh-7.5 cells ($p$=0.1039 for NKp46 and $p$=0.0301 for NKp30, Student's $t$ test; n=6, respectively; **Fig. 9**). Thus, HCV-NS3 reduces NK cell anti-viral functions through reduced expression of the activating receptors such as NKp46 and NKp30 on NK cells. Taken together these data suggest that HCV-NS3 might be associated with the impairment of NK cell anti-viral functions through downregulation of activating receptors on NK cells. And it is likely that HCV-NS3 could be the drug target and HCV-NS3 inhibitor could be useful for the restoration of NK cell functions that are impaired by HCV-infected cells.



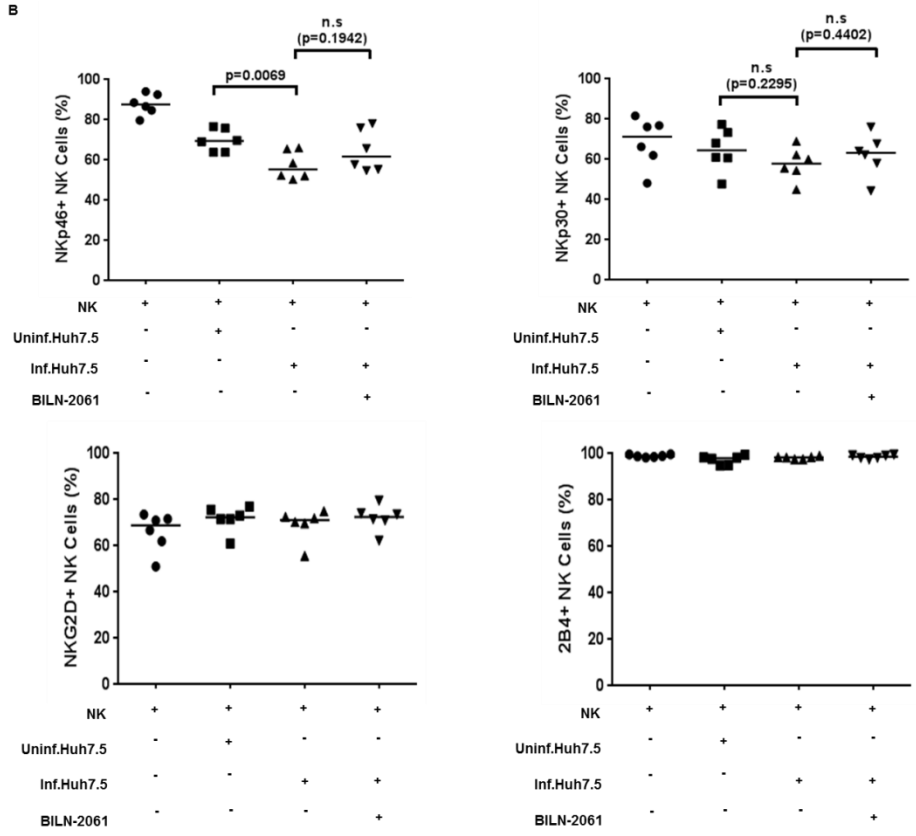
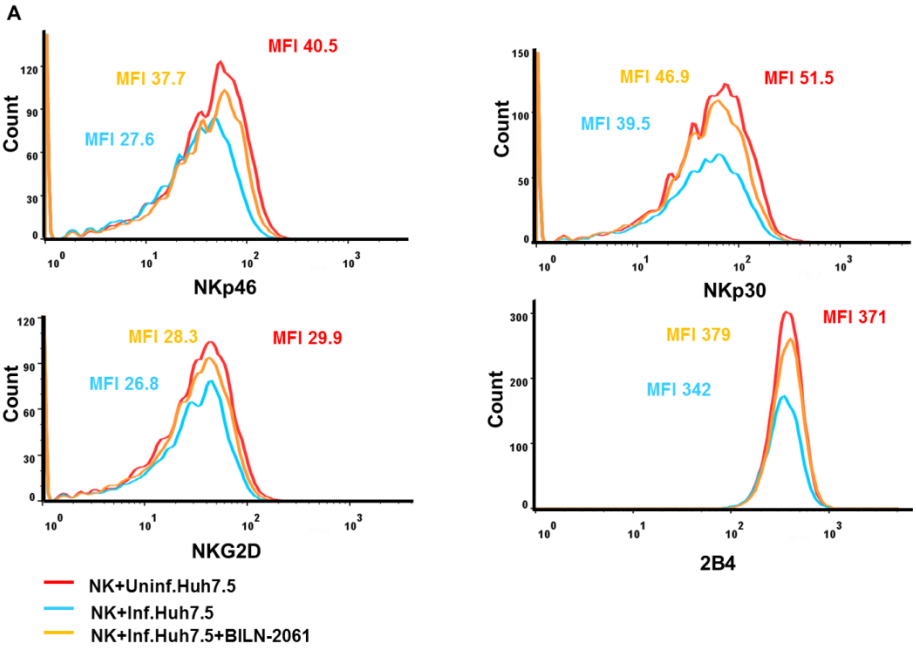



**Figure 8. Activating NK cell receptors expression after interaction with BILN-2061-treated HCV-infected Huh-7.5 cells.** NK cells were co-cultured with uninfected, HCV-infected, or BILN-2061-treated HCV-infected Huh-7.5 cells for 18 hr, and the frequency of activating NK cell receptors expressing NK cells was measured by flow cytometry. Frequencies of $NKp46^+$, $NKp30^+$, $NKG2D^+$, and $2B4^+$ NK cells are shown. (A) Representative pseudocolor plots of results from six independent individuals.



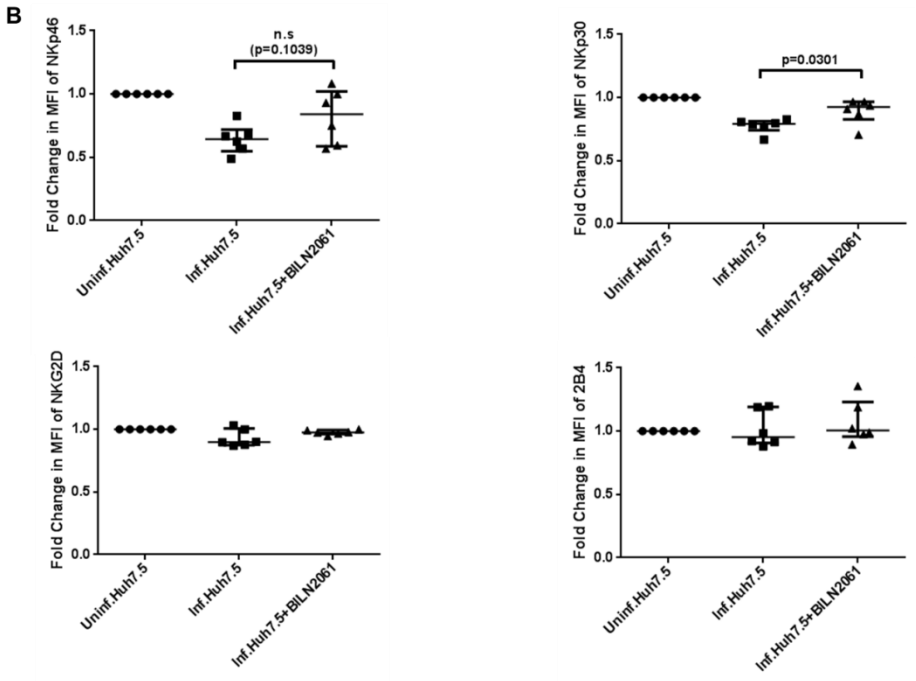



**Figure 9. Effect of BILN-2061-treated HCV-infected Huh-7.5 cells on the expression levels of activating receptors on NK cells.** NK cells were co-cultured with uninfected or HCV-infected or BILN-2061-treated HCV-infected Huh-7.5 cells for 18 hr. Fold change in mean fluorescence intensity (MFI) of NKp46, NKp30, NKG2D, and 2B4 on NK cells is shown as relative values to uninfected Huh-7.5 cells. Error bars indicate the median with interquartile range. (A) Representative histogram plots of results from six independent individuals.

## IV. DISCUSSION

Approximately 80% of untreated HCV-infected patients develop chronic hepatitis, making HCV infection a considerable public health burden.[25] This chronicity has been attributed to insufficient development of HCV-specific cytotoxic T lymphocytes owing to impairment of the innate immune response against the early phase of HCV infection.[5,50,51]

The evading mechanisms adopted by HCV to protect the virus from innate immune responses, notably NK cells, in the early phase of infection have not been clarified. The importance of NK cells in anti-viral immune responses has promoted studies on the interactions between NK cells and HCV, and several studies have suggested that NK cells play a role in the clearance of HCV. Genetic studies have demonstrated that genes encoding the inhibitory NK cell receptor KIR2DL3 and its human leukocyte antigen C group 1 (HLA-C1) ligand directly influence resolution of HCV infection.[52,53] On the contrary, some studies have reported that NK cell dysfunction is associated with chronic HCV infection. Increased NKG2A expression is a consistent finding in chronic HCV infection, suggesting the inhibition of NK cell functions.[54-56] Moreover, conflicting results have been reported for NCR expression levels in chronic HCV infection.[27,30,56,57] A previous study reported that the impairment of NK cell functions as a consequence of cell-to-cell interaction among NK

cells and HCV-infected Huh-7 and/or Huh-7.5 cells contributes to the chronicity of HCV infection.[33] This study, using HCV-NS replicon cells and an HCV-NS3 overexpression system, demonstrated that viral NS proteins could inhibit NK cell functions (**Fig. 3 and Fig. 4**). The changes in the surface expression of activating receptors on NK cells were also investigated upon cell-to-cell interaction with HCV-infected Huh-7.5 cells (**Fig. 8 and Fig 9**).

In this study, used was human hepatoma cell line Huh-7.5 cells, which is one of the well-known cell lines for highly permissive to infectious HCV. There are many human hepatoma cell lines available, such as Huh-7, Huh-7.5, Hep3B, HepG2, and PLC/PRF/5. However, only Huh-7 and Huh-7.5 cells are highly permissive for HCV infection *in vitro*. Therefore, many researchers who use the HCVcc system *in vitro* generally use Huh-7 and/or Huh-7.5 cell lines.[25,33,49,58,59] A recent study suggested Hep3B and PLC cells may also be permissive for HCV infection, but these cells show significantly less levels of infectious susceptibility than Huh-7 and Huh-7.5 cells.[60]

In this study, it was found that $CD107a^+$ and IFN-γ producing NK cells were decreased when they co-cultured with uninfected Huh-7.5 cells. Considering tumor cells mainly downregulate NK cell activating receptors or upregulate NK cell inhibitory receptors, these alterations may have induced the impairment of NK cell activity.[61,62] In previous studies, it was also found that HCV-uninfected Huh-7 or Huh-7.5 cells inhibit NK cell functions.[33,49]

Likewise, it was also observed that NK cell functional capacity decreased upon interaction with uninfected Huh-7.5 cells.

In accordance with a previous report,[33] co-cultivation of NK cells with HCV-infected Huh-7.5 cells significantly reduced the expression of CD107a against K-562 cells, as well as IFN-γ production. Also, NK cell cytotoxicity and IFN-γ productivity was reduced upon co-cultivation of NK cells with HCV-NS replicon cells expressing HCV-NS proteins. These observations indicate that HCV-infected cells regulate NK cell functions by cell-to-cell interaction and HCV-NS proteins may be involved in this modulation.

To investigate which HCV-NS protein is responsible for the reduced NK cell cytotoxicity and IFN-γ production, HCV-NS3 was mainly focused, because HCV-NS3/4A has a major key role in the immune evasion of HCV. When HCV-NS3/4A is overexpressed, it cleaves the adaptor molecules IPS-1 and TRIF, of RIG-I and TLR3,[5,44,63] thereby blocking RIG-I and TLR-3 signaling and inhibiting IFN-α and IFN-β secretion for neighboring hepatocyte's induction of anti-viral state. These findings suggest that HCV-NS3/4A plays a crucial role in the evasion mechanism against host innate immune responses. Additionally, the HCV-NS3 serine protease also cleaves the polyprotein of the HCV to generate individual non-structural (NS) proteins. Hence, it is important to understand the role of HCV-NS3 protease in the evasion mechanism against innate immune responses, especially NK cells. NK cell

functional capacity (cytotoxicity and IFN-γ production) was greatly reduced upon co-cultivation with HCV-NS3 expressing construct-transfected Huh-7.5 cells, as well as with HCV-NS replicon cells, suggesting that HCV-NS3 expressed in the HCV-infected cells might play a role in the modulation of NK cell functions.

To verify the role of HCV-NS3 in reduced NK cell degranulation and IFN-γ production, HCV-infected Huh-7.5 cells were treated with an HCV-NS3 inhibitor, BILN-2061. Treatment of HCV-infected Huh-7.5 cells with BILN-2061 restored NK cell degranulation and IFN-γ production, corroborating the hypothesis that HCV-NS3 plays a crucial role in the evasion mechanism against NK cell mediated innate immune response. It was also observed that the $NKp46^{+}$ and $NKp30^{+}$ expressing NK cells were restored after interaction with BILN-2061 treated HCV-infected Huh-7.5 cells. Thus, HCV-NS3 reduces the anti-viral functions NK cells by downregulating the expression of activating receptors, such as NKp46 and NKp30, on NK cells. BILN-2061 is a non-covalent competitive and macro-cyclic β-stranded inhibitor against HCV genotypes 1 and 2.[40,41,64] HCV-NS3 serine protease contains a classical catalytic triad, and BILN-2061 specifically acts against this active site.[40,64] BILN-2061 treatment restored functional impairment in NK cells co-cultivated with HCV-infected cells. Therefore, HCV-NS3 seems to be a

promising drug target, and HCV-NS3 inhibitors could be used to recover NK cell functions during HCV infection.

To confirm these findings, further investigation using HCV-NS3 mutants need to be conducted. NK cell dysfunction due to direct interaction between HCV-infected cells and NK cells may be explained by several mechanisms. First, HCV-infected cells also express HLA-E, which is a ligand for NKG2A/CD94 inhibitory receptors on NK cells.[49,65] However, other previous studies found that HLA-E expression on HCV-infected cells did not increased.[33,49] Furthermore, previous studies have demonstrated that a direct antagonistic interaction between human CMV protein pp65 and NKp30 activating receptor reduces NK cell cytotoxicity through dissociation of the linked CD3 ζ-chain adaptor protein from NKp30.[49,66] NKp46, NKp30 and CD16 are associated with the same ζ-chain adaptor molecule[67] and share the same signaling pathway.[68,69] Additionally, CD16 is associated with inhibition of antibody-dependent cell-mediated cytotoxicity (ADCC).[49,69] In a previous study, *ex vivo* NK cells from HIV-infected individuals showed reduced CD16 expression, leading to impaired NK cell functions through reduced NKp46 expression.[69] It is also possible that unknown antagonistic NKp46 and NKp30 ligands induced on HCV-infected cells inhibit NK cell functions. Unfortunately, many of the activating and inhibitory ligands for NKp46 and NKp30 remain uncharacterized. Therefore, the evasion mechanism of HCV

against NK cell responses in terms of the receptor-ligand interaction would be difficult clarified. This study prompts further investigation on the ligands of the NKp46 and NKp30 receptors in HCV infection.

Another study demonstrated that NS5A of HCV binds to TLR4 on monocytes, and induces IL-10 and TGF-β, while inhibiting IL-12 production, which downregulates NKG2D on NK cell surfaces and impairs NK cells.[45,70] These observations indicate that immunosuppressive cytokines might play a critical role in NK cell dysfunction in the early phase of HCV infection. HCV-NS3 protease may inhibit NK cell functions indirectly by modulating the expression of surface molecules on HCV-infected cells. On the other hand, a recent study showed that HCV-NS3 protease mimics TGF-β2 and binds to TGF-β type I receptor on Huh-7.5.1 cells enhancing liver fibrosis.[71] This might be another possible mechanism underlying HCV-NS3-mediated suppression of NK cells. Therefore, further studies are needed in order to reveal detailed mechanisms of HCV-NS3-mediated suppression of NK cell functions.

## V. CONCLUSION

This study revealed that NK cell functions are significantly impaired in the early phase of HCV infection *in vitro*, and this effect might be mediated via viral serine protease activity and downregulation of the activating receptors, NKp46 and NKp30.

Furthermore, it was demonstrated that HCV-infected cells modulated NK cell functions by cell-to-cell interaction, with HCV's non-structural protein (HCV-NS) modulating the functions as much as HCV-infected Huh-7.5 cells. It was also found that HCV-NS3 expressing construct-overexpressed in Huh-7.5 cells modulate NK cell functions in a manner similar to HCV-NS replicon cells. Downregulated NK cell surface expression of NKp46 and NKp30 might be associated with reduced NK cell functional capacity via HCV serine protease NS3. And BILN-2061-treated Huh-7.5 cells restored NK cell functions.

Therefore, in the early phase of HCV infection, viral serine protease, NS3 may be responsible for the impairment of NK cell functions. Further studies that investigate signaling processes developed by HCV-NS3 may elucidate the detailed evasive mechanisms against innate immune response, especially NK cells. HCV-NS3 might be a potential therapeutic target for HCV-infected patients, and these findings will be helpful for developing novel immune-based therapies related to NK cell activating receptors NKp46 and NKp30.

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**ABSTRACT (IN KOREAN)**

# C 형 간염바이러스의 NS3 세린 프로테아제를 통한 자연살해세포 활성의 억제

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양 창 모

C 형 간염바이러스(HCV) 감염은 내재면역 반응과 적응면역 반응의 손상으로 의해 만성 감염의 비율이 높은 것이 특징이다. C 형 간염 바이러스에 의한 자연살해세포(NK)의 기능 조절은 내재면역 반응의 손상을 유발시킨다. 그러나 감염 초기에 어떤 메커니즘을 통해 감염이 발생하는지, 이때 관여하는 C 형 간염바이러스의 단백질들의 역할은 아직 논쟁의 여지가 있다. 본 연구에서는 자연살해세포의 기능을 조절하는 C 형 간염바이러스 단백질을 연구하고 기전에 대하여 알아보았다. 사람의 간 암 세포주 중 하나인 Huh-7.5 세포에 배양을 통해 생산한 C 형 간염바이러스를 감염시키고, 사람에게서 자연살해세포를 분리하여 시험관에서 반응 시켰다. 감염된 간암 세포와 반응시킨 자연살해세포의 세포독성

능력과 인터페론 감마 생산 능력이 의미 있게 감소됨을 확인했고, C 형 간염바이러스의 단백질 중 자연살해세포의 활성화 상태에 영향을 주는 단백질을 규명하기 위해 C 형 간염바이러스의 레플리콘(replicon) 세포와 NS3 과발현(overexpression) 실험을 이용하여 측정했다. 리플리콘 세포와 NS3 를 형질주입 시킨 간암 세포는 C 형 간염바이러스로 감염시킨 자연살해세포와 동일하게 기능이 감소했다. 감소된 기능들은 C 형 간염바이러스로 감염시킨 Huh-7.5 세포에 NS3 의 세린 프로테아제(serine protease)기능 저해제인 BILN-2061 을 처리하면 자연살해세포 기능이 회복 되는 것을 확인했고, BILN-2061 을 감염된 간세포와 같이 배양 후, 자연살해세포를 처리하면 자연살해세포의 표적세포인 만성골수성백혈병 세포인 K-562 에 대한 자연살해세포의 탈과립반응이 의미 있게 증가하는 것을 확인했다. 인터페론 감마 생산도 BILN-2061 을 처리하지 않은 세포와 반응시킨 자연살해세포와 비교했을 때 의미 있게 증가함을 확인했다. 이러한 사실들과 함께 자연살해세포의 활성 수용체인 NKp46 과 NKp30 의 증가도 확인했다. 따라서 감염 초기에 C 형 간염바이러스의 세린 프로테아제 역할을 수행하는 바이러스 단백질인 NS3 가 자연살해세포의 NKp46 와 NKp30 수용체를 하향 조절 함으로써 자연살해세포의 기능을 저해시킨다고 할 수 있다. 이와 같은 결과를 종합하여 볼 때, 감염 초기에 C 형 간염바이러스의 NS3 가 자연살해세포의 기능을 손상시키는데 관여할 것이라 생각할 수 있다.

핵심되는 말: C 형 간염바이러스, 비구조단백질 NS3, 세린 프로테아제, 초기 감염, 자연살해세포, 활성 수용체, NKp46, NKp30

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