

THE ROLE OF HUMORAL IMMUNE RESPONSE IN HEPATITIS C INFECTION

by

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Submitted to the Graduate Faculty of
Graduate School of Public Health in partial fulfillment
of the requirements for the degree of
Master of Science

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH
GRADUATE SCHOOL OF PUBLIC HEALTH

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University of Pittsburgh, 2014

ABSTRACT

The World Health Organization (WHO) has estimated that about 3% of the world's population is currently infected with Hepatitis C Virus (HCV). Although spontaneous clearance of HCV infections occurs in 10-20% of patients, nearly 185 million people are still chronically infected by HCV, which is one of the leading risk factors for developing liver cirrhosis and/or liver cancers. In the HIV-1 infected population, chronic HCV infection has become one of the leading causes of non-AIDS specific morbidity and mortality. In the United States about 25% of HIV-1 positive individuals have co-infections with HCV. It has been observed that HIV-1 infection reduces the rate of spontaneous clearance and exacerbates the clinical course of HCV infection in HIV/HCV co-infected individuals, but the mechanisms driving this remain unknown. One of the major hurdles of studying the mechanisms of HCV infection over the last two decades has been the inability to grow and study this virus in a laboratory setting, as well as, the inability to develop reliable animal models. The development of the HCV pseudotyped viral particles (HCVpp) system was ground-breaking and became one of the most important *in vitro* study tools to test the neutralizing antibodies against virus entry and vaccine studies. Therefore, we used HCVpp to evaluate the role of neutralizing antibodies in an HIV-infected subject who spontaneously cleared HCV, but subsequently was re-infected with a different genotype of HCV.

In this study, we examined the role of HCV specific neutralizing antibodies in HCV clearance and chronicity in a HIV/HCV co-infected subject who was enrolled in Pittsburgh portion

of the Multicenter AIDS Cohort Study (MACS). The subject's records showed a 10-year history of HIV infection and treatment with antiretroviral drugs for 2 years prior to HCV infection. According to our previous studies, this person was consecutively infected with two different genotypes of HCV. By sequence analysis, the initial infecting HCV genotype was 3a and the subsequent infecting genotype, 1.5 years later, was HCV 1a. Sequence analysis of HCV with longitudinal samples from this individual shows that the initial infecting HCV 3a was cleared spontaneously after one and half year infection, but after the subsequent infecting HCV 1a established its chronic infection in this individual.

We hypothesized that neutralizing antibodies played an important role in controlling and clearing the HCV 3a infection, but had minimal impact on the HCV 1a infection. Using HCVpp containing autologous envelope proteins of HCV subtype 3a or 1a, we measured the neutralization activity of antibodies present in the subject's serum. We found that after pre-incubating genotype 3a specific HCVpp with the subject's serum collected 6 months after HCV 3a infection, there was a tendency of reduction in the genotype 3a specific HCVpp infectivity. However, we observed no reduction in infectivity of genotype 1a specific HCVpp, which were pre-incubated with the subject's serum collected after HCV 1a infection. Therefore, our results suggest that the development and presence of neutralizing antibodies may be important for the spontaneous clearance of HCV 3a in HIV co-infected individuals. This study contributes to our understanding of immune control of HCV, which may lead to the development of preventive vaccine against HCV and have a great Public Health impact in preventing HCV infection.

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PREFACE

I would like to thank my advisor Dr. Yue Chen for being a great mentor and giving me an opportunity to work on this project. This was very exciting and at the same time very challenging for me to work in molecular research field since every single technique and method were new to me, and without my advisor's valuable support and help it would not have been possible to accomplish all my goals. She has been a great listener and the most knowledgeable person for me who was willing to help and guide throughout my thesis project. I continue to admire her as a truly mentor and the person who dedicated her time and knowledge into the research field and teaching. I would also like to thank Dr. Gupta, Dr. Chengli Shen and Dr. Kelly Cole for guidance and expert opinions in regards to my project.

Another group of people who have been my supporters during my stay in this lab are Deena Ratner, Lori Caruso, Ming Ding, PhD students Soni and Anwasha, who are deserved the deepest admiration for making the work environment truly friendly and amazing. Also, I would like to thank special people I met in Pittsburgh, my peers in IDM program and great friends Suha Abdelbagi and Andrea Dobbs for making graduate student life memorable and for being such amazing friends.

1.0 INTRODUCTION

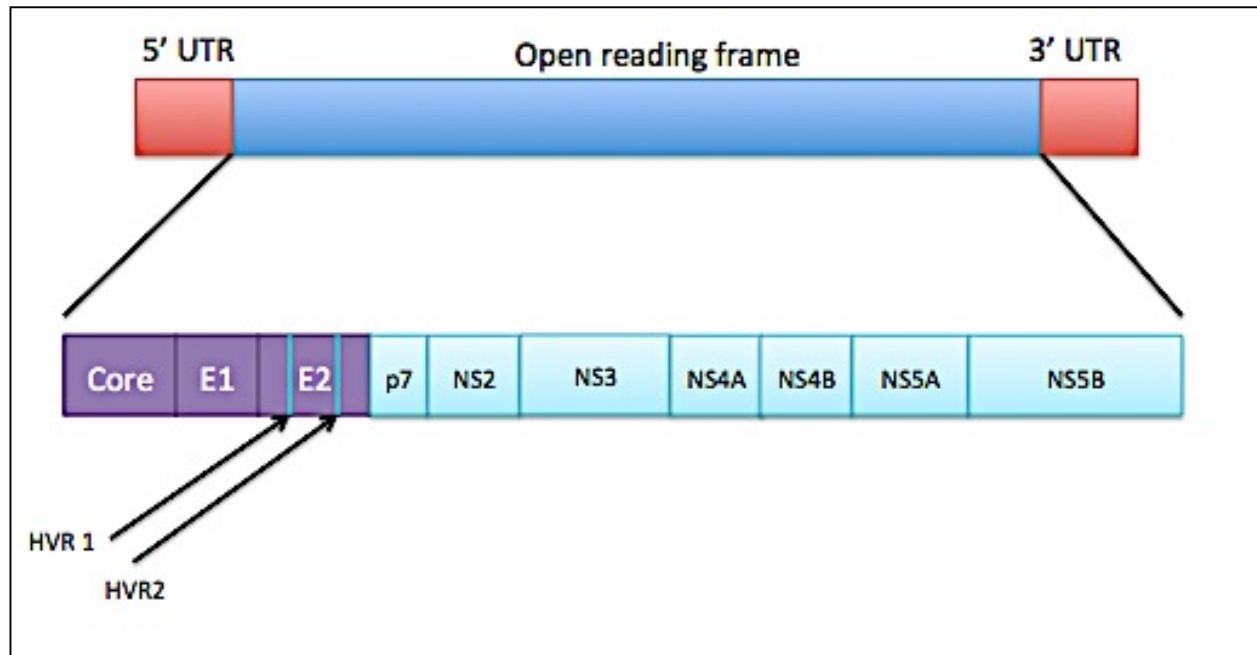
1.1 VIRAL HEPATITIS C INFECTION

1.1.1 HCV DISEASE BURDEN AND CURRENT ADVANCES

Chronic HCV infection is one of the leading causes of liver disease, cirrhosis and hepatocellular carcinoma. The prevalence of HCV according to 2014 WHO estimates is approximately 170-185 million worldwide with a US disease burden of about 4 million [1]. HCV is mainly spread by blood-blood contact with the primary routes of infection being injection-drug use and unscreened blood products used in transfusions. Until recently, the only “gold standard” treatment option available was pegylated interferon (pegIFN) and ribavirin, but this treatment was only effective in 40-80% of the HCV infected patients and carried many side effects [6, 7]. The latest developments in HCV antiviral drugs have been very promising. In 2011, protease inhibitors Boceprevir (Merck) and Telaprevir (Vertex) were approved by the FDA and are currently being used in a combination with pegIFN and ribavirin (ref). This combinational therapy has increased the rate of HCV clearance to 70-80% in treated patients. Another wave of new direct acting agents (DAAs), with an observed clearance rate of 90-99%, will be approved for treatment by 2015. It is predicted that these new DAAs will decrease the burden of HCV infection to half within 10-15

years [8]. However, there is still no preventive vaccine available, which still should be a priority to prevent and control the spread of HCV.

1.1.2 HCV GENOME STRUCTURE



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Figure 1. HCV Genome structure and viral proteins.

The HCV genome is ~9.6kb long with a single open reading frame that's translated into a single polypeptide. Co- and post-translationally this polypeptide is cleaved at specific sites to produce 10 viral proteins. There are 3 structural proteins (Core, E1, E2, p7) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B).

Hepatitis C virus is an enveloped, single-stranded positive sense RNA virus, that is the only member of the hepacivirus genus of the *Flaviviridae* family [9]. The genome is ~9.6 kilobases and contains 5' and 3' untranslated regions (UTR) that have important RNA structures required for replication. There is an internal ribosomal entry site (IRES) at the 5' UTR that forms a stable

pre-initiation complex required for efficient translation of the HCV 3000 amino acid long polyprotein. This polypeptide is cleaved by both cellular and the viral proteases to make 3 structural proteins, the envelope proteins E1 and E2, the core protein, p7, and six of non-structural proteins NS2, NS3, NS4A, NS5A and NS5B. NS5B. The first protein to be cleaved from the polyprotein is the 21kDa core protein that forms the nucleocapsid and is responsible for packaging the viral RNA. Between the core and E1 envelope protein is a signaling sequence that targets the polyprotein to the endoplasmic reticulum (ER). At the ER, the E1 protein is cleaved and translocated in the ER lumen for further post-translational processing and glycosylation. The envelope glycoprotein E1 and E2 are the essential components of the HCV virion and play an essential role in entry and fusion [10]. The p7 and NS2 proteins are thought to be important in the assembly of the virion. NS3 contains helicase activities that are essential for viral RNA replication and the NS5B contains the RNA-dependent RNA-polymerase, which is also an essential requirement for HCV genome replication. Both of these non-structural proteins have been ideal targets for current DAAs [11].

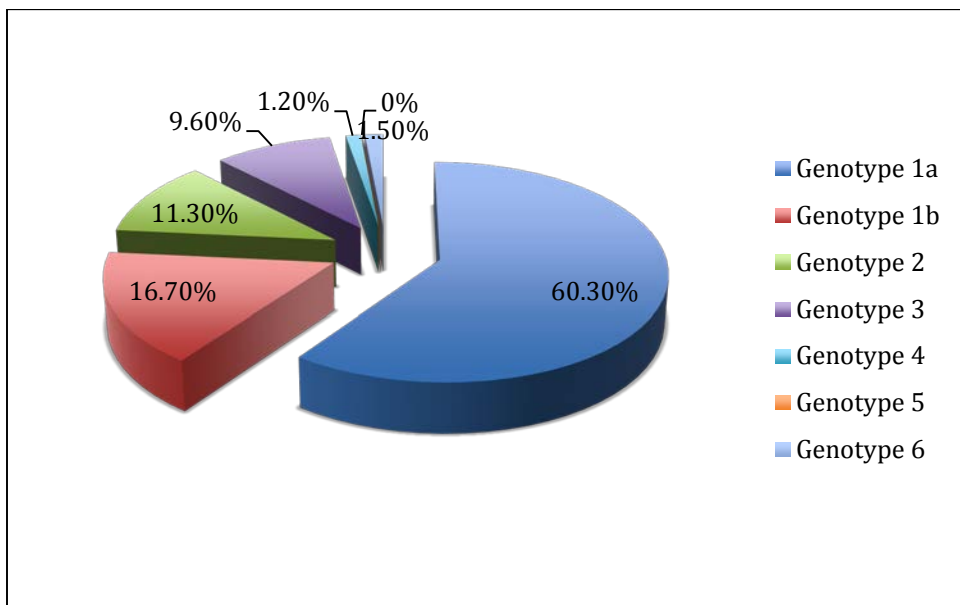
Initial attachment of the virus to hepatocytes requires cellular lipoproteins such as low density lipoproteins and apo-E proteins [12]. The essential cellular attachment molecules for HCV are CD81, scavenger receptor class B member (SRBI), claudin 1 (CLDN1), occludin (OCLN) and cholesterol absorption receptor Niemann-Pick C1-like 1 (NPC1L1). HCV Envelope protein E2 (HCV E2) directly binds to CD81, which is the main receptor that defines the tropism of HCV in human cells[13]. Many studies have shown that HCV E2 selectively binds with high affinity to CD81 in humans and chimpanzees[14]. Experimentally, blocking HCV E2 binding using an anti-CD81 antibody prevents the infection of cells in culture using HCVpp[15]. This experiment is part

of our rationale for investigating neutralizing antibodies that could prevent HCV attachment and entry by interfering with HCV binding to cellular receptors.

1.1.3 HCV GENOTYPE DIVERSITY

In 1989, investigators at the CDC identified HCV and shortly thereafter that same year sequenced and published its complete genome. Since the identification of HCV, investigators have been isolating and sequencing HCV from infected patients to build a data set of HCV genomic sequences, which can be accessed from the HCV Sequence Database (<http://s2as02.genes.nig.ac.jp/>). After isolating HCV from many patients with a variety of disease outcomes and from different geographical locations, it was observed that HCV is highly heterogeneous in nature [16]. The updated consensus of HCV classification in 2013 categorizes HCV into 7 major genotypes and 67 subtypes (Figure 2). Genotypes 1a and 1b are the most common genotypes, accounting for 60%-70% of all HCV infections worldwide. HCV infection with genotypes 1a or 1b is difficult to treat because the standard pegIFN and ribavirin regimen is not very effective overall. Genotype 3a and 2 are responsible for 20-25% of HCV infections in the US, but patients infected with these genotypes are twice likely to clear the infection while on the standard treatment regimen compared to those infected with genotypes 1a and 1b. Therefore, there is a poor prognosis for patients infected with HCV genotype 1 despite receiving the standard interferon treatment [17]. Sequence similarity among the different genotypes varies from 66% to 80%. There are three levels of genomic variations: types, subtypes, and quasispecies. The HCV genomes differ from 31-34% among genotypes, 21-23% among subtypes and 8-9% among quasispecies[18]. The HCV quasispecies found in a single individual are closely related to the original HCV genotype that established the initial infection. Due to selective pressure from the

host immune system and other factors, the virus evolves by inducing escape mutations to prevent recognition and neutralization [17]. These minor genetic mutations that occur spontaneously over time create many different quasispecies within the individual, but the genotype does not change. However, in some cases, individuals who receive multiple blood transfusions, under hemodialysis or who are IV-drug users can be infected with more than one genotype. This condition is referred to as a mixed-genotype infection [19].



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Figure 2. HCV genotype distribution in the US.

The most prevalent genotype 1a accounts for about 60% of all HCV infection cases.

HCV genotypes differ in their geographical distribution worldwide. In the United States, more than 60% of HCV infections are caused by HCV genotypes 1a (Figure 2), whereas in other parts of the world the HCV burden of disease may be caused by a different HCV genotype [20]. For

example, in Egypt HCV infections are only caused by genotype 4a while in South Africa HCV infections are only caused by genotype 5a [21].

1.1.4 HCV STUDY SYSTEMS

It was discovered in the 1980s that there was another virus other than Hepatitis A and B that was causing liver damage in individuals, but the etiological agent, HCV, wasn't discovered until 1989[22]. The discovery of hepatitis C virus was very difficult because isolating and growing this virus in culture is challenging, which has been a major hindrance in studying this new relatively new virus. Until 2005, there was no efficient method for growing and studying the replication of HCV in a cell culture system[23]. Many attempts have been taken to overcome the roadblocks in this field, but a lot of work still needs to be done to improve the current systems. One of the current available systems for in vitro study of HCV is using the human hepatoma cell line, Huh-7, which are transfected with genomic HCV RNA that is packaged and released as infectious HCV particles [4]. Although far from a perfect system due to limitations of the virus's ability to spread in culture cells, this is a major advancement that has significantly improved the ability to study HCV entry into host cells and replication.

Since HCV infection and replication are restricted to liver cells, the Huh-7 cell line, an immortal cell line derived from human hepatocyte cellular carcinoma, is the best permissive cell for HCV infection study *in vitro*[24]. Recent developments of self-replicating RNA's (HCV replicons) and a robust propagating cell culture system have made it possible to study the virus life cycle and DAA agents [25]. The lack of reliable and convenient animal models is still a major obstacle in the study of immune responses specific for HCV infections. The only available reliable

animal model is the chimpanzee, but the drawbacks to this model are ethical issues and economic costs. [26]. A newly developed transgenic mouse model that expresses HCV genes in the liver has provided a convenient model to study HCV pathogenesis [25]. However, this transgenic mouse model is not ideal to study immune responses during HCV infections because mice do not support HCV infection. Another available mouse model is the immunodeficient murine model SCID-Alb-uPA chimeric mice with xenografted human liver, which provides a good model to study virus entry and fitness, but the limitations for immunological studies is the need for the mice to have the same HLA type as the transplanted hepatocytes [27, 28]. Another system for studying HCV in vitro is HCV pseudoviral particle (HCVpp). After the development of HCVpp, scientists were able to study virus entry and neutralizing antibody responses during HCV infections[4]. HCVpp containing structural proteins of various HCV genotypes enable scientists to investigate humoral and cellular immune responses, and to expand their study objectives to encompass protective vaccine development against HCV infections.

1.2 IMMUNE RESPONSE DURING HCV INFECTION

1.2.1 HCV AND INNATE IMMUNE RESPONSE

The main characteristic of HCV infection is its ability to silently induce chronic infections such that most patients are unaware they have been infected. Even though about 30-50% of hepatocytes are infected with HCV, the infected cells exist in clusters around the liver, which suggests that the virus is transmitted from cell to cell [29]. The first line of defense against HCV is initiated by the innate immune system that recognizes specific HCV RNA structures by RNA-

dependent protein kinase R (PKR) or by RIG-I within the cytosol[30]. Recognition of a foreign invader by these sentries results in the activation of interferon regulatory factor-3 (IRF-3), which in turn activates the production of type I IFNs. This, in turn, induces a robust IFN response against the invading HCV. However, the main cellular source of IFN production is not clear, some investigators suggest that infected hepatocytes are the first cells to produce IFNs[30]. Then other cells and immune cells in response to this initial IFN production also induce IFN production and signaling [31]. Within the infected hepatocytes, RIG-I is thought to sense HCV RNA and activate caspases, which interacts with a mitochondrial membrane protein (MAVS) to mediate IRF-3, IRF-7 and NF- κ B activity. However, the viral proteases NS3/NS4A not only processes the viral polypeptide, but also blocks RIG-I mediated signaling by cleaving off the MAVS (or also called IPS-1). This is an immune escape mechanisms used by the virus to block the induction of an anti-viral IFN-response [32].

There is evidence that hepatocytes are not the only cells producing type I and type II IFNs in response to HCV infection because liver specific macrophages, called Kupffer cells, have also been observed to express IFN- β . Conventional dendritic cells (DCs) can also be responsible for the production of IFN- β . Through TLR3 signaling, DCs recognize dsRNA and initiate the production of IFN- β by recruiting TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN- β), which further activates downstream signaling to activate NF- κ B, IRF-3 and IRF-7 genes [33]. Viral proteases NS3/NS4A, as a means of immune evasion, abrogate this pathway by the cleavage of TRIF. This blocks the IFN signaling process and shuts down any IFN-response against HCV. These mechanisms of blocking IFN production during HCV infection could partially explain the impairment of IFN- α , β induction, but still it is not clear how HCV still persists under a robust immune response and interferon therapy [32]. Recent studies have shown that genetic

alteration of InterferonL3 (IFNL3) gene is associated with spontaneous clearance and interferon-based treatment outcome of HCV infection [34]. However, the mechanism of the Single Nucleotide Polymorphism (SNP) of IFNL3 gene is still not well understood. In the HCV infected patients with the rs12979860 CC-variant of IFNL3 gene, which is more prevalent in Asians and Europeans, there is a good prognosis when treated with the standard interferon treatment, whereas with other variants of IFNL3, such as the rs4803217 SNP TT variant, the prognosis for clearance isn't as promising [35].

The main players of innate immune response in liver are Natural Killer cells (NK), NKT cells and cell subsets from myeloid lineage including Kupffer cells. During HCV infection, the role of NKT cells is not well understood and there are some studies the report T cell functions are altered by the production of IL-10 and TGF- β [36]. NK cells are the key early responders during HCV infection [37]. They control viral infection by producing IFN- γ , lysing the HCV infected cells, and stimulating DC maturation. DC functions are impaired during HCV infections so they are unable to efficiently present viral antigens, which have a detrimental effect on mounting an effective adaptive immune response. Some authors speculate that this impairment of DCs is caused by HCV infection of these cells leading to a failure to properly mature. However, to date, no viral RNA has been detected within DCs isolated from infected animals [30]. Thus, the role innate immunity plays in protecting against HCV infections is not well understood, but there still a strong cause for the role certain IFNL3 gene in play in predicting successful outcomes to interferon therapy.

1.2.2 THE ROLE OF CELLULAR AND HUMORAL IMMUNE RESPONSE

Although the innate immune response and IFN production are very important during the early phase of HCV infection, HCV clearance requires a robust adaptive immune response. Numerous studies have reported the important role CD4⁺ T cell play in spontaneous clearance of HCV [38]. Usually HCV remains asymptomatic during the initial stages of infection. However, if the person develops signs and symptoms of an acute HCV infection, it usually corresponds strongly to eventual viral clearance. Studies have been performed to investigate how the immune system responds to acute HCV infection in the patients with a known time of exposure to HCV and in chimpanzee models [39]. Even in the presence of a high viral load, there are usually no symptoms of liver damage. HCV specific T cell responses can be detected earlier compared to HCV specific neutralizing antibodies. About 8-12 weeks after infection, an increase of liver transaminases in the blood indicates that there is a strong cytotoxic T cell activity against the infected hepatocytes, as shown in Figure 3A [40]. It has been reported that spontaneous clearance of HCV is related a robust T cell response, which targets multiple HCV epitopes [41]. An early CD4 T cell response is a strong marker used to predict the control of an acute HCV infection. In the absence of this robust response T-cell response, viremia levels rebound and a persistent infection is established [42]. A unique characteristic of HCV infections is the late development of the adaptive immune response. For 8-12 weeks post-infection, there is only an innate immune response detected by ISG's in the blood. The CD4⁺ and CD8⁺ T cell response wouldn't be detected until after the development of the acute stage of HCV infection. HCV-specific neutralizing antibodies appear even later about 16-18 weeks after onset of the initial infection and their appearance is usually followed by the resolution of HCV[43] . It has been reported that the small portion of patients who had self-limiting HCV infections mounted broad CD4⁺ T cell

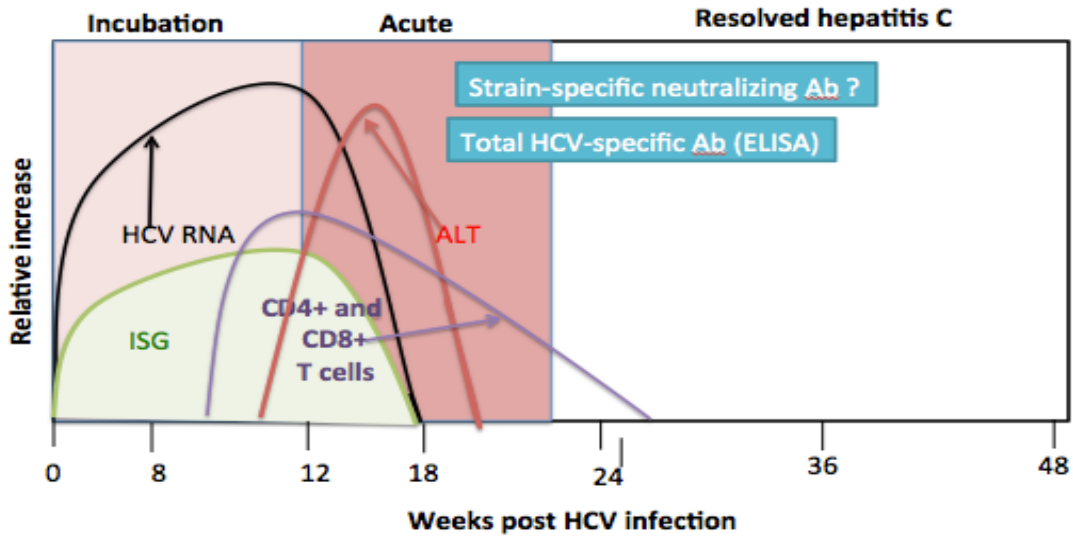
responses during the acute phase and had high levels of IL-2, TNF- α and IFN- γ , which are important cytokines for T-cell survival and proliferation[44].

Longitudinal studies have shown that there are a number of HCV epitopes targeted by T cells are similar between individuals who develop chronic infections and individuals who clear infections [45]. In an established chronic infection, the number of HCV specific CD8 T cells may be abundant, but they are not able to control the virus [46]. After the establishment of initial infection, about 36-38 weeks, escape mutants from neutralizing antibodies start to appear as well as escape mutations that can escape T cell activities, as shown in Figure 3B[43].

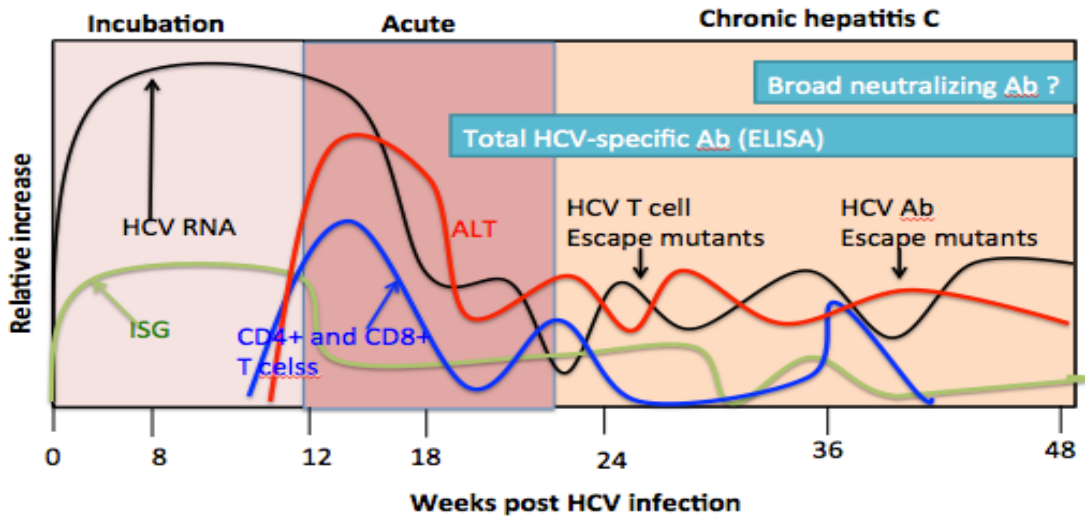
HCV evades the host's adaptive immune response, particularly the neutralizing antibodies (nAb), to establish chronic infections. Numerous studies have focused on T cell immunity and viral escape mutants [47], but the role of neutralizing antibodies in HCV clearance is not completely understood and is not very well studied. Vaccination studies performed on chimpanzees have shown they can successfully mount an HCV specific immune response [48]. According to previous studies on chimpanzees, the HCV E2 protein contains an extremely hypervariable region I (HVR1), which is a major target for nAb and high concentrations of HVR1 specific nAb in the serum are required to effectively neutralize the virus [49]. This region is also important for receptor binding and disease outcome. Many studies focused on HVR1 epitopes have found that it varies between different HCV genotypes. HVR1 seems to mask nAb epitopes within the E2 protein as mutation studies within the HVR1 region made HCV more susceptible for neutralization [50], [25]. There is strong evidence that nAb responses against E1/E2 epitopes could be protective as it has been associated with the resolution of HCV in some studies [51]. Even though, many studies focus mainly on the role of T cells in HCV clearance, the importance of the

neutralizing antibody responses should not be underestimated and should be the focus of more future studies.

A.



B.



Figures adopted with permission from *Immunity*, Volume 40, Issue 1, 2014, 13 – 24, Su-Hyung Park, Barbara Rehermann

Figure 3 Clinical, Virological, and Immunological course of Acute HCV infection.

A. Acute hepatitis C followed by recovery. B. Acute hepatitis C followed by chronic infection.

1.3 HIV/HCV COINFECTION

1.3.1 TRANSMISSION AND EVOLUTION OF HIV AND HCV

Human immunodeficiency virus (HIV) is an envelope RNA retrovirus that has overlapping routes of transmission with HCV. About 25% of HIV infected individuals are co-infected with HCV, which is about 1.2 million people in the US [52]. According to meta-analysis studies, individuals co-infected with HIV/HCV are three times more likely to develop liver complications such as cirrhosis and liver cancer compared to those only infected with HCV[53]. HIV/HCV co-infection also decrease the probability an individual will be able to spontaneously clear HCV [54]. Both HCV and HIV viruses produce “quasiespecies” over the course of chronic infections due to random mutations made during replication. In both infections, only a few founder viruses are required to establish systemic, chronic infections. This means the host immune system needs to successfully clear and control the virus early on in the infection to prevent establishment and systemic spread [55]. It is still not clear how the immune system controls viral replication during HIV/HCV co-infections, but it is a well-known that viral fitness in each infection depends on adaptive immune pressure leading to the development of escape mutations [53]. Most co-infection studies have been focused on the impacts of HCV infection on the natural history of HIV infections. There is limited knowledge about how the presence of HIV and the immune dysfunctions related to its infection effects the immune system’s ability to mount an effective nAb response against HCV.

2.0 OBJECTIVE

HYPOTHESIS

We hypothesize that the host immunity, especially HCV specific nAb plays an important role in controlling HCV 3a infection, but exerts a minimal impact on the elimination of HCV 1a infection.

SPECIFIC AIMS:

To test our hypothesis, we have the following two specific aims:

1. To construct and produce pseudoviruses carrying autologous HCV envelope proteins representing genotype 3a and genotype 1a derived from a study subject
2. To evaluate neutralizing activity of the patient's serum samples against the pseudoviruses carrying autologous HCV envelope proteins

2.1.1 A CASE-STUDY REPORT

The plasma/serum samples used in this study are from a participant of the Multicenter AIDS Cohort Study (MACS) in Pittsburgh. The MACS was designed to study the natural history of HIV infection and AIDS development and conducted at four cities, Los Angeles, Baltimore, Chicago and Pittsburgh. In this ongoing prospective cohort study homosexual and bisexual men were recruited to participate in the study. They have their regular checkup and give their biological specimens biannually.

Over the past two years our research group has been involved in the study of HCV transmission and evolution in HIV/HCV coinfecting MACS participants. Our study revealed one HIV/HCV coinfecting individual with unusual disease course, which provides a unique opportunity to study humoral immune response against two HCV subtypes under the same immune environment [5]. This patient has been infected with HIV since 1987 and went under antiretroviral therapy in 1996. In 1997 at his regular biannual visit 27, HCV was first detected in his plasma with very high HCV load/undetectable HIV load/low CD4 count (Table 1). Comprehensive HCV screening and sequence analysis with his plasma samples reveal that this patient harbored HCV 3a at visit 27. However, starting from visit 30 (one and half years from visit 27), HCV 3a was undetectable and instead high levels of HCV 1a was detected in this patient's plasma sample. Furthermore, HCV genotype 1a was consistently detected in his plasma samples in his subsequent visits (Table 1, Figure 4 and 5). This data suggest that HCV 3a was spontaneously cleared within 1.5 years post infection from this individual, but he was subsequently infected again with HCV 1a, which established chronic infection. It is unclear which host immunity factors contributed to HCV 3a clearance and why HCV 1a established chronic infection in the same host with the same immune environment.

Table 1. Patient's detailed records with laboratory data.

Visit - N	CD4 count	HIV (cp/ml)	HCV (cp/ml)	HCV genotype
27	171	Undetected	33,021,250	genotype 3a
30	272	Undetected	603,250	genotype 1a
31	491	Undetected	2,165,750	genotype 1a
32	333	Undetected	522,500	genotype 1a
33	264	Undetected	640,250	genotype 1a
40	352	Undetected	1,378,000	genotype 1a
43	321	Undetected	772,000	genotype 1a



Figure 4 Neighbor-joining tree of HCV E1/E2.

The phylogenetic tree was constructed from HCV E1/E2 sequences amplified from the plasma samples of visit 27, visit 30 and visit 32 of the study subject. The bottom empty squares representing the sequences from visit 27 were clustered separately from other sequences of three other visits. The circles shown in red, green and black on the top are representing the sequences from 3 different visits 30, 31, and 32, which were clustered into a separate clade.

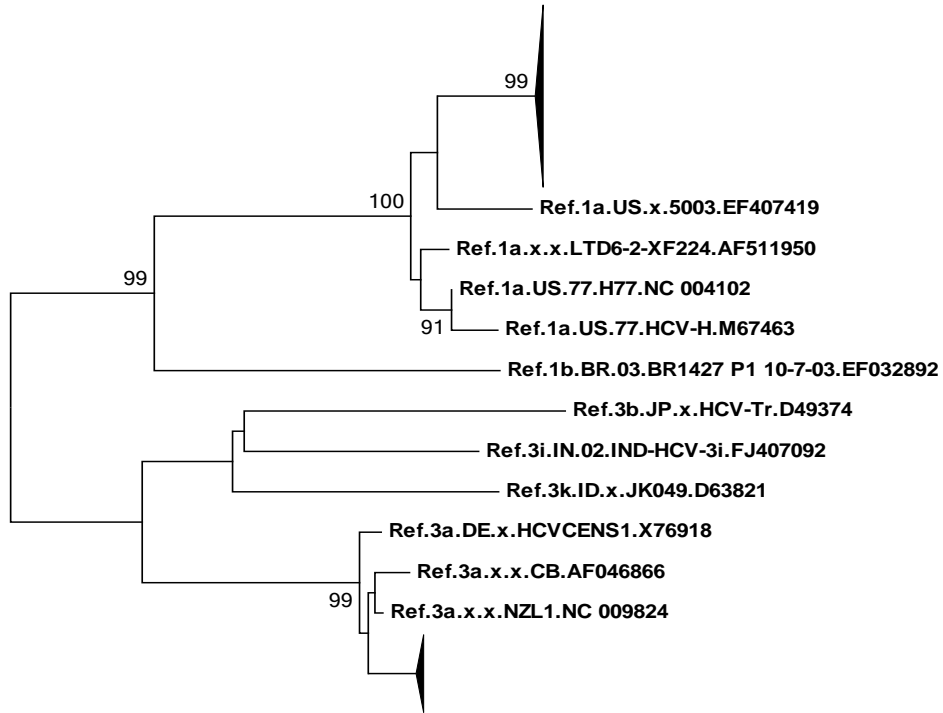


Figure 5 Phylogenetic tree of HCV E1/E2 sequences.

The E1/E2 sequences of HCV genotypes 3a and 1a retrieved from HCV sequence database were aligned against this subject's HCV E1/E2 sequences from visit 27 and visit 30, 31, and 32. Two clusters are formed: one with HCV genotype 3a at the bottom shown in black arrow and one with genotype 1a on top with black arrow.

3. MATERIALS AND METHODS

3.1. STUDY SUBJECT AND PLASMA/SERUM SAMPLES

Our previous study reveals one HIV/HCV co-infected Pittsburgh MACS participant with unique HCV infection course. In this study, we further investigated the immune response against HCV in this particular patient. The cryopreserved plasma/serum samples collected from this subject at his visit 27, visit 30 and visit 32 were used to amplify HCV envelope genes and perform the neutralization experiments.

3.2 CELLS

HCV permissive Huh-7.5.1 cells derived from human hepatocellular carcinoma were maintained in Dulbecco's Modified Eagle Medium with 10% heat inactivated fetal bovine serum (FBS), 0.1mM non-essential amino acids and penicillin/streptomycin at 37 °C and 5% CO₂.

Human epithelial kidney (HEK) 293T cells were propagated in Dulbecco's Modified Eagle Medium with 10% FBS and penicillin/streptomycin at 37 °C and 5% CO₂.

3.3. RNA EXTRACTION AND REVERSE TRANSCRIPTION

Total nucleic acids were extracted from the cryopreserved plasma samples using the automatic Nuclisense EasyMag nucleic Acid extraction machine (bioMerieux, Durham, NC). Extracted nucleic acids (25ul) were used for Reverse Transcription with 0.25µM HCV sequence-specific RT primer 5' GGGCAGDBCARRGTGTTGTTGCC. Reverse transcription reaction was

performed using SuperScript® III Reverse Transcriptase according to manufacturer's instruction (Applied Biosystems).

Table 2 HCV Core/E1/E2 primer sequences.

PRIMERS	SEQUENCES
HCV 3a Forward Primer	ATGAGCACACTTCCTAAACCTCAAAG
HCV 3a Reverse Primer	CGCTTCTGCTTGTGTGATATCATCAGC
HCV 1a Forward Primer	ATGAGCACGAATCCTAAACCTAAAG
HCV 1a Reverse Primer	CGCTCCGCTTGGGATATGA

3.4. PCR AMPLIFICATION OF HCV ENVELOPE GENE AND CLONING AND SEQUENCING THE PCR PRODUCT

The synthesized cDNA was used for PCR amplification of HCV envelope gene. To obtain a complete gene expression cassette, the translation start codon ATG was incorporated into the 5' primer and stop codons were included into 3' primer (Table 2).

The intact ORF of HCV core-E1-E2 was amplified from the cDNA with the designed primer pairs (Table 2), which generated 2.2kb PCR product. The PCR was performed by using high fidelity pfx DNA polymerase (Life Technologies) with the following thermo-cycling conditions: initially 95C for 10 minutes, then 35 cycles of denaturation 95C for 40 seconds, annealing 55C for 40 seconds, extension 68C for 2.5min with final extension 68C for 5minutes.

The PCR product was run in a 0.8% agarose gel and visualized under UV light. The 2.2kb DNA band in the agarose gel was excised and extracted using Ultra-Clean™ DNA purification kit

(name of the company) according to manufacturer's instructions. The extracted DNA was cloned into Topo blunt-end vector following manufacturer's protocols (Zero Blunt TOPO PCR cloning Kit, Life Technologies). Briefly, the TOPO vector and PCR product ligation mixture was incubated in room temperature for 20 minutes followed by transformation of the mixture into competent E. Coli DH5 α TM. The transformed cells were plated onto agar plate with Kanamycin, which was incubated overnight at 37C. The colonies grown on the agar plate were picked up and grew in LB broth with kanamycin overnight shaking in a warm room. Plasmids were purified with QIAprep Spin Miniprep Kit (Qiagen). The purified plasmid DNA was sent to University of Pittsburgh Genomics and Proteomics Core Laboratories for sequencing with commercial available M13 Forward and Reverse primers. The sequence results were analyzed with Vector-NTI program to check for the intactness of Core/E1/E2 ORF. There are no frame shift mutations or stop codons in the ORF of the envelope gene generated from all three visits' plasma

3.5. CLONING OF CORE/E1/E2 ORF INTO PROTEIN EXPRESSION VECTOR

The Topo vector plasmid with the 2.2kb HCV core-E1-E2 gene were subjected to enzyme digestion by Not I and Spe I, for which, there are unique cutting sites flanking the 2.2kb insert in the TOPO vector. The expression vector VR1012 (a gift from Dr. Bindong Liu, Meharry Medical College in Nashville) was digested with Xba I and Not I. All the digestion reactions were carried out for 1hour in 37⁰ C. Digested products was run in a 0.8% agarose gel, the 2.2kb DNA band from TOPO vector with insert and 4.9kb DNA band for the VR1012 were excised from the gel and the DNA was extracted with a Ultra-CleanTM DNA purification kit. The extracted 2.2kb and 4.9kb DNA was ligated to create the VR1012 with the 2.2kb insert with Fast-link DNA ligation kit (Illumina corporation) according to manufacturer's protocol. Then ligation mixture was

transformed into E.Coli DH5 α TM competent cells and plated on agar plate with kanamycin and incubated in 37⁰ C overnight. The colonies grown on the plate were picked up for colony PCR with the primers specific for Core/E1/E2 2.2kb insert. The 2,2kb positive colonies were cultured in LB broth followed by the plasmid extraction using QIAprep Spin Miniprep Kit. The purified plasmid DNA was digested with Not I and visualized after electrophoreses in a 0.8% agarose gel to check the size of the plasmid after ligation (Figure 6).

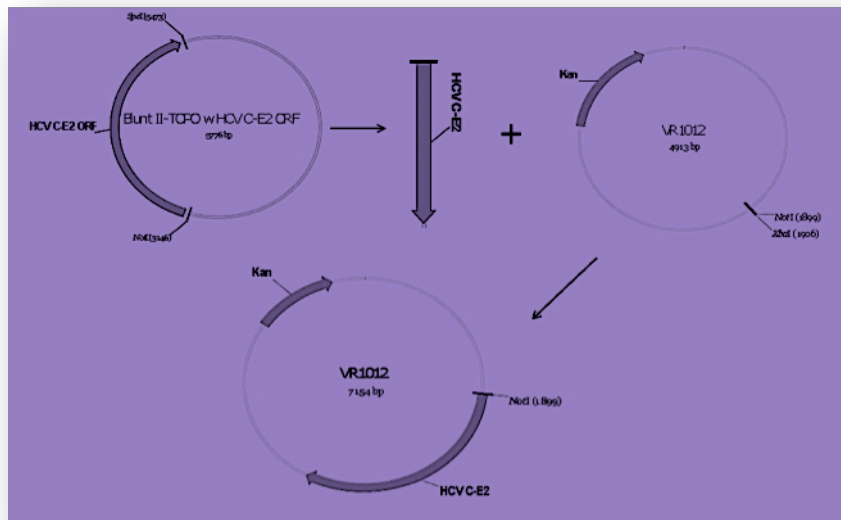


Figure 6 Schematic diagram for plasmid construction.

3.6. TRANSFECTION AND MEASUREMENT OF HCV PROTEIN EXPRESSION

HEK 293T Cells were transfected with the cloned VR1012 expression vectors carrying HCV core/E1/E2 gene in the presence of Lipofectamin-2000. The cells were seeded in the 6-well plates with 10⁶ cells/per well one day prior to transfection in DMEM supplemented with 10% FBS

without antibiotics. Equal amount (2.4ug) of plasmid DNA was used for all constructed plasmids to achieve the equal expression levels of HCV envelope proteins. In addition, the plasmid containing GFP gene was used as a control for monitoring the transfection efficiency. The plasmid transfections using Lipofectamin 2000 were performed following the protocol provided by the manufacturer (Life Technologies). After 6 hours' incubation in an incubator, the media was changed with fresh 10% DMEM/FBS containing pen/strep and the transfected cells were incubated in 37 °C and 5% CO₂ for additional 72 hours. Then the transfected cells were lysed and measured for HCV protein expression by Western blotting.

The cell lysate was prepared by lysing the cells with 1x SDS lysing buffer and 2x loading buffer. The samples were boiled for 5 minutes and run in a 12% pre-cast gels (Mini-PROTEAN® TX- Precast Gels, Biorad), followed by transferring the proteins onto a nitrocellulose membrane. The membranes were blocked in the blocking buffer (phosphate-buffer saline (PBS) containing 0.1% Tween-20 and 5% non-fat dried milk) for one hour in room temperature. Then the membranes were incubated overnight with rocking at 4C with C7-50, the mouse anti HCV core antigen monoclonal antibody (Pierce Thermo Scientific) in 1:1000 dilution with PBS containing 0.1% tween-20 and 1% non-fat dried milk. After washing with PBS and 0.1% tween-20, the membranes were incubated with secondary antibody, goat anti-mouse IgG (Pierce Thermo Scientific) with 1:15000 dilution for one hour followed by washing. The protein bands were detected with SuperSignal chemiluminescent reagent (Pierce, Rockford, IL) and visualized by exposing to X-ray film. The amount of α -tubulin was also visualized in the same membrane and used as a loading control.

3.7. HCVpp PRODUCTION AND INFECTIVITY ASSAY

The expression plasmid VR1012 carrying HCV Core/E1/E2 gene together with the plasmids in second generation lentiviral packaging system including dr8.2 CMV encoding HIV gag/pol proteins and pTrip-luc was transfected into 293T cells using Lipofectamin-2000 (Figure 4). The plasmid expressing GFP was used as a transfection control and VR1012 only as a negative control for HCVpp infectivity assays.

The 293T cells were seeded into the 6-well plate with 1×10^6 cells /per well one day before transfection in DMEM supplemented with 10% FBS without antibiotics. The plasmids were transfected into 293T cells in the presence of Lipofectamin-2000. After 6 hours' incubation, medium was changed with 10% DMEM/FBS and pen/strep media and the cells were incubated in 37°C and 5% CO_2 . Culture supernatant was collected at 24hours, 48hours and final 72hours post transfection for HIV p24 antigen measurement. Also, at 48hours and 72hours culture supernatant was collected, filtered through a $0.45\text{-}\mu\text{m}$ membrane and stored at -80C for HCVpp infectivity assay.

To measure the HCVpp infectivity, Huh-7.5.1 cells (susceptible for HCV infection in vitro) were plated at 2×10^3 cells per well in a 96 well plate 24h before infection in DMEM supplemented with 10% FBS, 1% nonessential amino acids and antibiotics. The culture supernatant containing HCVpp was added to the Huh7.5.1 cells with $4\mu\text{g/ml}$ polybrene and incubated for 48h in 37°C . Then, the medium was removed and the cells were lysed with 1x cell lysis buffer (Promega, Madison, WI). After addition of luciferase substrate into the cell lysate, the firefly luciferase activity was measured in a luminometer (Veritas, Microplate Luminometer).

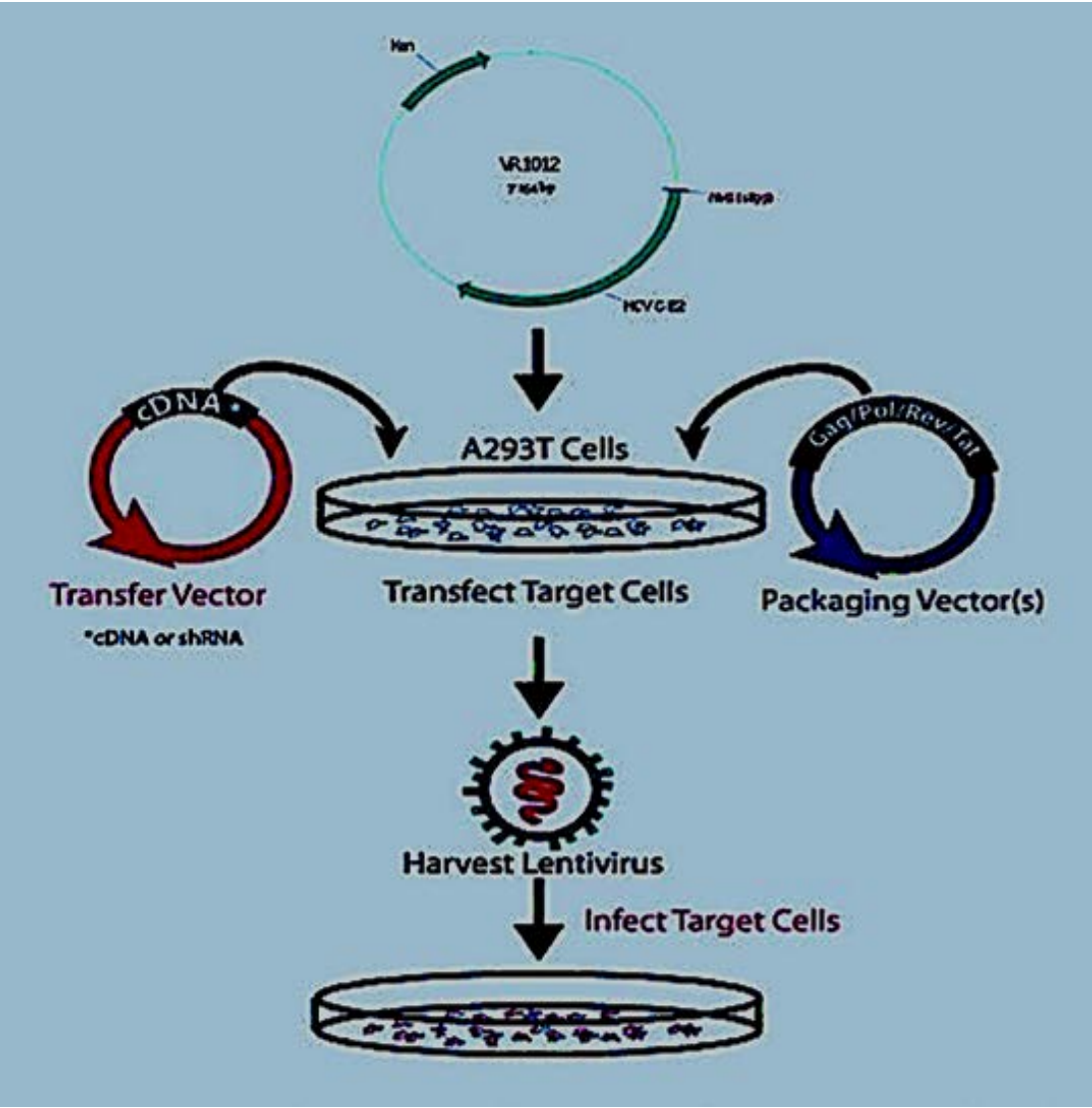


Figure 7 Schematic picture for HCV pseudovirus production

3.8. NEUTRALIZATION ASSAY WITH HCV_{pp} BEARING AUTOLOGOUS HCV ENVELOPE PROTEINS AND PATIENT'S SERA

Serum neutralization activity was assessed by the luciferase activity in the Huh7.5.1 cells infected by HCV_{pp} with or without pre-incubation with autologous serum. Huh-7.5.1 cells were seeded into a 96 well plate with 2×10^3 cells per well 24h prior to the infection. The serial 10 fold diluted heat-inactivated serum starting from 1:10 dilution was incubated with equal amount of HCV_{pp} supernatant at 37C for 1 hr. After incubation the virus and serum mixture were added to the Huh-7.5.1 cells and incubated for 48h in 37C. Then the medium was removed and the cells were lysed with the cell lysing buffer (Promega, Madison, WI). After addition of luciferase substrate into the cell lysate, the firefly luciferase activity was measured in a luminometer. All the samples were run in duplicates or triplicates and the average of each sample's RLU was calculated.

5.0 RESULTS

Specific AIM-1: Construct and produce pseudoviruses carrying autologous HCV envelope proteins representing genotype 3a and genotype 1a

5.1 CLONING OF HCV CORE/E1/E2 GENE INTO EXPRESSION VECTOR

The schematic diagram of constructing the expression plasmids carrying HCV envelope gene is shown in Figure 6. Expression vector VR1012 (a gift from Dr. Bindong Liu, Meharry Medical College in Nashville) was used in the construction. Plasma RNA was isolated from the patient's plasma samples collected at visit 27, visit 30 and visit 31 and was subjected to PCR to amplify HCV core/E1/E2 region with primers containing ATG and TAG at the respective ends. The 2.2kb PCR product containing HCV core/E1/E2 gene (Figure 8) was cloned into TOPO vector (Zero-blunt, Topo, Life Technologies) and sequenced with commercially available M13 forward/reverse primers. The sequence analysis showed there were no frameshift mutations or stop codons within the cloned Core/E1/E2 ORF in all 3 visits.

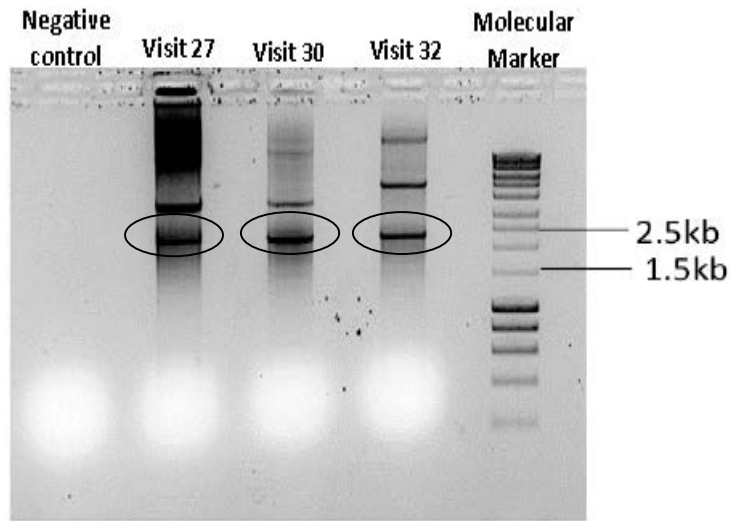


Figure 8 The PCR products of 2.2kb of HCV Core/E1/E2

The Topo vector carrying the core/E1/E2 gene was digested with restriction enzymes Not I and Spe I (Figure 6). The protein expression vector VR1012 was digested with Not I and XbaI. Then the digested products of 2.2kb core/E1/E2 gene and 4.9kb linearized vector VR1012 (Figure 9) were ligated to form the expression vector carrying HCV core/E1/E2 ORF (Figure 6). Then, the ligation mixtures were transformed into competent cells and the colony PCR was performed in the following day with the primers amplifying the 2.2kb insert. A 2.2kb PCR product was confirmed in all 3 plasmids (Figure 10). Subsequently, All 3 purified expression plasmids carrying core/E1/E2 ORF from Visit 27, visit 30 and visit 32 were digested with restriction enzyme Not I to confirm the ligation (7.2 kb) (Figure 11).

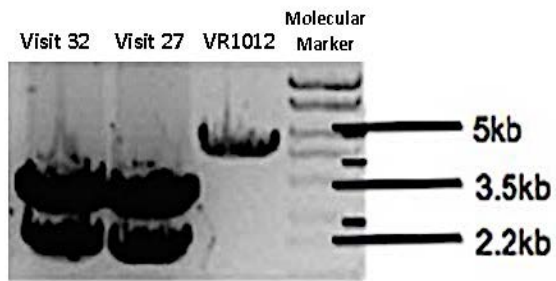


Figure 9 Enzymatic digestion of plasmids 2.2kb and 5kb

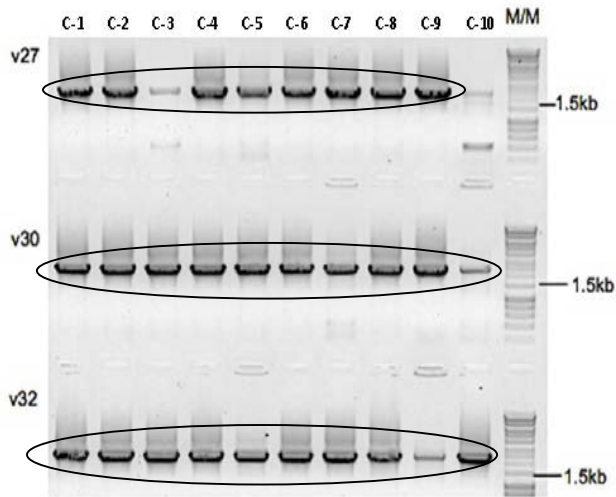


Figure 10 Colony PCR for 2.2kb insert with all 3 ligated plasmids from v27, v30 and v32

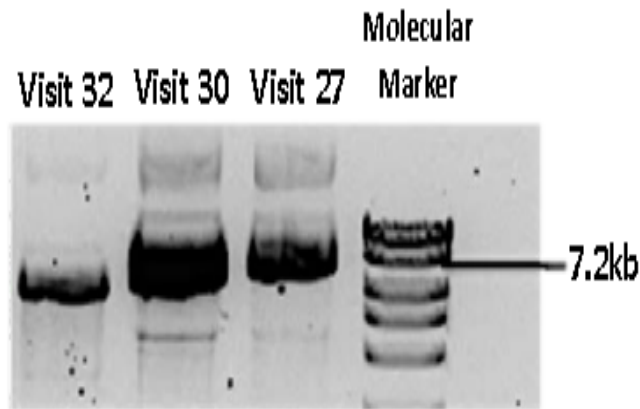


Figure 11 Enzyme digestion of expression plasmid after ligation

5.2 HCV PROTEIN EXPRESSION IN 293T CELLS

Transfection of the constructed expression plasmids bearing HCV Core/E1/E2 into HEK 293T cells was performed to monitor HCV protein expression in the transfected cells. Equal amounts of individual plasmid DNA was used for transfecting 293T cells in the presence of Lipofectamin-2000. GFP plasmid was also included in the experiment as a transfection control to make sure that DNA was getting transfected into 293T cells. At 48h post transfection, 60-70% of the cells were positive for GFP as shown in Figure 12. After 72 hours post transfection, the transfected cells were lysed and Western Blot was performed with the cell lysate with anti-HCV core antibody (Figure 13). Nearly equal amount of α -tubulin was detected in all the samples in the Western Blot (Figure 13). This result confirms that HCV proteins were produced and processed in the transfected cells.

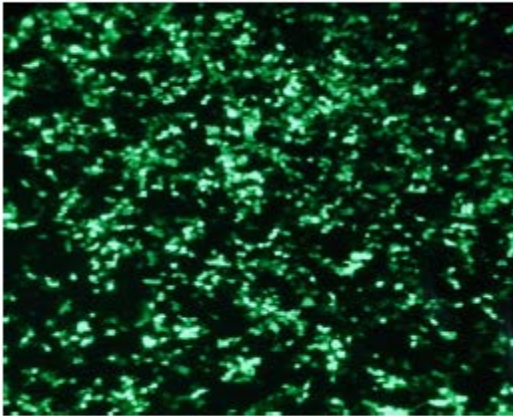


Figure 12 293T transfected cells after 48h with GFP expression in a separate well

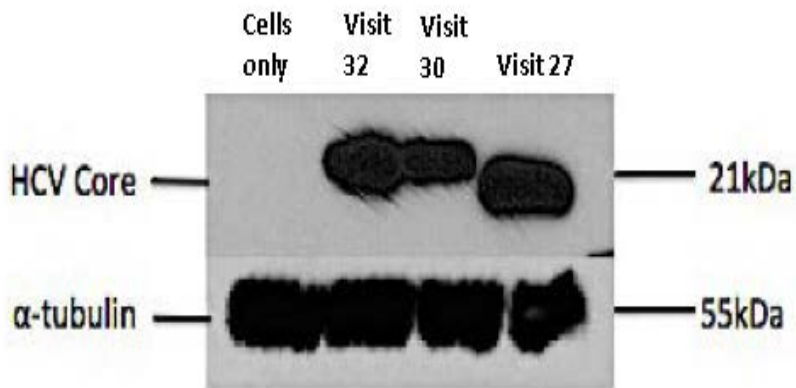


Figure 13 HCV Core (21kDa) protein expression.

Expression plasmids carrying HCV Core/E1/E2 from all 3 visits were transfected into 293T cells and the cells were lysed after 72h incubation. Western Blot was performed with the cell lysate to detect HCV Core (21kDa) . Subsequently, the Western blot membrane was stripped and re-probed with α -tubulin (55kDa).

5.3.HCV PSEUDOPARTICLE PRODUCTION (HCVpp)

To produce HCV pseudoparticles bearing HCV envelope (HCVpp), expression plasmid with Core/E1/E2 genes were cotransfected with lentiviral expression plasmids pCMV Δ R8.2 and pTrip into 293T as described in the Materials and Methods. The plasmid pCMV Δ R8.2 encoding HIV Gag and Pol proteins is responsible for particle budding at the plasma membrane. Whereas the plasmid pTrip contains a reporter protein Luciferase gene and the third plasmid is our constructed expression plasmid carrying patient's HCV envelope gene, which provides envelope proteins for the pseudovirus. The vector control was used in this transfection experiment and it was co-transfected with pCMV Δ R8.2 and pTrip as well. Cell culture supernatant was collected at 24hrs, 48hrs and 72hrs post transfection and measured for HIV p24 antigen production by ELISA.

The mean p24 values from 2 independent experiments are shown in Figure 13. This result indicates that HCVpp was produced, and the production of HCVpp in supernatants was peaked at 72h post transfection. Overall the levels of HIV- p24 production from all 3 plasmids were very compatible (Figure 14).

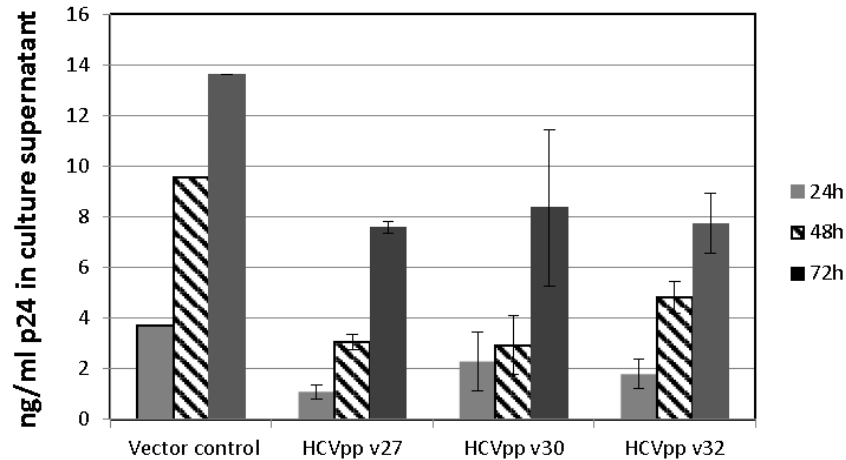


Figure 14 HIV p24 production from lentiviral packaging system.

Average quantifications of p24 from 2 independent transfection experiments are shown. Supernatants were collected at 24h, 48h and 72h post transfection.

5.2 SPECIFIC AIM 2 RESULTS

Specific AIM-2: To evaluate neutralizing activity of the patient’s serum samples with the produced HCV pseudoparticles carrying autologous envelope proteins

5.2.1 Evaluation of HCVpp Infectivity in Huh7.5.1 cells

To determine the infectivity of the produced pseudoparticles carrying autologous HCV Core/E1/E2 proteins, the culture supernatants containing HCVpp were tested for infectivity in HCV susceptible cells. The culture supernatant containing HCVpp was incubated with Huh-7.5.1 cells in the presence of polybrene for 48hrs followed by measurement of luciferase activity in the cell lysate. The results are shown in Figure 15.

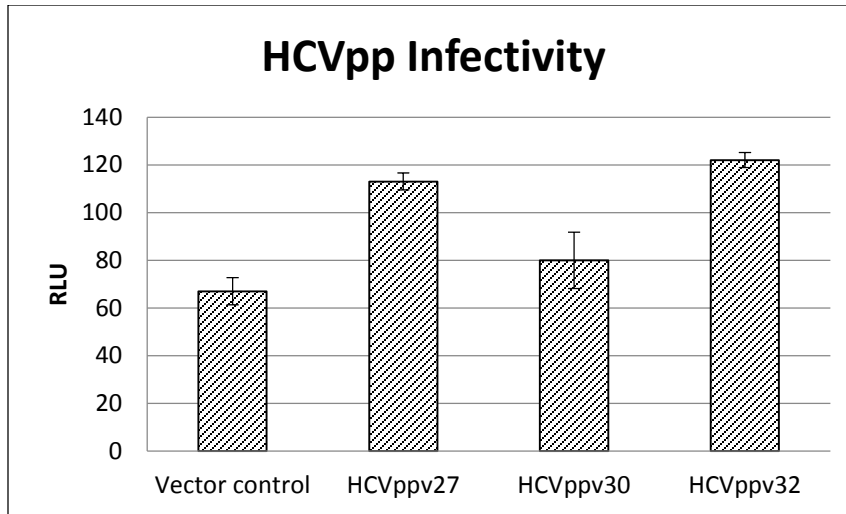


Figure 15 HCVpp infectivity on Huh-7.5.1 cells.

50ul of culture supernatant containing HCVpp from 3 different visits were individually added to Huh-7.5.1 cells with 80% confluency in the presence of polybrene. The Relative Light Unit (RLU) was measured in cell lysate 48hrs post transfection. The mean quantities of RLU from of 6 independent experiments are shown.

5.2.2 Evaluation of neutralizing activity of the patient’s serum against the produced HCVpp with autologous HCV envelopes.

In order to evaluate the neutralizing activity in patient’ serum, HCVpp from each visit was used to infect Huh 7.5.1 cells with or without pre-incubation with three corresponding visit serum samples individually as shown in Figure 16. Relative Light Unit (RLU) was measured for calculating the neutralization activity of the serum against the HCVpp,

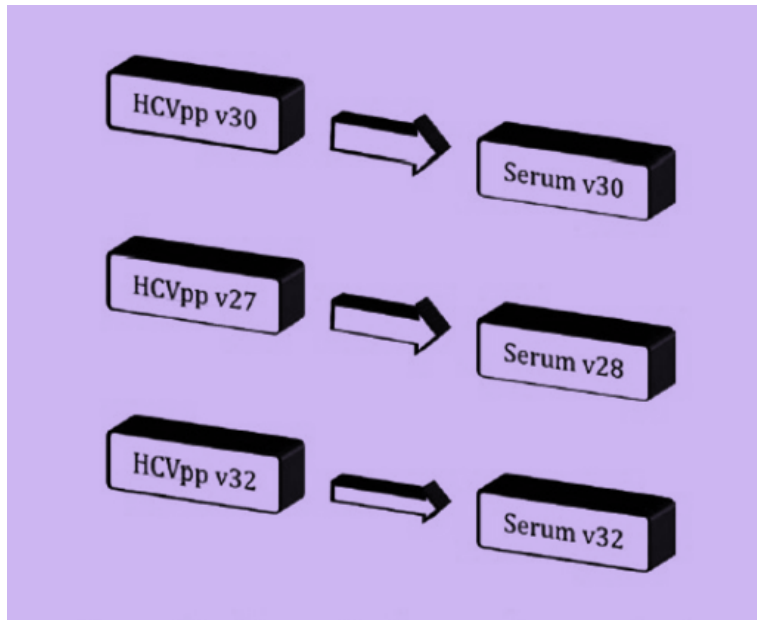


Figure 16 Schematic diagram of Neutralization experiment

The same amount of HCVpp was pre-incubated at 37C for 1 hour with serial 10 fold dilutions of serum samples. The mixture was added to Huh-7.5.1 cells and incubated for additional 48hrs and then the cells were lysed for luciferase activity measurement. Neutralization activity was calculated by the reduction of RLU. For each serum dilution, the neutralization experiments were run in duplicates.

The results in Figure 17 show that RLU detected in the HCVpp infected cells were not significantly different with or without presence of serum, which indicates that there are no significant neutralizing activity in all the serum against the HCVpp. However, there are some reductions in RLU when HCVpp v27 was pre-incubated with v28 serum. Serum at visit 28 was still HCV 3a and we used that sera to measure neutralization activity against HCVpp from v27 represented genotype 3a (Figure 17).

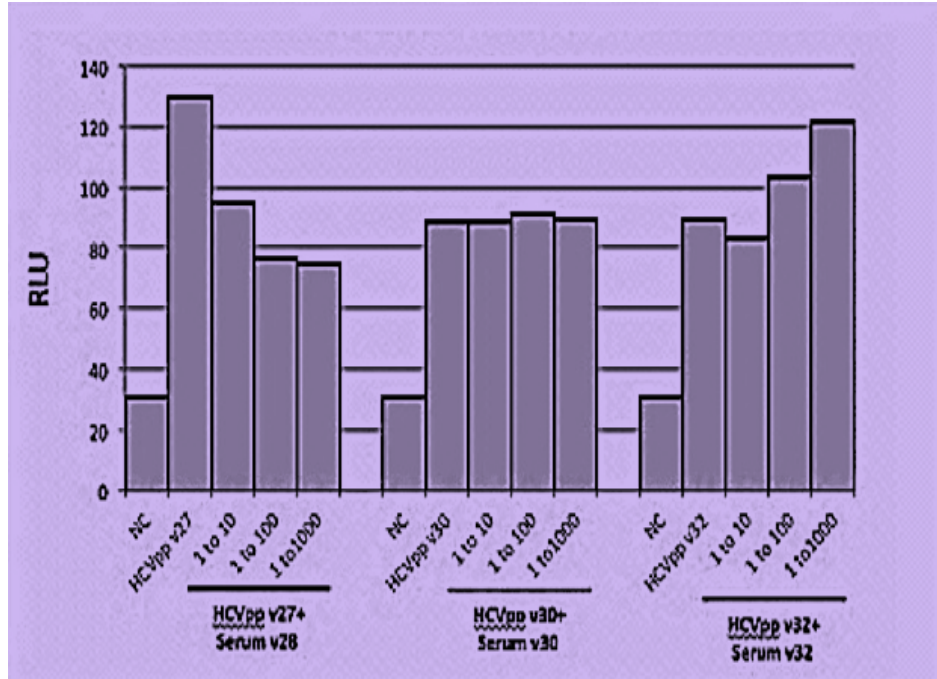


Figure 17 Neutralization of HCVpp with serially diluted serum samples.

Serial 10 fold dilution of sera obtained at visit28, visit30 and visit32 were tested against corresponding HCVpp.

Average reduction in RLU from one experiment in duplicates is shown

6.0 DISCUSSION

During acute HCV infection HCV-specific nAb's develop late after T cell immune response suggesting their role in HCV clearance questionable. There is a big debate among HCV scientists regarding to the role of humoral immune response in HCV infection and clearance. Some authors have shown the importance of nAb's in virus clearance, whereas others provide evidence for minimal role of nAb's in the resolution of HCV infection. According to early chimpanzee studies, mammalian cell-derived recombinant HCV E1 and E2 glycoproteins were able to induce an effective protective immune response. After a high dose of viral challenge, the immunized chimpanzees were able to clear the virus before developing a chronic or acute HCV infection [56]. Other previous studies have also shown the importance of neutralizing antibodies in the protection against HCV infections. When viral inoculum was pre-incubated with human anti-HCV sera and immunoglobulin and injected into chimpanzee models, the monkeys did not develop HCV infection in response to this viral challenge [57, 58]. These findings imply that broadly neutralizing antibodies play an important role in preventing the monkeys from HCV infection.

Some authors reported that nAb's are not required for HCV clearance [59]. According to chimpanzee studies during acute HCV infection the response against HCV hypervariable region in E2 leads to chronic infection, instead of viral clearance . Also, other studies have shown that the broadly neutralizing antibodies were present in the serum of chronically infected patients suggesting that they do not mediate HCV clearance[60-62].

Approximately 25% of HCV infected people spontaneously clear the infection, which provides the opportunity to study how human immune system effectively eliminate the virus. In this study, the HCV 3a were cleared, whereas subsequent HCV 1a infection established a chronic

infection in the same co-HIV/HCV infected subject, which provides us a chance to study the immune response against two HCV genotype infections with different infection outcomes. Comparing the host immune responses against these two genotype viruses may lead to the significant findings of the host factors responsible for the HCV clearance.

The development of *in vitro* study systems for studying HCV life cycle and replication has been a long process with limited progress since the isolation of viral cDNA in 1989 [22] and the availability of first HCV cDNA clone in 1997[63]. Currently, with few exceptions, HCV still could not efficiently infect and replicate in the target cells in *in vitro* condition. Through the work of HCV protein's structural biology the full-length genomic HCV replicons were developed, but they were unable to infect susceptible cell lines[64]. In 2005, the isolation of HCV RNA from a patient with Japanese fulminant hepatitis genotype 2a enabled the researchers to construct an infectious molecular clone of HCV (JFH-1) for studying HCV replication cycle in susceptible cells[23]. Followed by this advances, the number of other infection systems have been advanced too[11]. However, for some unclear reasons, these *in vitro* produced HCV have lower infectivity compared to JFH-1 and quickly develop cell culture-adaptive mutations [65].

Another breakthrough in HCV study models was the development of HCV pseudoparticles (HCVpp)[4]. This system works by incorporating unmodified HCV glycoproteins E1 and E2 onto retroviral particles, which bypasses the hurdle of growing HCV in the lab [66] and enables scientists to study HCV entry and antibody neutralization activity *in vitro*. With the HCVpp system, reproducible results have been acquired in studying the neutralizing antibody responses in HCV infections [4], suggesting that HCVpp system is suitable for studying immune responses against HCV. Up to now, in most of the studies, the HCVpp was produced by incorporating E1 and E2 of H77 or other available HCV into retroviral particles, which is not optimal for measuring

anti HCV activity in patient serum since each individual patient harbors its unique HCV E1 and E2 sequence.

In this study we investigated the role of neutralizing antibodies in HIV/HCV co-infected individual where HCV genotype 3a was naturally resolved, even though it has been proved that HIV infected people have less chance to clear HCV infection [3]. However, the subsequent infection with HCV genotype 1a led to chronicity. It is more likely that in this particular HIV/HCV co-infected individual, the host immunity handles differently the two sequential HCV infections with genotypes 1a and 3a. To accurately measure the antibody responses against these two sequential HCV infections, we constructed and produced the HCVpp by incorporating autologous HCV E1 and E2 of 3a and 1a detected in this patient's plasma instead of using the commercial available H77 envelope genes. Then we measured the neutralization activity of patient's serum with the produced autologous HCVpp.

We produced autologous HCVpp representing HCV genotypes 3a and 1a extracted from patient's plasma as shown in schematic diagram in Figure 7. The infectivity of the produced HCVpp was observed in Huh-7.5.1 cells suggesting that the HCVpp enters the cells similar to the entry step of the HCV life cycle and could be used for neutralization experiments (Figure 15). Following the confirmation of infectivity on susceptible cell lines we proceeded to measure the neutralizing activity of this patient's serum against the autologous HCVpp represented HCV genotype 3a and 1a. Our results showed that there is some, but non-significant neutralizing activity detected in patient's serum against HCVpp genotype 3a, whereas for HCVpp genotype 1a, no neutralizing activity was detected in patient's serum (Figure 17). This result is consistent with previous reports that for some unknown mechanism, there is a higher spontaneous clearance rate of HCV 3a infection than that of 1a [67].

In conclusion, the neutralizing activity against autologous HCV in patient's serum could be studied more accurately by constructing and producing the HCVpp carrying autologous HCV E1 and E2 proteins. Using this assay, we were able to demonstrate that in this HIV/HCV co-infected person's serum low level of neutralizing activity against HCVpp genotype 3a was detected, but no neutralizing activity was detected against HCV genotype 1a. By using this experimental approach we could expand our knowledge in the future in obtaining new information on the interactions between HCV and neutralizing antibodies.

7.0 LIMITATIONS AND FUTURE DIRECTIONS

Despite the fact that HCVpp were successfully produced with patient's autologous HCV envelope proteins of genotype 1a and 3a, their infectivity on Huh-7.5.1 cells was not high although multiple transfections followed by infection had been performed. However, other researchers also reported similar low infectivity of HCVpp. It has been speculated that the 293T cells used for producing HCVpp lack lipid metabolism as a result HCVpp do not have association with any host lipoproteins like in serum-derived HCV virions [12, 68], which leads to the low infectivity. If the HCVpp infectivity remains low in the future study, the primary human liver cells will be used to increase the infectivity for accurate measurement of neutralizing ability of sera [24]. In the future studies, the patient's serum samples from different visits will also be tested for neutralizing activity against all constructed HCVpp to evaluate the neutralization activity in the serum with a broader range.

8.0 PUBLIC HEALTH SIGNIFICANCE

Currently there are no vaccines available against HCV infection and it should be prioritized to explore the vaccine development approaches by understanding the mechanisms of the natural HCV clearance. In 2010 Food and Drug Administration approved the use of direct acting antiviral agents against HCV proteases, and in 2014 new effective drugs became available against HCV infection [69]. However, due to its high cost (\$84-130.000 per course of treatment) it will be a high economic burden and will be impossible to provide all infected patients with the new therapy. Therefore, the development of an effective and low cost vaccine will be the ideal method to prevent HCV infection. In this current study, the role of neutralizing antibodies during natural resolution of HCV infection is studied. The results reported in this study showed the minimal neutralizing activity in the patient's serum with the HCV clearance, which needs to be further explored in order to fully understand its mechanisms.

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