Natural Killer Cell Responses in Chronic Hepatitis C Virus Infection

by

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Dedication:
For my parents, whose patience and support have been limitless.

ABSTRACT
Characterization of Natural Killer Cell Responses
in Chronic Hepatitis C Virus Infection

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Background: Hepatitis C virus (HCV) has infected 170 million people worldwide and is a leading cause of chronic viral hepatitis, liver cirrhosis, and hepatocellular carcinoma. Liver injury and disease progression in hepatitis C virus infection is driven by the host immune response. Previous research has largely focused on the contribution of virus-specific T cells to this process. However, in chronic HCV infection, T cells are functionally exhausted and do not control viremia. Thus, it is likely that immune cells other than HCV-specific T cells contribute to disease pathogenesis.

Natural killer (NK) cells constitute the major innate immune cell population in the liver. They are capable of producing cytokines and utilize multiple mechanisms of cytotoxicity. The high prevalence of NK cells in the liver, along with their potential for viral specificity, suggested we study NK cell function in the setting of chronic hepatitis C infection. An improved understanding of the endogenous mechanisms that regulate liver inflammation and disease pathogenesis in chronic hepatitis C infection may result in a new focus for therapeutic options to decrease the rate of disease progression in individuals who are unable to clear infection with antiviral agents.

Objective: Here we sought to characterize whether natural killer cells from individuals chronically-infected with hepatitis C virus respond in chronic HCV infection and to compare the frequencies of HCV-specific NK cell responses with corresponding virus-specific T cell responses.

Methods: We tested the NK cell function of chronically HCV-infected patients not currently on antiviral treatment using a whole blood activation assay. In this assay, heparin-anticoagulated whole blood from 39 anti-HCV positive HCV patients and 10 healthy blood donors was stimulated for 8 hours with pools of overlapping 18 amino acid peptides (OLPs) comprising the HCV envelope (E1, E2) and nonstructural (NS3) sequences. Specificity controls consisted of a pool of overlapping hepatitis D virus peptides and samples without peptide addition. As a readout for NK and T cell function,

the frequency of IFNγ+ NK cells and T cells was assessed by multicolor flow cytometry. In addition, cytotoxicity was assessed by the frequency of NK or T cells expressing CD107a, a marker of cellular degranulation. Variance between responses in HCV-infected versus healthy donors and variance between NK versus T cell responses were analyzed by matched two-way ANOVA with multiple comparisons and Bonferroni post-test using Graphpad Prism software.

Results: This study demonstrates that NK cells from chronically HCV-infected individuals are activated in HCV infection in a whole blood peptide stimulation assay with both structural and nonstructural HCV proteins, including E1 and E2 envelope proteins and NS3 nonstructural protein. NK cells secrete cytokines assessed here by release of IFNγ, as well as NK cell degranulation, and degranulated, assessed by CD107a expression. As expected, there were minimal NK cell or T cell responses in healthy donor blood. NK cell cytokine responses were 4-6 times greater than HCV-specific T cell cytokine responses.

Conclusion: This study demonstrates that NK cell cytokine production and degranulation is greater than that of corresponding T cells. Further research is needed to characterize the mechanism of these NK cell responses to understand antiviral mechanisms and factors influencing disease progression.

TABLE OF CONTENTS

PRIOR PRESENTATIONS

PRESENTATIONS AND POSTERS:

1. KA Bowman, LE Holz, and B Rehermann. "Antigen-specific natural killer cell responses in chronic hepatitis C infection." Poster presentation: Immunology 2013, Annual Meeting of American Academy of Immunologists, Honolulu, HI. May, 2013.

2. KA Bowman. "Bridging the Gap Between Innate and Adaptive Immunity: Antigen-specific Natural Killer Cell Responses in Chronic Hepatitis C Infection." Oral Presentation. NIH Medical Research Scholars Scientific Symposium. May, 2013.

3. KA Bowman, LE Holz, and B Rehermann. "Antigen-specific natural killer cell responses in chronic hepatitis C infection." Poster presentation: UT Southwestern 52nd Annual Medical Student Research Forum. Feb 4, 2014.

CHAPTER 1

Introduction

HCV epidemiology and disease progression

Hepatitis C virus (HCV) has infected 170 million people (~2-3% of the global population) and is a leading cause of chronic viral hepatitis, liver cirrhosis, and hepatocellular carcinoma[1]. Of those acutely infected, 60-80% will develop persistent infection (> 6 months) and chronic disease. Among chronically-infected individuals, 20% will develop progressive liver fibrosis and cirrhosis over the subsequent two to three decades and 1-5% will develop hepatocellular carcinoma[2]. HCV-related liver disease is the most common indication for liver transplantation in the United States and Europe and the majority of patients demonstrate persistent viremia and recurrent liver injury after transplantation[3,4]. In addition to cirrhosis and hepatocellular carcinoma, chronic infection is associated with multiple extrahepatic manifestations, including an increased prevalence of membranoproliferative glomerulonephritis, cryoglobulinemia, Non-Hodgkin's Lymphoma (NHL) and dermatological manifestations including lichen planus, vitiligo, and porphyria cutanea tarda[5].

There are seven major genotypes of HCV, with significant differences of ~30% at the nucleotide level[6]. These are further subcategorized into greater than 50 subtypes that vary by 20-25% of nucleotide sequence[6]. Genotype 1 and genotype 2 are the most common in the United States and Europe[7]. Adding to this viral diversity, HCV exists within each infected individual as a heterogeneous population of differing viral isolates or quasispecies[8]. This viral heterogeneity is multifactorial and poses a considerable challenge for vaccine development. One major contributor to heterogeneity is the viral

RNA polymerase (NS5B), which is highly error-prone and lacks proofreading activity, with estimated mutation rates of ~10^{-4} to 10^{-5}/nucleotide/round of replication[9,10]. This high error rate leads to the generation of frequent viral escape mutants which allow for effective evasion of T cell responses and the establishment of chronic infection.

Transmission of HCV is primarily horizontal during adulthood through parenteral routes. Intravenous drug use (IVDU) accounts for the majority of new infections in the United States[11]. Since the introduction of screening of blood products for HCV antibodies in 1992, transfusion transmission has become rare in the United States, but remains a significant source of infection in countries without routine screening of blood products[7]. Perinatal transmission is uncommon, occurring in 2-8% of infants of HCV-infected women, and sexual transmission between monogamous partners is rare, although the prevalence of HCV infection is higher among individuals with multiple sexual partners[7,11].

HCV Treatment and Screening

HCV is the only hepatitis virus for which no effective vaccine has been developed. Until recently, a significant percentage of patients (40-50% genotype 1) did not achieve sustained virologic response (SVR) to the mainstay of therapy (pegylated-IFNα, ribavirin), although this response improved to ~70% with the addition of nucleoside analogues, including boceprevir and telaprevir[12,13]. The recent development of a new directly acting antiviral, sofosbuvir, which inhibits the viral RNA polymerase NS5B, has drastically improved outcomes among all genotypes. New goals of therapy will be achievement of SVR on interferon-free, all-oral regimens. Currently, multiple

phase II and III clinical studies with a single directly acting antiviral (DAA), sofosbuvir, plus ribavirin demonstrate improvement in SVR for genotypes 2 and 3 over peginterferon-based therapies[14,15]. For genotype 1 patients, several clinical trials using two DAAs plus ribavirin have shown improvements in SVR, including high rates SVR in treatment-naïve patients and improvements in SVR in prior non-responders[16]. However, cost and access to drug therapy remains an issue for many people in the US and abroad, underscoring the continuing need for development of a prophylactic vaccine.

Because chronic HCV infection is generally asymptomatic, an estimated 45-85% of chronically infected individuals are unaware that they are infected[17]. As a result, recognition and subsequent treatment of infection requires appropriate screening for risk factors, which should prompt HCV testing if found. These risk factors include: injection or intranasal illicit drug use, long-term hemodialysis, healthcare-associated exposures, incarceration, or recipients of blood/organs prior to 1992, HIV infection, or unexplained chronic liver disease and chronic hepatitis including alanine aminotransferase (ALT) elevations. However, based on the high rate of failure (50%) of risk-based screening to identify HCV infections, the Centers for Disease Control (CDC) and US Preventive Services Task Force (USPSTF) expanded HCV screening guidelines in 2012 with a recommendation for a one-time HCV antibody test for all persons born between 1945 and 1965, regardless of other HCV risk factors[17-19]. This 1945-1965 birth cohort accounts for nearly three quarters of HCV infections.

HCV structure and life cycle

HCV is an enveloped positive-sense single-stranded RNA virus of the

flaviviridae family which infects hepatocytes of humans and chimpanzees. Its genome, which does not integrate into the host cell DNA, is approximately 9.6kb in length and encodes a single long polyprotein within a single ORF[20]. This polyprotein is processed into 10 proteins including structural (core, E1, and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins by viral enzymes (NS2/3 and NS3/4A serine protease) and cellular enzymes (signal peptidase and signal peptide peptidase). The two envelope glycoproteins, E1 and E2, are displayed on the viral surface and appear to be responsible for receptor binding. The HCV nonstructural proteins (NS3, NS4A, NS4B, NS5A, and NS5B) are used for replication. Notably, the NS3/NS4A serine protease is the target of two directly-acting antivirals (DAAs), telaprevir and boceprevir. The NS5B functions as the viral RNA-dependent RNA polymerase and is the target for the most recent DAAs, NS5B polymerase inhibitors including sofosbuvir.

HCV viral entry into hepatocytes is mediated by binding of the virus to several surface-expressed proteins, including tetraspanin CD81, the scavenger receptor class B type I (SR-B1), two tight junction proteins – occluding (OCLN) and claudin (CLDN1), and the cholesterol absorption receptor Niemann-Pick C1-like 1 (NPC1L1), as well as several low-affinity attachment receptors[21-24]. Subsequent viral entry by clathrin-mediated endocytosis leads to HCV translation and replication in the cytosol as discussed above. Capsid proteins and genomic RNA forms a nucleocapsid and are released from cytoplasmic vesicles fusing with the plasma membrane through the secretory pathway[11].

Since the identification of HCV in 1989, HCV research has been complicated by the virus' species specificity for humans and chimpanzees and its cellular tropism for

hepatocytes. The initial lack of efficient cell culture systems and small animal models slowed research on the viral life cycle and necessitated development of alternative research models. Although functional cDNA clones of HCV were generated in 1997 which infected in chimpanzees, these clones did not replicate well in cell culture. The first breakthrough for cell culture of HCV came with the development of selectable subgenomic HCV replicons that were able to efficiently replicate in cell culture[25,26]. Unfortunately, adaptive mutations that allowed replicons to replicate well in transfected hepatoma cells in culture were not infectious, limiting a complete study of the viral life cycle. However, in 2005, the cloning of a genotype 2a HCV isolate from a Japanese patient with fulminant hepatitis (JFH1 strain) generated an isolate which both efficiently replicates in human hepatoma cell line (Huh) and produces infectious virions *in vitro* and *in vivo*[27-29]. Efforts to address the need for small animal models for *in vivo* studies have included the use of immunodeficient mice transplanted with human hepatocytes and, more recently, the development of genetically humanized mice permissive to HCV infection[30]. However, these models, while useful for study of the viral lifecycle, do not allow adequate analysis of immune cell responses[30].

Anatomy of the Liver and its Immune system

A brief review of the structural organization of the liver and its immune components is critical to an understanding of the immunopathology of chronic HCV and especially important to shed light on the limitations of immunologic assays using non-hepatic tissue or cell culture[31]. The liver provides an immunologically complex setting wherein antigen-rich blood from the splanchnic venous system circulates through a

sinusoidal network, thereby allowing extended contact with liver resident lymphocytes and antigen-presenting cells. The liver is composed of a diverse array of nonparenchymal cells in addition to hepatocytes including Kupffer cells, stellate cells, intrahepatic lymphocytes, sinusoidal endothelial cells (LSEC), and biliary cells. Intrahepatic lymphocytes include innate populations, including NK cells, CD1d+ NKT cells, and gamma-delta T cells, as well as adaptive populations, including B cells, CD4+ and CD8+ T cells[31]. The liver also contains an array of resident antigen presenting cells (APC) that capture antigen from blood circulating through the liver or released when infected hepatocytes die. These APCs include Kupffer cells (liver resident macrophages), liver sinusoidal endothelial cells (LSEC), and dendritic cells (DCs). These APCs appear to play a significant role in maintaining tolerance and their role in T cell priming in immune responses to infection is not well defined[31].

Immunology of Hepatitis C

Liver injury and disease progression in HCV infection is driven by the host immune response. Previous research has focused mainly on the role of virus-specific T cells in this process, which are critical for viral clearance. However, only 20-30% of acutely infected individuals will mount an effective T cell response and 70-85% of individuals with acute infection will develop chronic infection[10]. In chronic HCV infection, T cells are functionally exhausted, demonstrating markedly impaired proliferative ability, cytotoxicity, and cytokine production, and do not control viremia[32]. This T cell exhaustion is due to high and persistent levels of viral antigen, which lead to upregulation of programmed death-1 (PD-1) on HCV-specific T cells. This PD-1 binds

its ligand PD-L1, expressed on the surface of liver sinusoidal endothelial cells (LSECs), Kupffer cells, and stellate cells in the liver, and leads to the decreased functionality that characterizes the exhausted phenotype[33]. Additionally, neutralizing antibodies are not necessary to clear HCV and appear only after chronic infection has been established[34]. Neutralizing antibodies select escape variants, rendering them ineffective, and their titers decay over time[34]. In the setting of a downregulated T cell response and ineffective, declining neutralizing antibody titers, it is unclear what causes the intrahepatic inflammatory response that drives disease pathogenesis.

An introduction to natural killer cells

Natural Killer (NK) cells are effector cells of the innate immune system, which can recognize and kill virally infected cells or tumor cells and produce antiviral cytokines, including IFNγ and TNFα[35]. Unlike cells of the adaptive immune system, they do not require priming, lack a T cell receptor and do not produce immunoglobulins. Additionally, unlike T cells, they can recognize non-self or infected cells in the absence of antibodies and MHC. NK cells directly kill through release of constitutively-expressed, pre-formed intracellular granules containing perforin (a cytolytic protein) and granzyme (a serine protease), which lead to apoptosis in the target cell. NK cells require activation by type I interferons (IFNα and IFNβ), or pro-inflammatory cytokines, including interleukin-15 (IL-15), IL-12, and IL-18, or they may directly recognize and respond to virally infected cells. NK cells do not express CD3, but do express CD56 and CD16 (FcγIII receptor), thus their basic phenotype is CD3-CD56+ lymphocytes. NK cells may be divided into $CD56^{bright}$ cells, which produce IFNγ and contribute to CD4+ T

cell type 1 priming, and a fully mature $CD56^{dim}$ subset which is more highly cytotoxic and capable of antibody-dependent cellular cytotoxicity using its FcγIII receptor (CD16) expressed on its surface[35-37]. Additionally, a dysfunctional and rare CD56-CD16+ has also been described. In addition to an array of cytokine receptors, NK cells express both activating and inhibitory cell surface receptors, including killer cell immunoglobulin-like receptors (KIRs), lectin-like receptors (NKG2A-F), and natural cytotoxicity receptors (NKp30, NKp44, NKp46).

NK cells in chronic HCV infection

NK cells constitute the major innate immune cell population in the liver, composing 30% of resident lymphocytes in a healthy liver, compared to 5-20% of peripheral blood lymphocytes, and their frequency is further enriched in the liver in chronic HCV infection[38]. In the context of hepatitis, the intrahepatic NK cell population may constitute up to 90% of liver resident lymphocytes[27,30]. Both their high frequency in the liver at baseline and further enrichment in the setting of chronic HCV, as well as their multiple mechanisms of cytotoxicity against virally-infected hepatocytes, make NK cells potentially important effector cells in acute and chronic HCV infection. Further, immunogenetic studies have demonstrated that KIR/HLA haplotypes affect the outcomes of acute and chronic HCV infection, likely as a result of differential NK cell activation, with some KIR/HLA haplotypes associated with increased likelihood of viral clearance (KIR2DL3 and HLA-C group 1 allelic homozygosity), while others (KIR2DS3) have been associated with increased liver injury in chronic infection [39-41].

NK cell activation results in cytotoxicity as well as release of antiviral cytokines

such as IFNγ. NK cells may utilize multiple mechanisms of cytotoxicity, including antibody-dependent cellular cytotoxicity (ADCC), which depends on NK cell CD16 (FcγRIII) recognition of circulating antigen-specific immunoglobulins bound to virally-infected cells, with specificity contributed by the antibody.

In chronic HCV infection, intrahepatic NK cells are more activated than their counterparts in the peripheral blood[40]. In patients with chronic infection, the activating receptor NKG2C, NKp44, and TRAIL, which are all markers of activated NK cells, as well as CD122, a subunit of the IL-2 receptor, which is important in NK cell activation, were expressed on a higher percentage of NK cells than in healthy controls[40]. The upregulation of these activating receptors, and the downregulation of inhibitor receptors, demonstrates that NK cells are activated in chronic infection compared to healthy controls.

The activated NK cell phenotype demonstrated in chronic HCV does not result in equal increases in cytotoxicity and cytokine production. Intrahepatic NK cells are polarized towards cytotoxicity, indicated by increased degranulation and TRAIL production, without significantly increased IFNγ production[40,42]. This polarization towards cytotoxicity appears to be induced by chronic exposure to HCV-induced type I interferons in chronic infection [40]. The absence of IFNγ may contributes to viral persistence because IFNγ suppresses HCV replication, and IFNγ –mediated viral clearance is more effective and efficient than individual 1:1 killing of infected hepatocytes by cytotoxic mechanisms[43]. The relative importance of IFNγ has been further suggested by the observation that the increase in intrahepatic IFNγ-mRNA levels in the liver corresponds with clearance of acute HCV infection in chimpanzee models[44].

Objectives / Hypothesis

The high prevalence of NK cells in the liver, along with their potential for viral specificity and multiple mechanisms of activation and cytotoxicity, suggested we study NK cell function in the setting of chronic hepatitis C infection. Given the recent interest in antibody-dependent cellular cytotoxicity, we sought to understand whether NK cells respond in chronic HCV and if so, how these responses compared to corresponding T cell responses. An improved understanding of the endogenous mechanisms that regulate liver inflammation and disease pathogenesis in chronic hepatitis C infection may result in a new focus for therapeutic options to decrease the rate of disease progression in those individuals who are unable to clear infection with antiviral agents.

CHAPTER 2: MATERIALS AND METHODS

Study Cohort

Peripheral blood NK cells and T cells were studied in thirty-seven individuals with chronic hepatitis C infection and ten HCV-uninfected healthy blood donors (Table 1) at the National Institute of Diabetes, Digestive and Kidney Diseases. Inclusion criteria included patients with chronic infection (> 6 months) with genotype 1a or 1b HCV only, who were not currently or recently (>2 years) on therapy for HCV. Study subjects include both patients who failed to respond to previous therapy (non-responders) and treatment-naïve patients. Reasons for exclusion included co-infection with HIV or more than one genotype of HCV. Whole blood samples were drawn in sodium heparin tubes. All subjects gave written informed consent for research testing under protocols approved by the National Institute for Diabetes and Digestive and Kidney Diseases Institutional Review Board and the National Cancer Institute Institutional Review Board.

Synthetic HCV peptides

Overlapping peptides (OLPs; 18mers overlapping by 10 to 11 amino acids) spanning HCV genotype 1 sequence for each of three proteins, HCV E1, E2, and NS3, were chosen and arranged in pools. To make each pool, 50uL of each 18mer was combined and volume was adjusted with PBS/5% DMSO to a volume of 2100 uL (1mg/mL – 24 ug/mL each). The peptides were synthesized by the NIAID NIH AIDS Research Reagents Program.

Whole-blood intracellular cytokine staining (ICS) and NK cell degranulation assay

The frequencies of NK cells and T cells producing interferon-gamma (IFNγ) were determined by stimulating from HCV-infected subjects or healthy donors with peptide pools. 400uL of whole blood, collected in sodium heparin Vacutainer tubes, was stimulated with HCV E1, E2, and NS3 peptide pools at a final concentration of 2ug/mL in the presence of costimulatory anti-CD28 and anti-CD49d (BD Biosciences) and Brefeldin A (10ug/mL; Sigma-Aldrich). Specificity controls included a tube with no peptide added, as well as a positive control tube with stimulation by PMA-ionomycin (1ug/mL and 0.5 ug/mL; Sigma-Aldrich), both in the presence of costimulatory molecules and Brefeldin A. Samples were incubated for 6 hours at 37C at 5% CO2 in dark. Following incubation, red blood cells were lysed using red blood cell lysis buffer (145 mM NH4Cl, 0.1mM EDTA, 12mM NaHCO3 in distilled water). Cells were washed, and stained with ethidium monoazide (EMA, Sigma) at 4 degrees under light, followed by surface staining for 30 minutes in the dark at 4 degrees with the following panel: anti-CD14/ CD19 Cy5PE (CD14 Cy5-phycoerythrin, BIORAD; CD19 Cy5-phycoerythrin, BD Biosciences), CD56-Cy7PE (Cy7 phycoerythrin, BD Biosciences), CD3 Alexa700 (Alexa Fluor 700, BD Pharmingen), CD57 Pacific Blue (Biolegend), and CD16 Violet500 (BD Horizon). After fixation/permeabilization (eBioscience), cells were intracellularly stained for 30 minutes in the dark at 4C for TNFα FITC (fluorescein isothiocyanate, BD Pharmingen) and IFNγ PE (phycoerythrin, BD Pharmingen). Cells were then washed and resuspended in phosphate-buffered saline with 5% FBS and DNAse ($3x10^{-4}$ U/mL final concentration). Samples were acquired within 36 hours of

fixation on the LSR II flow cytometer (BD Biosciences), with filtration of cells (50um pore) directly prior to acquisition. 800,000 to 1.5 million events were acquired for each sample.

NK cell degranulation in response to stimulation with HCV peptides was studied using whole blood stimulated with HCV peptide pools in the presence of monensin (GolgiStop, BD Biosciences) and anti-CD107a PE (BD Pharmingen) in addition to Brefeldin A and costimulatory anti-CD28 and anti-CD49d (BD Biosciences) as described previously. Peptide stimulations were initiated within 12 hours of blood draw on 10 HCV infected patients and 10 healthy donors in parallel with cytokine release assays. A negative specificity control tube with no peptide added was included for each patient sample and a positive control stimulated with PMA-I was included in each experiment, as in the cytokine assay.

Samples were analyzed by multicolor flow cytometry using an LSRII FacsDiva Version 6.1.3 (BD Biosciences, San Jose, CA) and flow cytometry data was analyzed using FlowJo software version 8.8.6 (Tree Star, Ashland, OR). Gating strategy involved an initial time gate, followed by exclusion of doublet cells (FSC-A vs. FSC-H), a gate on a normal lymphocyte population (SSC-A vs. FSC-A), and a gate to exclude monocytes, B cells, and dead cells using CD14+, CD19+, and EMA to purify the lymphocyte population (Fig 1). Simultaneous gating of CD56+ CD3- NK cells and CD3+ CD56- T cells allowed for parallel analysis of NK cells and T cells from each whole blood sample. In the cytokine release assays, gating on IFNγ and TNFα allowed for selection of single and double-positive populations. Analysis of degranulation involved gating on CD107a+ NK cell and T cell populations.

Laboratory Tests

Serum immunoglobulin levels (IgG), alanine aminotransferase (ALT), and HCV RNA were tested at the NIH Clinical Center and lab testing was conducted by the NIH Department of Laboratory Medicine. Serum HCV RNA was tested by quantitative and qualitative reverse transcriptase polymerase chain reaction (Amplicor; Roche Molecular Systems, Pleasanton, CA, USA) (>100 viral copies/mL; 50 IU/mL). Results of these lab tests were accessed using the NIH Clinical Research Information System and only lab results from the same day as whole blood collection for corresponding NK and T cell assays were utilized.

HCV Genotyping

Genotyping for genotype 1a or 1b was conducted by the NIH Clinical Center. Patient records were accessed using the NIH Clinical Research Information System and were searched for a previously recorded viral genotype and genotyping was ordered for those patients without previously recorded genotype.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism Version 6.0 (GraphPad Software, Inc, San Diego, CA) and Microsoft Excel Version 14.3.2 (Microsoft Corporation). A matched:stacked 2-way Analysis of Variance (ANOVA) with multiple comparisons followed by Bonferroni posttest was used to compare peripheral blood NK cells from HCV-infected samples and healthy donor samples. The appropriateness of

using a matched:stacked 2-way ANOVA was verified by a low p value demonstrating significant variation between individual subjects. Wilcoxon matched paired test was used to compare ALT values. A P value < 0.05 was considered significant.

CHAPTER 3

Results

Cytokine production

The frequency of IFNγ-producing NK cell responses from HCV-infected individuals was significantly greater compared with healthy controls (variance $p < 0.05$) (Fig 2). The frequencies of IFNγ+ NK cells in whole blood stimulated with HCV E2 peptides ($p < 0.01$) and HCV NS3 peptides ($p < 0.05$) were statistically greater than the frequency of IFNγ+ NK cells in whole blood stimulated with the HDV overlapping peptide pool (Fig 2). However, a parallel analysis of T cell responses from the same peptide-stimulated whole blood samples shows a very low frequency of T cell cytokine responses, with frequency of IFNγ+ responses approximating baseline levels seen in unstimulated HCV+ or healthy whole blood (Fig 3). The frequencies of T cell IFNγ responses were small with no significant variance between HCV-infected and HCV-uninfected samples. On comparison of NK cell IFNγ production with T cell IFNγ production in the same whole blood HCV peptide stimulation assays, the frequency of IFNγ+ NK cell responses was 2-5 fold greater than corresponding IFNγ+ T cell responses, with significant variance $p < 0.0001$ between IFNγ+ responses in NK cells and T cells in whole blood stimulated with HCV E1, E2, or NS3 peptides (Fig 4).

Degranulation/cytotoxicity

In addition to cytokine production, we also assessed the cytotoxic activity of NK and T cells in the whole blood peptide stimulation assay, using CD107a surface expression as a marker of degranulation. The frequency of cytotoxic NK cell responses

was increased in HCV-infected samples (n=10) over healthy controls (n=10) after stimulation of whole blood with structural (E1, E2) and nonstructural (NS3) peptides (variance $p < 0.01$) (Fig 5). The frequency of CD107a+ T cells in response to peptide stimulation was higher in HCV+ whole blood than in healthy controls, demonstrating some HCV-stimulated T cell degranulation. However, on comparison of NK cell degranulation to corresponding T cell degranulation responses in the whole blood assay, NK cell degranulation in response to HCV E1 and E2 were significantly larger than T cell responses to these peptides (variance $p<0.05$). Notably, there is considerable variability of responses to individual HCV peptide pools between subjects. NK cell responses to each HCV protein tested varied between subjects. Notably, NK cell responses did not correlate with T cell responses, liver transaminase levels, or viremia.

CHAPTER 4

Conclusions and Discussion

This study demonstrates that NK cells degranulate and produce cytokines in chronic HCV infection. These NK cell responses are detectable with HCV peptide stimulation of whole blood.

NK cell cytotoxic and cytokine responses were larger than corresponding T cell responses. Consistent with previous observations of dampened T cell responses in chronic infection, this study demonstrated that T cells were producing cytokines and degranulating in response to HCV peptide stimulation at low frequencies in these chronically infected individuals. The significantly more robust NK cell response underscores the importance of understanding the role of NK cells in chronic infection, both for understanding antiviral mechanisms and disease progression.

Several mechanisms may be considered to explain these findings:

First, it is possible that NK cells from a chronically HCV-infected patient may elicit a non-specific increased baseline activation due to inflammatory environment of chronic infection. However, low NK cell responses without peptide stimulation were subtracted from corresponding stimulated samples and blood stimulated with HDV overlapping peptides generated very low frequencies of responding NK cells.

Second, NK cells can mediate cytotoxicity through ADCC, using surface-expressed CD16 (FcγRIII), to recognize circulating HCV-specific immunoglobulins which could bind HCV proteins on the surface of virally-infected cells. In this case, specificity for HCV-infected cells is contributed by the antibody and depends on the presence of HCV proteins on the surface of infected hepatocytes. While viral proteins

are not expressed on the surface of infected hepatocytes to the same degree as in many infections, Dumonceaux et al. demonstrated cell-surface expressed E1/E2 heterodimers in cells transfected with HCV envelope-encoding sequences[45]. The limited prior research on HCV-specific NK cell activation through ADCC has focused on demonstration of NK-mediated ADCC responses to surface-expressed HCV E2 peptide on a hepatoma cell line, and have not yet demonstrated whether NK cell responses occur in chronic HCV directly in response to HCV peptide stimulation[46]. Our study demonstrates NK cell responses resulting from stimulation of whole blood, not isolated NK cells, with both structural (E1 and E2) as well as nonstructural (NS3) peptides. However, because in these experiments, HCV proteins were added as overlapping peptides, constituting linear rather than conformational epitopes, this study does not demonstrate in what context or presentation these peptides would be important *in vivo.* Additionally, it is still unclear whether E1 or NS3 can be transiently expressed on the surface of infected hepatocytes and, if so, whether it is present as a whole protein.

Third, other factors in whole blood which could lead to NK cell activation and/or degranulation include: IL-2 from activated CD4+ T cells, IL-12, IL-15, or IL-18 form monocytes, IL-12 or type 1 IFNs from dendritic cells, and IL-2 or IL-12 from induced natural killer T cells (iNKTs). Granulocytes may potentially also contribute to NK cell activation; however, no role for granulocytes in NK cell activation has been previously described. Additionally, NK cells in the whole blood could dampen HCV-specific CD8+ T cell responses through the release of IL-10. Future studies include IL-2 blocking with anti-IL-2 antibodies in the whole blood assay to assess for decreased frequency of NK cell responses.

This study includes several limitations. First, conclusions of this study are limited by the artificial nature of any *in vitro* experiment. Additionally, as described in the introduction, the liver constitutes an immunologically unique environment through its structure and enrichment of resident immune cells. Thus, this study using peripheral blood may not give an accurate representation of NK cell responses in the chronically HCV-infected liver. Experimentally, the overlapping peptide pools used for stimulation constitute linear epitopes rather than conformational epitopes, which would provide more effective, physiologic binding sites for antibodies.

A large amount of research has recently demonstrated that NK cells play an important role in the immunology of chronic HCV, with potential for driving pathology as well as antiviral activity through multiple diverse mechanisms. However, additional research is needed to clearly understand how the NK cell response might be modulated to eliminate HCV and reduce liver damage. Additionally, with the ongoing need for a prophylactic HCV vaccine, it is unclear whether modulation of NK cell responses could assist in vaccine development.

LIST OF TABLES

Table 1: Chronic HCV+ Cohort.

	Patients with chronic HCV infection
Subjects	39
Age (median year)	59
Gender (male: female)	28:11
HCV RNA (median copies/mL)	15,417,000 (869,400-67,770,000)
ALT (median IU/L)	84 (15-610)
Serum IgG (median mg/dL)	1700 (918-2700)

LIST OF FIGURES

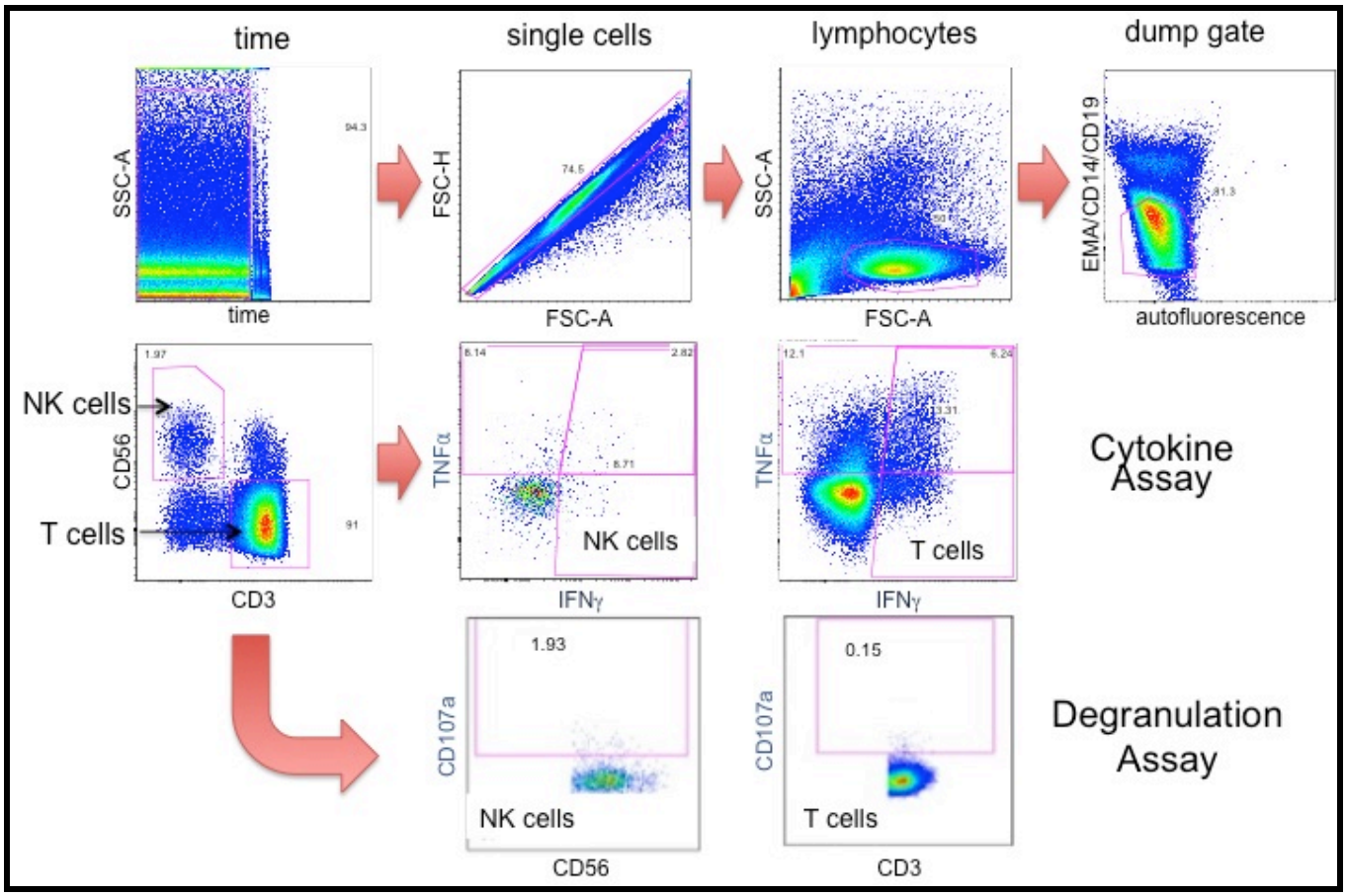


Figure 1. Flow Cytometry Gating Strategy: Stained, washed cells from whole blood HCV peptide stimulation assay were run on LSRII FacsDiva Version 6.1.3. Flow cytometry data was analyzed using the above gating strategy. (1) A time gate to gate out any obvious machine or operator errors. (2) Single cell gate to exclude any cells stuck together. (3) Within a SSC-A vs. FSC-A plot, lymphocytes are gated using established size and complexity. (4) Lymphocyte population is further purified with plot to exclude dead cells, monocytes, and B cells (EMA, CD14, CD19) and autofluorescence. (5) Within the lymphocyte population, NK cells (CD56+CD3-) and T cells (CD3+CD56-) are differentiated and individually gated. At this point two different assays analyses were done depending on the staining done. For the cytokine assay, NK cells and T cells were analyzed in parallel for expression of TNFα and IFNγ. For the degranulation assay, NK cells and T cells were analyzed in parallel for expression of CD107a, a surface marker for degranulating cells.

Figure 2. Natural killer cell activation (IFNγ+) in HCV-infected patients vs. healthy controls in whole blood peptide stimulation assay. Natural killer cell IFNγ production was assessed in HCV-infected individuals and healthy controls using the whole blood assay with stimulation with either HCV E1, E2, NS3 or HDV OLPs. Baseline responses seen in whole blood sample without peptide stimulation were subtracted from responses in peptide-stimulated samples for corresponding blood samples. Matched:stacked 2way ANOVA demonstrates significant variance ($p < 0.05$) between NK cell IFNγ production in HCV+ whole blood stimulated with HCV peptides in HCV-infected vs. healthy whole blood. Frequencies of IFNγ+ NK cells stimulated with HCV E2 peptides ($p < 0.01$) and HCV NS3 peptides ($p < 0.05$) were statistically greater than the frequency of IFNγ+ NK cells stimulated with the HDV peptide pool.

Abbreviations: Interferon γ (IFNγ); Hepatitis C virus (HCV); Natural killer cell (NK cell); Envelope 1 (E1) protein; Envelope 2 (E2) protein; Non-structural 3 (NS3) protein; Hepatitis D virus (HDV); Overlapping peptides (OLPs); Analysis of Variance (ANOVA)

* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

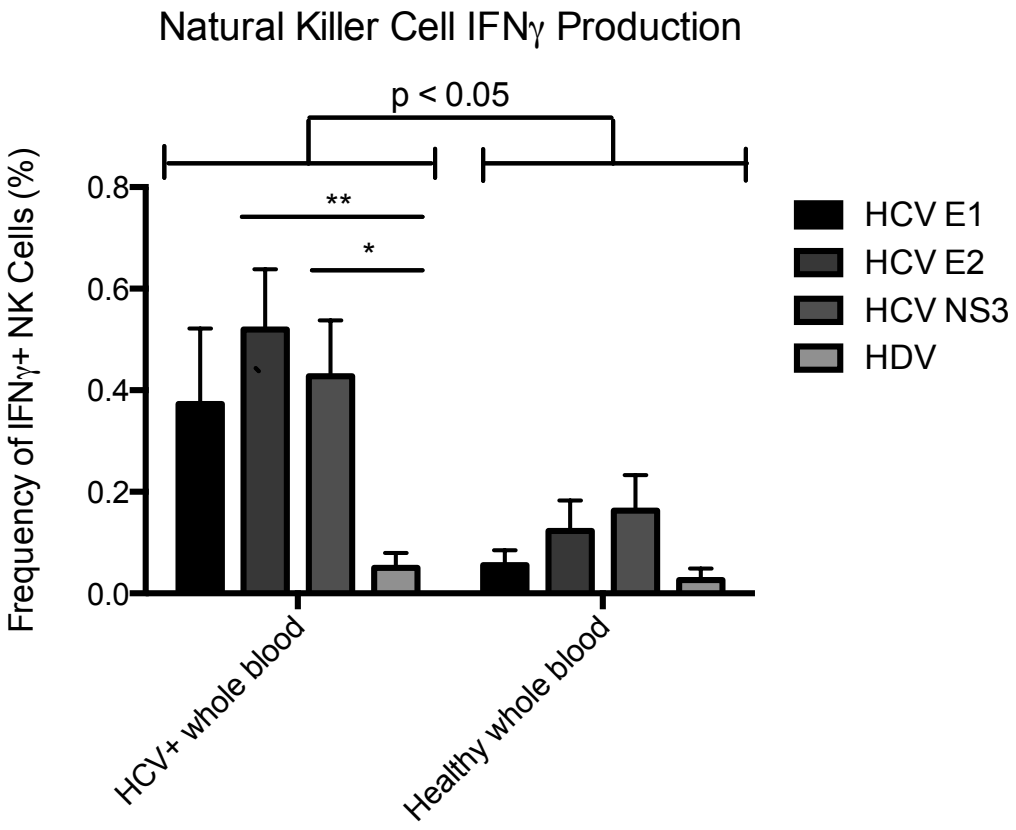


Figure 3. T cell activation (IFNγ+) in HCV-infected patients vs. healthy controls in whole blood peptide stimulation assay. Frequencies of T cell IFNγ responses were small with no significant difference between HCV-infected and HCV-uninfected samples on matched:stacked 2way ANOVA. Subgroup comparisons revealed significantly greater ($p < 0.05$) T cell IFNγ production on whole blood stimulation with HCV E1 than HCV NS3. Baseline T cell responses to unstimulated whole blood samples for each individual were subtracted from corresponding T cell responses in peptide-stimulated samples.
Abbreviations: Interferon γ (IFNγ); Hepatitis C virus (HCV); Envelope 1 (E1) protein; Envelope 2 (E2) protein; Non-structural 3 (NS3) protein; Hepatitis D virus (HDV); Overlapping peptides (OLPs); Analysis of Variance (ANOVA)
* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

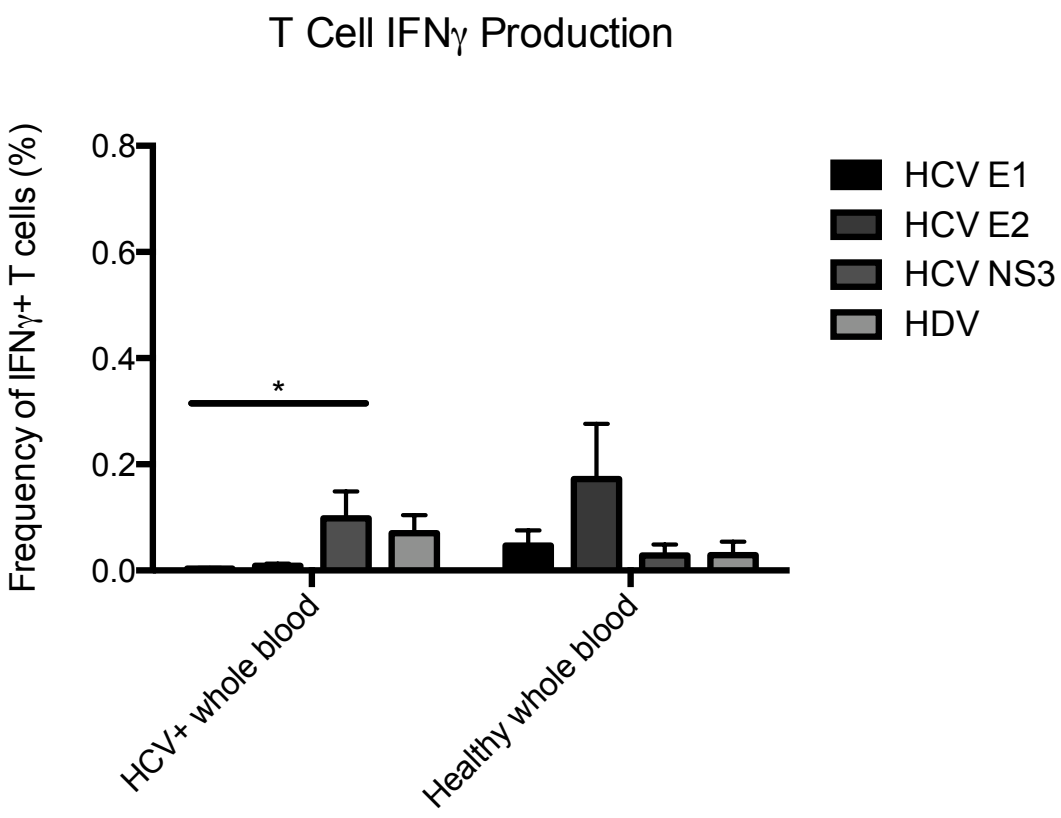


Figure 4. NK cell vs. T cell activation (IFNγ+) in HCV-infected patients in a whole blood peptide stimulation assay. Matched:stacked 2way ANOVA demonstrates significantly greater ($p < 0.0001$) frequency of NK cell IFNγ production than T cell IFNγ production in the same whole blood assays in samples stimulated with HCV E1, E2, or NS3 OLPs. There is no significant difference between NK cell and T cell IFNγ production in whole blood stimulated with HDV OLPs.
Abbreviations: Interferon γ (IFNγ); Hepatitis C virus (HCV); Natural killer cell (NK cell); Envelope 1 (E1) protein; Envelope 2 (E2) protein; Non-structural 3 (NS3) protein; Hepatitis D virus (HDV); Overlapping peptides (OLPs); Analysis of Variance (ANOVA).
* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

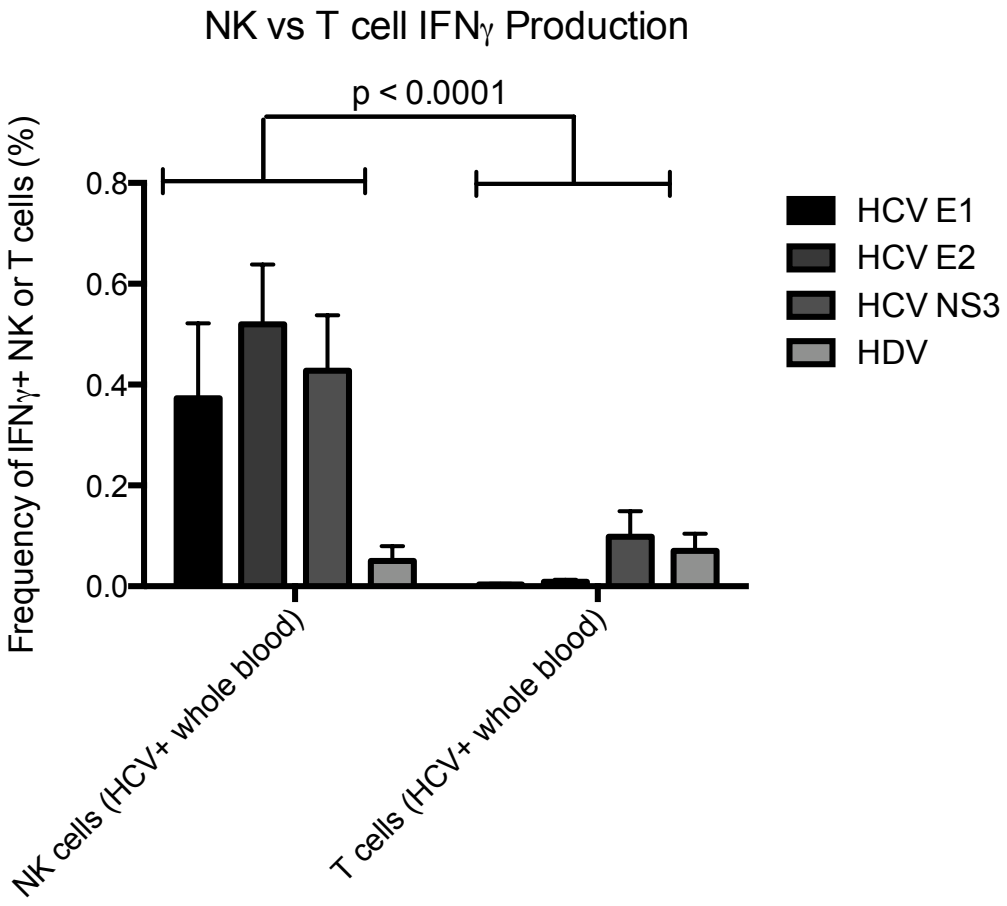


Figure 5. Natural killer cell degranulation (CD107a+) in HCV-infected patients vs. healthy controls in whole blood peptide stimulation assay. CD107a expression is a marker for degranulation (cytotoxicity). The frequency of cytotoxic NK cell responses is increased in HCV-infected samples (n=10) over healthy controls (n=10) after stimulation with structural (E1, E2) and nonstructural (NS3) peptides ($p < 0.01$).
Abbreviations: Interferon γ (IFNγ); Hepatitis C virus (HCV); Natural killer cell (NK cell); Envelope 1 (E1) protein; Envelope 2 (E2) protein; Non-structural 3 (NS3) protein; Hepatitis D virus (HDV); Analysis of Variance (ANOVA).

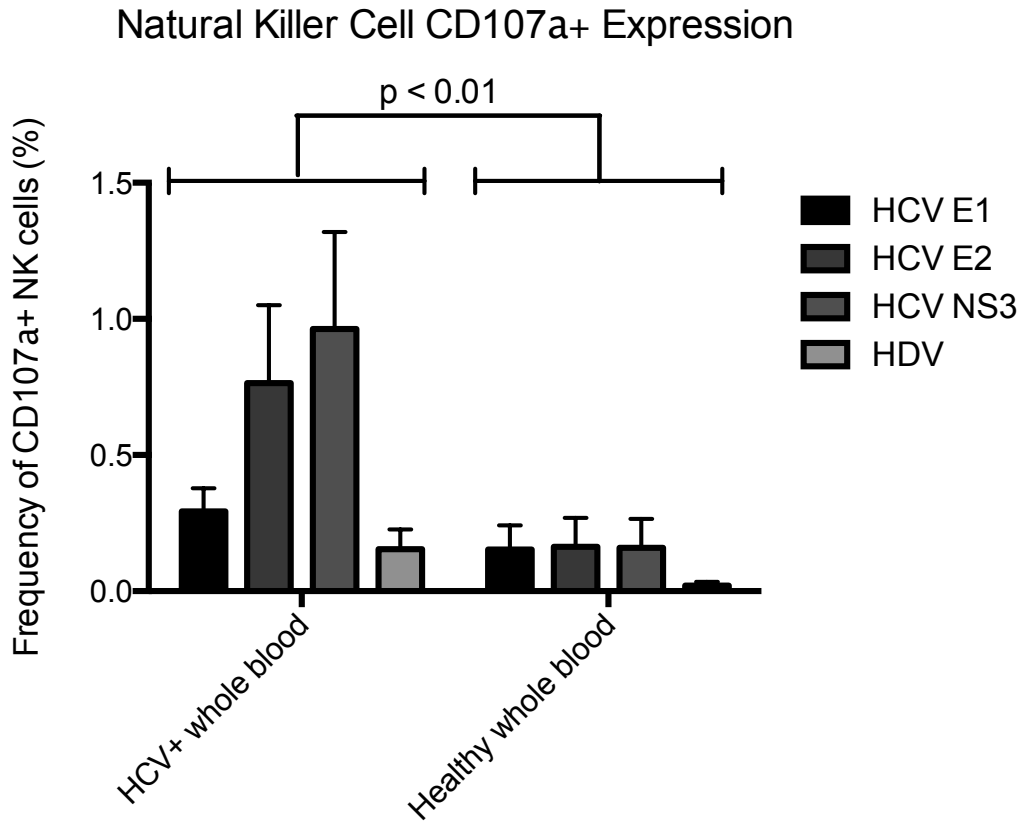


Figure 6. T cell degranulation (CD107a+) in HCV-infected patients vs. healthy controls in whole blood peptide stimulation assay. There is no significant variance between frequency of T cell degranulation in HCV+ whole blood versus healthy control whole blood by matched:stacked 2-way ANOVA. On multiple comparisons, the frequency of CD107a+ T cells in whole blood stimulated by NS3 is greater ($p < 0.001$) than the frequency of CD107a+ T cells in E1-stimulated whole blood. T cell responses in healthy whole blood were near baseline regardless of peptide stimulation.
Abbreviations: Interferon γ (IFNγ); Hepatitis C virus (HCV); Natural killer cell (NK cell); Envelope 1 (E1) protein; Envelope 2 (E2) protein; Non-structural 3 (NS3) protein; Hepatitis D virus (HDV); Analysis of Variance (ANOVA).
* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

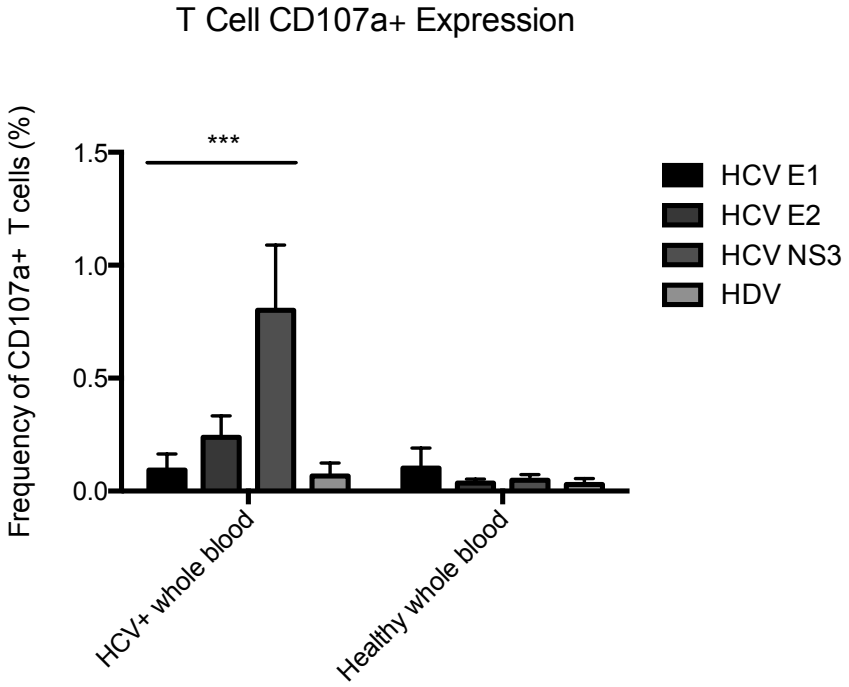
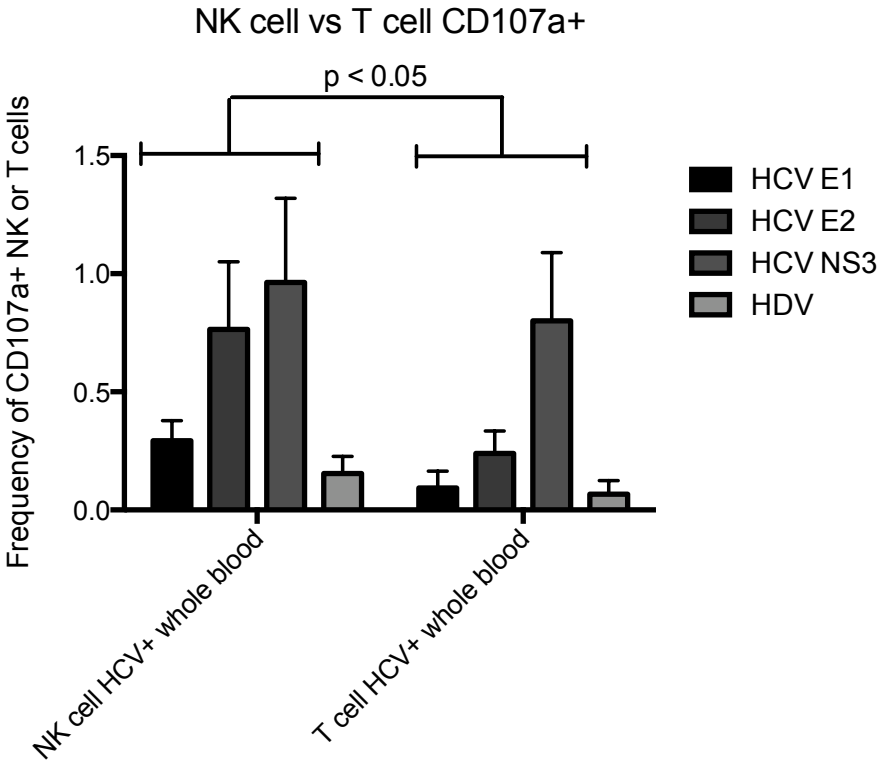


Figure 7. NK cell vs. T cell degranulation (CD107a+) in HCV-infected patients in a whole blood peptide stimulation assay. The frequency of NK cell degranulation in response to whole blood stimulation with HCV E1, E2, and NS3 is greater (variance $p < 0.05$) than the frequency of T cell degranulation in whole blood stimulated with these peptides. Frequencies of degranulating NK or T cells in whole blood stimulated with HDV OLPs, which were used as a negative control, are not statistically different. Baseline responses from these whole blood samples and not stimulated with peptides were subtracted from these responses.
Abbreviations: Interferon γ (IFNγ); Hepatitis C virus (HCV); Natural killer cell (NK cell); Envelope 1 (E1) protein; Envelope 2 (E2) protein; Non-structural 3 (NS3) protein; Hepatitis D virus (HDV); Analysis of Variance (ANOVA).
* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$



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VITAE

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REFERENCES

1 Park, S. H. & Rehermann, B. Immune responses to HCV and other hepatitis viruses. *Immunity* **40**, 13-24, doi:10.1016/j.immuni.2013.12.010 (2014).

2 Hoofnagle, J. H. Course and outcome of hepatitis C. *Hepatology* **36**, S21-29, doi:10.1053/jhep.2002.36227 (2002).

3 Martin, P., DiMartini, A., Feng, S., Brown, Jr., R., Fallon, M. Evaluation for Liver Transplantationin Adults: 2013 Practice Guideline by the American Association for the Study of Liver Diseaes and the American Society of Transplantation. *Hepatology* **59**, 1144-1165, doi:10.1002/hep.26972 (2013).

4 Burra, P. Hepatitis C. *Semin Liver Dis* **29**, 53-65, doi:10.1055/s-0029-1192055 (2009).

5 El-Serag, H. B., Hampel, H., Yeh, C. & Rabeneck, L. Extrahepatic manifestations of hepatitis C among United States male veterans. *Hepatology* **36**, 1439-1445, doi:10.1053/jhep.2002.37191 (2002).

6 Simmonds, P. *et al.* Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* **42**, 962-973, doi:10.1002/hep.20819 (2005).

7 Ghany, M. G., Strader, D. B., Thomas, D. L. & Seeff, L. B. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* **49**, 1335-1374, doi:10.1002/hep.22759 (2009).

8 Murakawa, K., Esumi, M., Kato, T., Kambara, H. & Shikata, T. Heterogeneity within the nonstructural protein 5-encoding region of hepatitis C viruses from a single patient. *Gene* **117**, 229-232 (1992).

9 Ogata, N., Alter, H. J., Miller, R. H. & Purcell, R. H. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. *Proc Natl Acad Sci U S A* **88**, 3392-3396 (1991).

10 Rehermann, B. Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. *Nat Med* **19**, 859-868, doi:10.1038/nm.3251 (2013).

11 Rehermann, B. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. *J Clin Invest* **119**, 1745-1754, doi:10.1172/JCI39133 (2009).

12 Jacobson, I. M., J.G. McHutchinson, G. Dusheiko, A.M. Di Bisceglie, K.R. Reddy, N. H. Bzowej, P. Marcellin, A.J. Muir, P.Ferenci, R. Flisiak, J. Goerge, M. Rizzetto, D. Shouval, R. Sola, R. A. Terg, E.M. Yoshida, N. Adda, L. Bengtsson, A.J. Sankoh, T.L. Kieffer, S. George, R.S. Kauffman, S. Zeuzem. Telaprevir for previously untreated chronic hepatitis C virus infection. *N. Eng. J. Med.* **364**, 2405-2416 (2011).

13 Poordad, F., J. McCOne, Jr., B. R. Bacon, S. Bruno, M.P. Manns, M.S. Sulkowski, I.M. Jacobson, K.R. Reddy, Z.D. Goodman, N. Boparai, M.J. DiNubile, V.Sniukiene, C. A. Brass, J.K. Albrecht, and J.P. Bronowicki Boceprevir for untreated chronic HCV genotype 1 infection. *N. Eng. J. Med.* **364**, 1195-1206 (2011).

14 Lawitz, E. & Gane, E. J. Sofosbuvir for previously untreated chronic hepatitis C infection. *N Engl J Med* **369**, 678-679, doi:10.1056/NEJMc1307641 (2013).

15 Osinusi, A. *et al.* Sofosbuvir and ribavirin for hepatitis C genotype 1 in patients with unfavorable treatment characteristics: a randomized clinical trial. *Jama* **310**, 804-811, doi:10.1001/jama.2013.109309 (2013).

16 Zeuzem S, B. T., Gane E, Ferenci P, Foster GR, Fried MW, Hezode C, Hirschfield GM, Jacobson I, Nikitin I, Pockros PJ, Poordad F, Scott J, Lenz O, Peeters M, Sekar V, De Smedt G, Sinha R, Beumont-Mauviel M. Simeprevir increases rate of sustained virologic response among treatment-experienced patients with HCV genotype-1 infection: a phase IIb trial. *Gastroenterology* **146**, 430-441 (2014).

17 Smith, B. D. *et al.* Recommendations for the identification of chronic hepatitis C virus infection among persons born during 1945-1965. *MMWR Recomm Rep* **61**, 1-32 (2012).

18 Testing for HCV infection: an update of guidance for clinicians and laboratorians. *MMWR Morb Mortal Wkly Rep* **62**, 362-365 (2013).

19 Recommendations for prevention and control of hepatitis C virus (HCV) infection and HCV-related chronic disease. Centers for Disease Control and Prevention. *MMWR Recomm Rep* **47**, 1-39 (1998).

20 Rice, C. M. New insights into HCV replication: potential antiviral targets. *Top Antivir Med* **19**,

117-120 (2011).
21 Lindenbach, B. D. & Rice, C. M. The ins and outs of hepatitis C virus entry and assembly. *Nat Rev Microbiol* **11**, 688-700, doi:10.1038/nrmicro3098 (2013).
22 Liu, S. *et al.* Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. *J Virol* **83**, 2011-2014, doi:10.1128/JVI.01888-08 (2009).
23 Evans, M. J. *et al.* Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**, 801-805, doi:10.1038/nature05654 (2007).
24 Scarselli, E. *et al.* The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *Embo J* **21**, 5017-5025 (2002).
25 Lohmann, V. *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110-113 (1999).
26 Lohmann, V. & Bartenschlager, R. On the history of hepatitis C virus cell culture systems. *J Med Chem* **57**, 1627-1642, doi:10.1021/jm401401n (2014).
27 Lindenbach, B. D. *et al.* Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. *Proc Natl Acad Sci U S A* **103**, 3805-3809, doi:10.1073/pnas.0511218103 (2006).
28 Lindenbach, B. D. *et al.* Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623-626, doi:10.1126/science.1114016 (2005).
29 Wakita, T. *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**, 791-796, doi:10.1038/nm1268 (2005).
30 Dorner, M. *et al.* Completion of the entire hepatitis C virus life cycle in genetically humanized mice. *Nature* **501**, 237-241, doi:10.1038/nature12427 (2013).
31 Racanelli, V. & Rehermann, B. The liver as an immunological organ. *Hepatology* **43**, S54-62, doi:10.1002/hep.21060 (2006).
32 Wedemeyer, H. *et al.* Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* **169**, 3447-3458 (2002).
33 Raghuraman, S. *et al.* Spontaneous clearance of chronic hepatitis C virus infection is associated with appearance of neutralizing antibodies and reversal of T-cell exhaustion. *J Infect Dis* **205**, 763-771, doi:10.1093/infdis/jir835 (2012).
34 al., v. H. T. e. Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection *in vivo*. *Gastroenterology* **132**, 667-678 (2007).
35 Ahlenstiel, G. The Natural Killer Cell Response to HCV Infection. *Immune Netw* **13**, 168-176, doi:10.4110/in.2013.13.5.168 (2013).
36 Moretta, L. *et al.* Human natural killer cells: their origin, receptors and function. *Eur J Immunol* **32**, 1205-1211, doi:10.1002/1521-4141(200205)32:5<1205::AID-IMMU1205>3.0.CO;2-Y (2002).
37 Moretta, A., Bottino, C., Mingari, M. C., Biassoni, R. & Moretta, L. What is a natural killer cell? *Nat Immunol* **3**, 6-8, doi:10.1038/ni0102-6 (2002).
38 Doherty, D. G. & O'Farrelly, C. Innate and adaptive lymphoid cells in the human liver. *Immunol Rev* **174**, 5-20 (2000).
39 Khakoo, S. I. *et al.* HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* **305**, 872-874, doi:10.1126/science.1097670 (2004).
40 Ahlenstiel, G. *et al.* Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner. *Gastroenterology* **138**, 325-335 e321-322, doi:10.1053/j.gastro.2009.08.066 (2010).
41 Ahlenstiel, G., Martin, M. P., Gao, X., Carrington, M. & Rehermann, B. Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses. *J Clin Invest* **118**, 1017-1026, doi:10.1172/JCI32400 (2008).
42 Oliviero, B. *et al.* Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. *Gastroenterology* **137**, 1151-1160, 1160 e1151-1157, doi:10.1053/j.gastro.2009.05.047 (2009).
43 FV, G. L. a. C. Immunobiology and Pathogenesis of Viral Hepatitis. *Annu. Rev. Pathol. Mech. Dis.* **1**, 23-61 (2006).
44 Thimme, R. *et al.* Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci U S A* **99**, 15661-15668,

doi:10.1073/pnas.202608299 (2002).

45 Dumonceaux, J. *et al.* Expression of unmodified hepatitis C virus envelope glycoprotein-coding sequences leads to cryptic intron excision and cell surface expression of E1/E2 heterodimers comprising full-length and partially deleted E1. *J Virol* **77**, 13418-13424 (2003).

46 Nattermann, J. *et al.* Serum antibodies against the hepatitis C virus E2 protein mediate antibody-dependent cellular cytotoxicity (ADCC). *J Hepatol* **42**, 499-504, doi:10.1016/j.jhep.2004.12.018 (2005).