Université de Montréal

Correlates of Long-term Immune Protection in Hepatitis C Virus (HCV) Exposed Non-reinfected Individuals

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Résumé

La majorité des personnes infectées par le virus de l'hépatite C (VHC) développent une virémie persistante. Malheureusement, notre connaissance des paramètres de l'immunité protectrice est limitée. La génération et la maintenance des cellules T CD4+ et T CD8+ spécifiques au VHC et polyfonctionnelles, caractérisées par la sécrétion de plusieurs cytokines comme l'IFN-γ, le TNF-α et l'IL-2, sont associées à la résolution spontanée du virus. De plus, la présence d'anticorps neutralisants est associée à une charge virale plus faible et à une durée de virémie réduite suivant une réinfection. Dans ce projet, nous avons l'intention de définir les paramètres de l'immunité protectrice contre le VHC chez les personnes s'injectant des drogues (PID) qui ont déjà été infectés par le VHC. Ces personnes sont exposées au virus à répétition, mais sont résistantes à une seconde infection. En outre, nous avons l'intention de comparer l'immunité protectrice chez les personnes ayant résolu leur infection spontanément versus ceux qui ont reçu un traitement antiviral. Nous émettons l'hypothèse que les PID résistantes à la réinfection développent une réponse immunitaire supérieure, caractérisée par une augmentation de la fréquence de cellules T mémoires spécifiques au VHC; du nombre d'épitopes ciblés par ces cellules; du nombre de fonctions effectrices par cellule; ainsi qu'une réponse élevée des anticorps neutralisant. En utilisant les techniques « Enzyme-linked immunospot » (ELISPOT), le marquage intracellulaire des cytokines (*ICS*), ainsi qu'un test de neutralisation de pseudoparticules (HCVpp), nous avons examiné la réponse cellulaire et humorale chez les PID exposées mais non-réinfectées versus les PID réinfectées. Nous avons observé une plus grande fréquence des cellules T spécifiques au VHC chez les PID qui n'ont pas été réinfectées pendant ≥ 2 ans. Nous n'avons pas observé une différence significative pour la neutralisation par les anticorps, ni pour la fonctionnalité de cellules T spécifiques. Nos résultats suggèrent que la protection contre la réinfection chez les PID est corrélée à une haute fréquence de cellules T spécifiques au HCV soutenue dans le temps.

Mots-clés : virus de l'hépatite C (VHC), réinfection, immunité protectrice, vaccins, personnes s'injectant des drogues (PID)

Abstract

The majority of HCV infected individuals develop persistent viremia. Correlates of long-term protective immunity remain undefined. Generation and maintenance of polyfunctional CD4 and CD8 HCV-specific T cells that produce multiple cytokines like IFN-γ, TNF-α and IL-2 correlates with spontaneous resolution of acute primary HCV and can be predictive of long-term protection. The presence of neutralizing antibodies (nAb) was associated with reduced magnitude and length of viremia upon reinfection. The aims of this project were: 1) to define correlates of protective immunity in HCV-resolved high risk people who inject drugs (PWIDs) repeatedly exposed to but resistant to reinfections; and 2) to compare protective immune responses in PWIDs with spontaneous versus treatment-induced clearance. We hypothesized that PWIDs resistant to reinfections harbor an immune response of high magnitude, breadth and quality of the HCV-specific memory T cells as well as higher nAb responses. Using IFN-γ enzyme-linked immunospot (ELISPOT), intracellular staining for cytokines, and pseudoparticle neutralization assays, we examined cellular and humoral immune responses in long-term protected versus reinfected PWIDs. We observed long-lived HCV-specific T cell responses in spontaneously resolved and treatment resolved PWIDs who are protected against reinfection. We did not observe a difference in the nAb responses nor in the functionality of HCV-specific T cells. Our results suggest that protection from reinfection in PWIDs is associated with a sustained high frequency of HCV-specific cellular immune responses.

Keywords: HCV, Reinfection, Protective Immunity, Vaccines, PWIDs

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List of abbreviations

+ss	Positive single stranded RNA
Ab	Antibody
Ago	Argonaute
CLDN1	Claudin-1
CMI	Cell-Mediated Immunity
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte associated antigen-4
DAA	Direct-acting antiviral
DC	Dendritic cell
E1-E2	Envelope glycoprotein 1- envelope glycoprotein 2
EC50	Half maximal effective concentration
eIF2α	Eukaryotic initiation factor 2α
ELISPOT	Enzyme-linked ImmunoSpot
ESCRT	Endosomal-sorting complex required for transport
EVC	Early virological clearance
EVR	Early virological response
HCC	Hepatocellular carcinoma
HCVpp	Hepatitis C virus pseudoparticle
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HVR	Hypervariable region
IFN	Interferon

IL	Interleukin
IRES	Internal ribosome entry site
ISDR	Interferon sensitivity-determining region
ISG	Interferon-stimulated gene
JFH	Japanese fulminant hepatitis 1
Kb	Kilobase
LCMV	Lymphocytic choriomeningitis virus
LDL	Low-density lipoproteins
LDs	Lipid droplets
MAVS	Mitochondrial antiviral signalling protein
mDC	Myeloid dendritic cell
MHC I,II	Major histocompatibility complex class I, class II
miR	MicroRNA
MTP	Microsomal triglyceride transfer protein
nAb	Neutralizing antibody
NI	Nucleotide inhibitor
NK	Natural killer
NNI	Non-nucleotide inhibitor
NR	Non-responder
NS	Non-structural
OCLN	Occludin
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern

PBMC	Peripheral blood mononuclear cells
PD-1	Programmed death 1
pDC	Plasmacytoid dendritic cell
PEG	Polyethylene glycol
PHH	Primary human hepatocyte
PI	Protease inhibitor
PKR	Protein-kinase R
PRR	Pattern recognition receptor
PWID	People who inject drugs
RdRp	RNA-dependent RNA-polymerase
RIG-I	Retinoic-acid-inducible gene I
RLR	RIG-I-like receptor
SCID	Severe combined immunodeficiency
SNP	Single nucleotide polymorphism
SOCS3	Suppressor of cytokine signalling 3
SR	Spontaneous Resolver
SRB1	Scavenger receptor B1
STING	Stimulator of interferon gene
SVR	Sustained virologic response
Tfh	T follicular helper cell
Th1,2	Helper T cell subtype 1,2
Tim-3	T cell immunoglobulin and mucin domain 3
TLR	Toll-like receptor

TNA	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
TRIF	TIR domain-containing adapter inducing IFN
UTRs	Un-translated regions
VLDL	Very-low density lipoproteins

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Chapter 1: Literature Review

1.1. Historical Introduction

The hepatitis C virus (HCV) is the causative agent of approximately 185 million chronic infections worldwide [1]. The first traces of this virus were documented in 1975 when twenty-two patients showed symptoms of transfusion-associated hepatitis without exhibiting serological markers of hepatitis A and B viruses [2], hence adopting the term, non-A, non-B viral hepatitis (NANBH). In the majority of patients, NANBH resulted in persistent liver damage and caused liver cirrhosis in 20% of chronically infected patients [3]. Following an arduous screening process of millions of bacterial cDNA clones derived from experimentally infected chimpanzees, a single HCV clone was discovered by a team of scientists lead by Michael Houghton at the Chiron Corporation in 1989 [3]. This discovery unveiled the 50-80 nm enveloped positive sense single stranded RNA virus of about 10,000 nucleotides [4]. Within the past 25 years, from its discovery to the development of effective therapies, significant breakthroughs have been accomplished, however HCV prevails as a major global problem as an astounding ≈ 3% of humans remain chronically infected [5].

1.2. The virus

1.2.1. Genome

HCV is a non-cytopathic virus infecting only hepatocytes of humans and chimpanzees. The HCV genome is comprised of an uncapped positive single-stranded RNA (+ssRNA) of about 9.6 kb-pairs [4]. The genome consists of an uninterrupted open reading frame (ORF) encoding a 3000 amino acid polyprotein precursor [4]. Following co- and post-translational modifications by cellular and viral proteases, the polyprotein is cleaved into three structural (Core, E1, and E2) and seven non-structural (NS) proteins (P7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Figure 1.1). The ORF is flanked by 5' and 3' un-translated regions (UTRs) that harbour secondary RNA structures important for viral replication. The 5' UTR contains an internal ribosome entry site (IRES) that facilitates the initiation of protein translation [6].

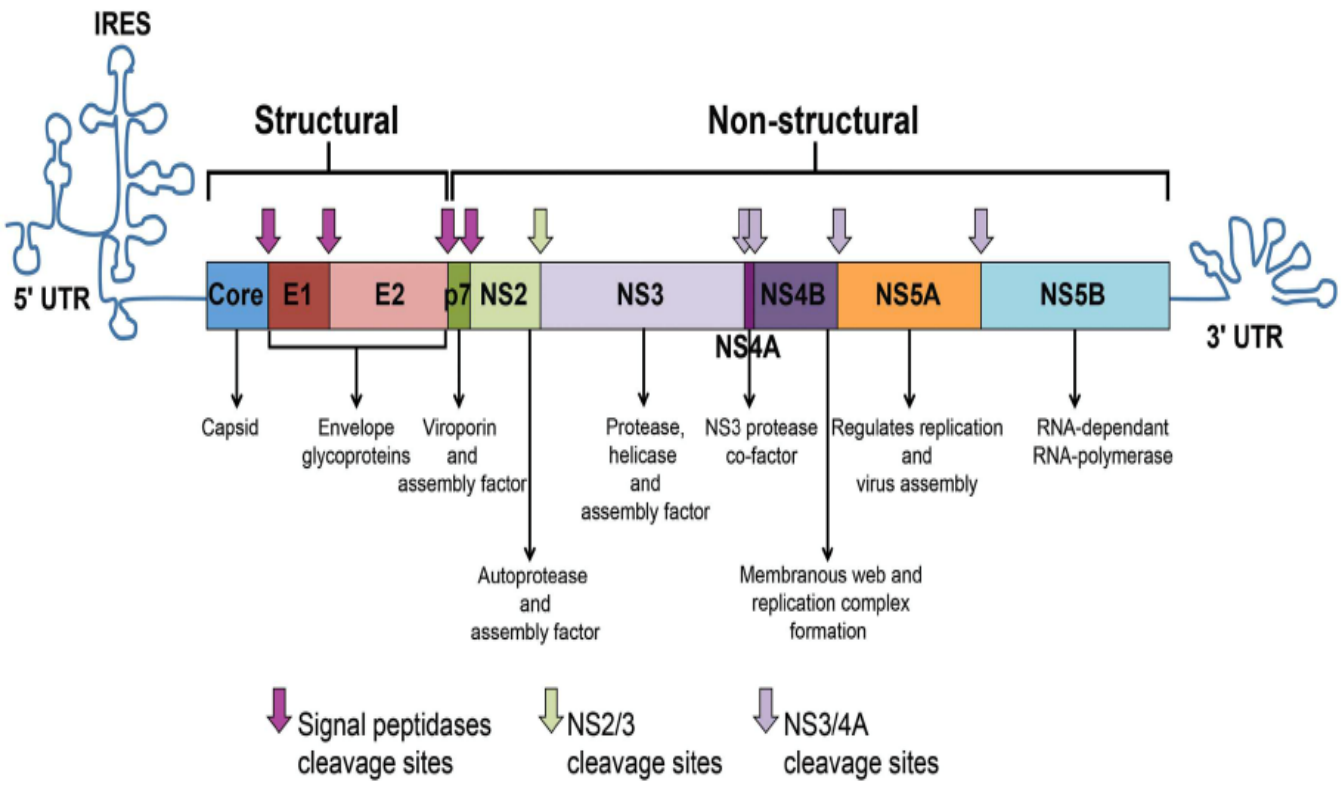

Figure 1.1.: Genomic Organization of the HCV Virus. HCV RNA is translated into a precursor polyprotein molecule that undergoes further post-translational processing into HCV proteins and enzymes. (Adapted from Abdel-Hakeem et al., 2014 [7])

1.2.2. The viral proteins

The core protein is the scaffolding unit of the viral nucleocapsid. The E1 and E2 glycoproteins make contacts with receptors on target cells and mediate entry of the virus. E2 harbours hypervariable regions (HVR) that are the targets of neutralizing antibodies (nAbs). P7, a small hydrophobic polypeptide, acts as a viroporin or ion channel. The NS2 protein bears an autoprotease activity essential for the cleavage of the HCV polyprotein between NS2 and NS3. NS3 is a multifunctional protein, and together with the cofactor NS4A, act as an NS3-NS4A serine protease that catalyzes the processing of the HCV polyprotein. NS3 also possesses RNA helicase/NTPase activity that loosens RNA-RNA substrates and is vital for RNA replication. The functions of NS4B and NS5A are not well defined. However, some evidence suggests that NS4B causes the formation of a membranous web wherein viral replication is allowed to take place [8]. NS5A was suggested to have an important role in enhancing viral replications [9, 10] and was also shown to contain a region important for alpha interferon (IFN-α) therapy susceptibility known as the interferon sensitivity-determining region (ISDR) [11]. NS5B is the viral RNA-dependent RNA-polymerase (RdRp) that allows HCV-RNA replication [6]. The HCV RdRp enzyme has an absence of proofreading ability and is consequently error prone, resulting in the emergence of varying viral populations present in a patient's blood as a mosaic of related sequences, collectively termed "quasispecies" [6]

1.2.3. Classification and Genetic variability

The HCV virus is classified under the Hepacivirus genus within the Flaviviridae family, which also encompasses the yellow fever, dengue and the west Nile virus [4]. HCV shows extensive genetic diversity and is classified into seven genotypes (1-7) based on phylogenetic and sequence analysis of the viral genome [12]. These different genotypes diverge at approximately 30-35% of nucleotide sequences. In addition, each genotype is further subdivided into 67 confirmed subtypes, and strains in the same subtype differ at less than 15% of nucleotide sites [13]. Interestingly, it is well established that genotypes show geographically variance, and certain genotypes such as subtypes 1a, 1b, 2a and 3a are extensively spread across the globe and are endemic in high-income countries [14].

1.2.4. HCV Life Cycle

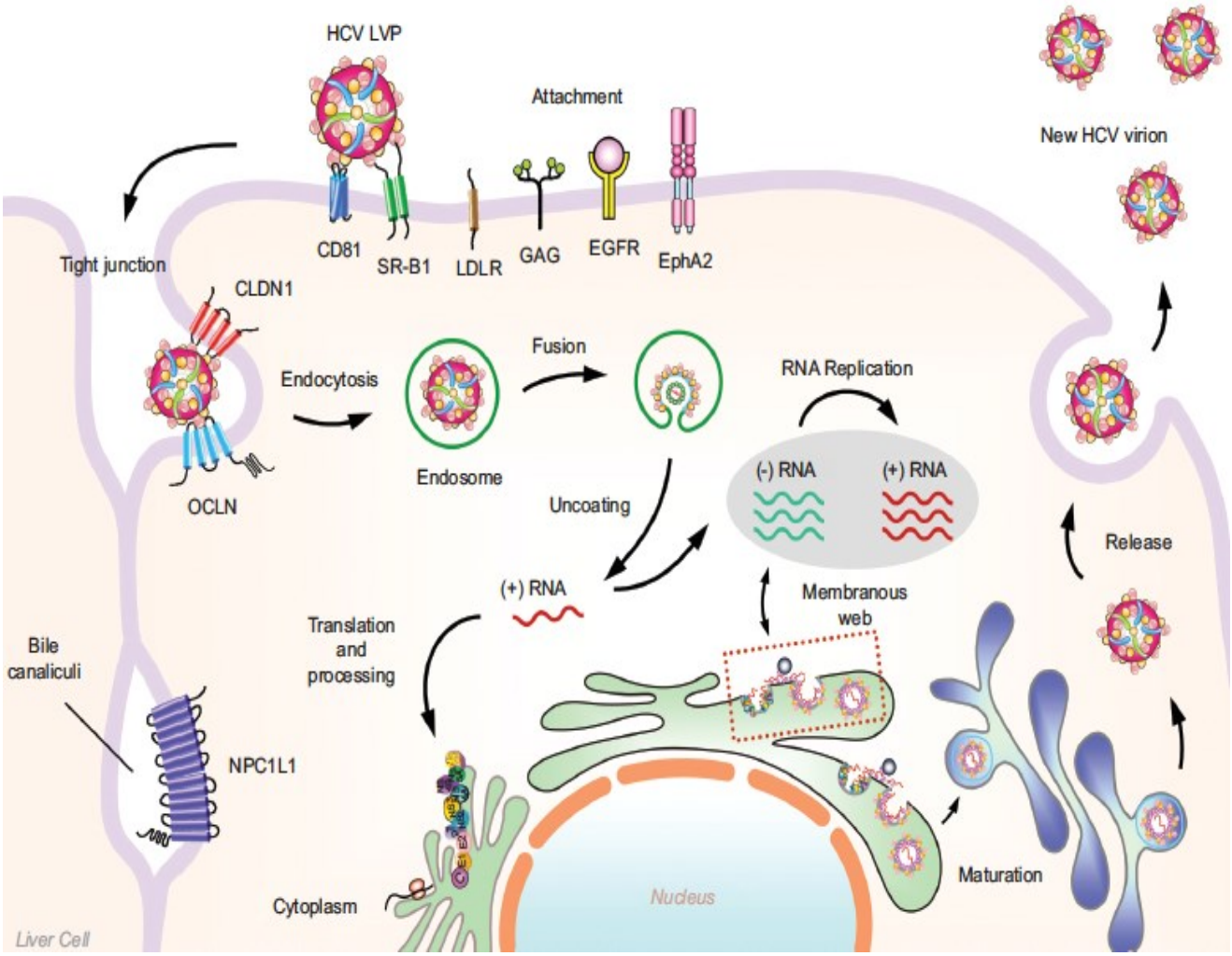

Figure 1.2. Summary of HCV replication cycle. The HCV lipoviral particle (LVP) anchors to SRB1 and CD81 as well as claduin-1 and occluding and enters the cell via receptor-mediated endocytosis. Following internalization and fusion of the viral envelope with endosomes, the viral genome is uncoated and released into the cytoplasm. In the ER, the viral RNA is translated and the HCV polyprotein is produced and cleaved into mature structural and non-structural (NS) proteins. In collaboration with host factors, viral NS proteins form a membranous web wherein viral replication takes place. Viral assembly is suggested to take place in proximity the ER. Lastly, HCV particles are secreted into the extracellular environment through the secretory pathway. **(**Adapted from Wong et al.. 2016 [15])

1.2.4.1. Virus Composition and Viral Entry

HCV virions are 50-80 nm in diameter and contain a single-stranded (ss) RNA genome [16]. The genome is enclosed by a nucleocapsid composed of core protein. The nucleocapsid is then enclosed within a lipid membrane that constitutes the viral envelope. The envelope glycoproteins E1 and E2 are anchored within the lipid-laden viral envelope. The HCV virion is closely associated with lipoproteins and apolipoproteins such as apoE and apoB. The lipid composition of the viral envelope is similar to very-low density lipoproteins (VLDL) and low-density lipoproteins (LDL) and cholesteryl esters make up for approximately half of total HCV lipids [17]. The E1 and E1 envelope glycoproteins play an essential role in receptor attachment and mediate the fusion between the viral envelope and the target cell membrane.

During the onset of an HCV infection, HCV virions are transported by the blood and after crossing the endothelium of liver sinusoids, they establish direct contact with the basolateral surface of hepatocytes in the space of Disse. The first attachment of HCV particles onto hepatocytes is made possible by the heparin sulfate proteoglycan syndecan-1 or syndecan-4 [18, 19] or by the scavenger receptor B1 (SRB1) [20]. The steps immediately following the initial attachment of the virion are partially understood and are thought to involve four important cellular factors. Among these are SRB1, tetraspanin CD81, tight-junction protein claudin-1 (CLDN1) and occludin (OCLN) [21]. It has been suggested that SRB1 may be the first entry factor that interacts with the virion after cell attachment due to its dual interaction with E2 and lipoproteins. An interesting hypothesis the remains to be confirmed is that SRB1 modifies the lipid composition of the virion enabling the unveiling of the CD81 binding site on the E2 glycoprotein [20, 22]. HCV is

endocytosed in a clathrin-dependent process into Rab5a positive early endosomes where fusion takes place. Once released into the cytosol, the HCV genome is translated to produce viral proteins and start viral replication. Interestingly, in the liver of HCV patients, infected cells are found in clusters, therefore suggesting that cell-to-cell spread accounts for the main mechanism of HCV transmission. The exact mechanisms governing this mode of transmission remain to be investigated, but may implicate the role of exosomes [23].

1.2.4.2. Translation and replication

HCV RNA translation begins with the aid of cellular factors [24]. The main outcome of HCV ORF translation is a single large polyprotein that is subsequently cleaved and processed into 10 mature proteins mentioned earlier. The junctions between the structural proteins are cleaved by host signal peptidases from the ER [6]. NS3 performs the cleavage of NS4A from itself and NS4B. NS4A then partners with NS3 resulting in the NS3/NS4A complex ready to cleave at the NS4B/5A and NS5A/NS5B junctions. The NS2/3 autoprotease achieves the cleavage between NS2 and NS3 [25].

Once translation is complete, the HCV proteins are associated with endoplasmic reticulum-derived membranes. Collectively, the NS3/4A, NS4B, NS5A, NS5B proteins comprise the viral proteins of the replication machinery, that is responsible for replicating the positive sense RNA genome from a negative strand intermediate [26]. The NS5B RdRp is the principal enzyme for RNA synthesis and HCV replication is dependent on a liver-specific microRNA (miR) called miR-122 that recruits the Argonaute (Ago) 2 protein to the 5’ end of the viral genome [27]. This attachment of Ago to the viral genome retards the degradation of the HCV genome by the 5’ exonuclease Xrn1 [28].

1.2.4.3. HCV Virion Assembly and Release

The morphogenesis of the HCV virion requires the aggregation of viral structural proteins and genomic RNA that are brought to proximity in a timely and spatially organized condition [29]. It is difficult to detect assembling, budding, or egressing virions in infected cells, implying that these mechanisms are either rare or extremely rapid. An interesting peculiarity of HCV assembly is its close connection to the lipid metabolism pathway. An important component of the viral particle is the core protein, which constitutes the nucleocapsid harbouring the HCV genome. Upon synthesis and cleavage in the ER membrane, the core protein homodimerizes [30] and is then displaced to lipid droplets (LDs) [31, 32]. This interaction between the core protein and the LDs is suggested to be imperative for the recruitment of other key components in HCV assembly [33].

Another crucial component of the HCV virion is the envelope glycoprotein complex whereby E1 and E2 glycoproteins form a non-covalent heterodimer in the ER [34] that needs to migrate close to the LDs where assembly occurs [33]. It has been reported that NS2 interacts with E1, E2 and p7, establishing essential interactions for the migration of the E1E2 heterodimer to the virion assembly site [35-38]. The disulphide bridges between E1 and E2 at the surface of the HCV virion are suggested to play an active role in the budding step of the HCV particle [39].

HCV particle biogenesis is believed to have a close connection the VLDL assembly pathway. It has been reported that blocking microsomal triglyceride transfer protein (MTP), a protein implicated in VLDL biogenesis, inhibits the formation of HCV viral particles [40-42].

The endosomal-sorting complex required for transport (ESCRT) pathway has been suggested to have a key role in HCV budding [43-45]. The ESCRT pathway is involved in the budding and the fission of vesicles out from the cytoplasm, and is exploited by many enveloped viruses for their release from infected cells [46].

Once assembly and budding have been completed in the ER, HCV particles transit through the secretory pathway and are released from infected cells [47]. In this process, the HCV virions attain their particular low buoyant density [40, 48]. Lastly, during egress, it is believed that HCV virions are dependent on p7 for neutralizing the acidic compartments within the secretory pathway [49].

1.2.5. Difficulties in Studying HCV and Experimental Models

Humans and chimpanzees are the only two species that can be infected with HCV. Early studies of HCV immunity used the chimpanzee model to generate important findings as the timing and dynamics of infection can be controlled, and hepatic tissues could be isolated. However, research using chimpanzees is now restricted, forcing scientists to innovate newer models to probe further investigations.

1.2.5.1. *In vitro* Models

1.2.5.1.1. Huh-7 cells and replicons

The HuH-7 cell line, isolated from an HCC tumour of a Japanese man, proved to be instrumental in the early HCV research [50]. This cell line was used to create a replicon that could express and replicate HCV RNA continuously. This development of a stable cell line expressing the HCV RNA genome was indeed a remarkable achievement as it allowed for drug screening and discovery of potent direct-acting antivirals against HCV [51]. Despite this major advancement, this replicon model lacked the ability to be a fully infectious model *in vitro*. It was reported that the increased replication rate of the replicon was conferred by mutations in the HCV genome, which rendered it incapable of producing infectious virions in chimpanzees [52]. Consequently, it was agreed upon that the optimal method to develop an *in vitro* model would focus on wild type HCV genomes. Finally in 2005, the first successful attempt at generating an infectious *in vitro* model of HCV infection was achieved with the discovery of the Japanese fulminant hepatitis 1 (JFH1) genotype 2a clone. This viral strain was obtained from a Japanese patient having developed fulminant hepatitis [53]. Upon transfection of this newly isolated strain into HuH-7.5 cells,

infectious HCV virions were produced and could be used to infect chimpanzees [54]. In 2006, more infectious isolates were developed for HCV genotype 1a [55] as well as for genotypes 1b, 3a and 4a [56, 57].

1.2.5.1.2 HCV pseudoparticles (HCVpp)

Lentiviruses easily forms pseudotypes with the envelope proteins of countless viruses [58]. Accordingly, HCV pseudoparticles (HCVpp) developed from an HIV backbone expressing HCV glycoproteins are infectious for hepatocytes and hepatoma cell lines [58, 59], and facilitated research on HCV entry as well as measuring nAb responses for the first time. The infectivity of HCVpp is pH-dependent and can be neutralized by many E2-specific mAbs.

1.2.5.1.3. Primary human hepatocytes (PHH)

PHHs are considered the gold standard for studying hepatocyte function. These normal cells are isolated from adjacent tumour tissue in patients undergoing liver resection. PHHs can also be obtained from the fetal livers of aborted embryos and can serve as the substrate for infection with HCV [60]. Once plated, PHHs do not divide and have a limited lifespan of approximately 1-2 weeks. Several studies have observed productive infection of PHHs after culture, including cells from HCV-infected patients [61-63].

1.2.5.2. *In vivo* Models

Due to the asymptomatic nature of acute HCV infection, a very small number of patients seek medical consultation in the acute phase. Consequently, it is not possible to determine the exact date of infection or exposure and to figure out the exact infecting strain of virus. The majority of the early knowledge of acute infection is derived from chimpanzee

experiments or from studies following high-risk exposures such as needle-stick accidents in health care workers and blood transfusions, and the rare cases of symptomatic acute HCV. There have also been important efforts in establishing cohorts for the monitoring of high-risk individuals such as people who inject drugs (PWIDs) who represent the main reservoir of HCV in developed countries.

1.2.5.2.1. Mouse models

The generation of a successful mouse model for the study of HCV has been slow and difficult as compared to other hepatic viruses such as HBV. Mouse hepatocytes are a poor model for HCV infection due to the differences in many host factors present in human hepatocytes [64]. To overcome the hurdle of this species-specific phenomenon, humanized mice were generated through the xenotransplantation of PHHs. For this system to be effective, the mouse has to be immunodeficient for it to accept human PHHs and replace the normal mouse hepatocytes. The first successful use of this strategy was attained in the severe combined immunodeficiency (SCID) mice carrying a transgene activated by a liver-specific albumin promoter [65]. Expression of this transgene allows for acute hepatotoxicity, which can then be rescued by the transplantation of injected PHHs, the target for inducing HCV infection [65]. Another model wherein hepatotoxicity can be controlled consists of using *Fah*-knockout mice where the liver stays healthy with the administration of a molecule called NTBC. When this molecule is no longer administered, mice hepatocytes develop toxicity and PHHs can be implanted into the mice liver. Moreover, another strategy to develop humanized mice requires genetically adding human genes to express human entry factors required for HCV infection. With the discovery of HCV entry factors CD81 and occludin, mice were engineered to express human

homologues of these proteins and viral entry was reported for the first time in a proof-of-concept study [66]. Subsequently, HCV infection was successfully reported in a follow-up study [67], however, viral replication was low and the mice failed to develop liver disease, indicating that this model does not fully exemplify an HCV infection. Another group used a similar approach in mice with a different genetic background and were able to achieve sustained viremia with progression to fibrosis and cirrhosis [68], suggesting a more promising model.

Humanized mouse models may constitute a reasonable substitute to chimpanzees and additional efforts are required to replicate the natural history of human immune responses to HCV [69]. These mouse models may also be useful to test vaccine efficacy and more efforts are needed to expand their use in the vaccine community [70].

1. 3. The disease

1.3.1. Epidemiology

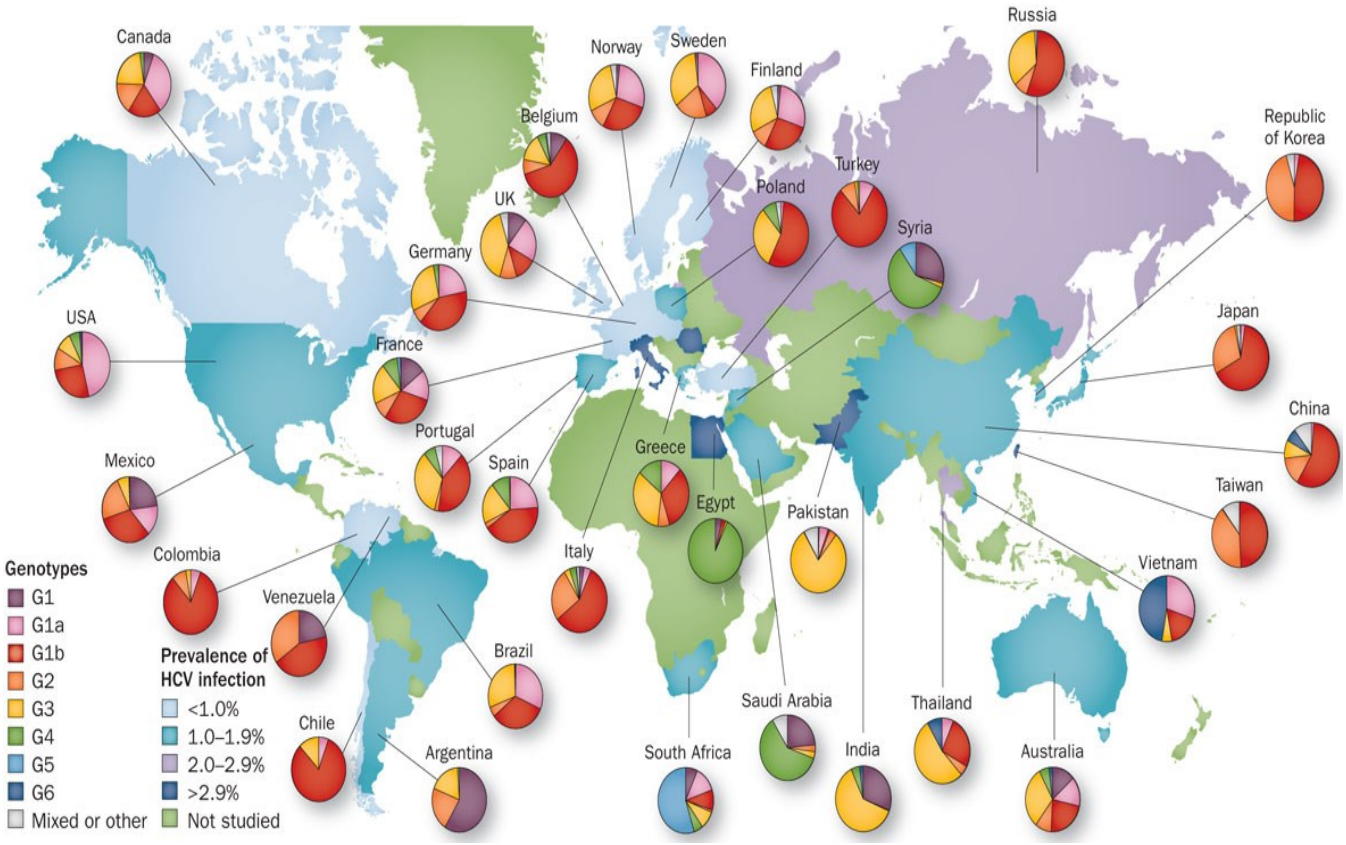

Figure 1. 3: The estimated prevalence of HCV infection and the global distribution of HCV genotypes. The highest prevalence rates of HCV are in Egypt, Pakistan, Greece, and Russia. **(**Adapted from Hajarizadeh et al., 2013[71])

The HCV virus infects approximately 180 million individuals are worldwide, which translates to a 3% prevalence worldwide [1]. HCV distribution across countries can range from <1 % to more than 10% [72, 73]. The highest number of infections is in the developing countries of Africa and the Middle East, whereas higher income countries such as the Americas, Australia, Northern and Western Europe have a lower prevalence [72, 73]. Egypt and Cameroon have the highest prevalence of HCV at > 10% of the population [74, 75]. The countries with the highest absolute numbers of HCV infections are China (29.8 million), India (18.2 million), Egypt (11.8 million), Pakistan (9.4 million) and Indonesia (9.4 million) [72].

1.3.2. Transmission

HCV is most efficiently transmitted by percutaneous exposure to contaminated blood, such as by blood donation, hemodialysis and injection drug use [76, 77]. HCV transmission from mother to child is rare (2% to 10%) [78]. The exact details are not known, however maternal HIV co-infection, ruptured membranes and/or high HCV RNA levels augment the risk of transmission [78, 79]. The predominant mode of transmission in developed countries such as USA, Australia and Europe is through intravenous drug use [80]. In Canada, between 70 and 80% of newly acquired acute HCV infections are attributable to injection drug use [81]. Conversely, in developing countries, the majority of transmissions of HCV occur through iatrogenic exposure. For example, the main source of the HCV surge in Egypt was due to unsafe mass parenteral therapy campaigns against schistosomiasis from 1920s to 1980s [82].

1.3.3. Natural history of HCV infection

An unfortunate feature of HCV infection is its predisposition to establish a chronic infection. Nearly 70% (55-85%) of acute infections progress to a persistent infection [83]. Moreover, HCV has an extremely rapid turnover with a half-life of 3 hours, generating up to 10^{12} virions daily [84], resulting in exponential serum titres by the end of the first week of infection [85, 86]. Furthermore, physical manifestations such as jaundice only result in one third of acute infections, leaving the rest of the patients largely asymptomatic and undiagnosed for years until serious liver disease symptoms become apparent [87]. Due to its asymptomatic nature, the spread of infection and loss of opportunity for early intervention are major consequences.

Approximately 20% of chronically infected HCV patients develop end-stage cirrhosis, liver failure and hepatocellular carcinoma (HCC) [88], comprising a quarter of the worldwide cirrhosis and HCC cases [89]. Accordingly, HCV is the most frequent cause of liver transplantation (40-50%) [90]. As HCV is a non-cytopathic virus, several reports investigated its contribution to the immunopathogenesis of the liver. Proposed mechanisms include direct cytotoxic T lymphocyte (CTL)-mediated killing of hepatocytes [91], and continuous secretion of inflammatory cytokines resulting in tissue impairment [92]. Chronic HCV is also linked to metabolic dysfunction such as insulin resistance, type 2 diabetes, lipid disorder, and steatosis [93]. Suggested mechanisms for metabolic dysfunction consist of the down-regulation of hepatocyte insulin receptor substrate 1 [94] as well as the glucose transporter, and the increased expression of PP2A [95].

1.4. The Immune Response to HCV

1.4.1. Innate Immunity

The innate immune system is the host's first line of defense against viral infections whereby the interferons (IFNs) are produced for mounting an antiviral state in infected cells [96]. Depending on their antiviral properties, IFNs are divided into three classes: type I, type II and type III IFNs [97]. In humans, type I IFNs consist mainly of IFN-α and IFN-β [98]. IFN-α and IFN-β target viruses directly by blocking viral replication or indirectly by generating innate immune responses [97]. IFN-γ is the only member of the type II IFN group and unlike type I IFNs that are induced directly in response to viral infection; IFN-γ is secreted by NK cells as well as mitogenically activated T cells [97]. It has been demonstrated that IFN-γ downregulates claudin-1 and CD81, thereby inhibiting HCV infection [99]. Type III IFNs are comprised of three members including IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). Similar to Type I IFNs, type III IFNs are also directly activated by viral infection and are secreted mainly in the liver during HCV infection [97]. In addition to infected hepatocytes, type I and type III IFNs can also be secreted by Kupffer cells [100], BDCA3+ myeloid cells [101] and as plasmacytoid DCs (pDCs) [102, 103].

The innate immune response begins with the recognition of foreign RNA molecular patterns shared by related pathogens, called pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) including TLRs and intracellular nucleic acid binding proteins [104-106]. Upon HCV entry into hepatocytes, the main PRRs that are activated are TLR3, protein-kinase R (PKR), and retinoic-acid-inducible gene I (RIG-I). Signalling through TLR3 is relayed through the TIR domain-containing adapter

inducing IFN (TRIF), while PKR and RIG-I signalling is relayed via the mitochondrial antiviral signalling protein (MAVS) [107]. These adaptor proteins lead to signalling cascades ultimately inducing the secretion of type I IFNs [107]. The auto- and paracrine binding of type I IFNs to their cognate receptors relays signalling via the JAK-STAT pathway which ultimately leads to the expression of hundreds of ISGs within the infected and neighbouring cells [108]. The expression of ISGs establishes a general antiviral state in the liver whereby HCV RNA replication and cell-to-cell viral spread is restricted [106]. Certain ISGs expressed in response to HCV are proteins with known antiviral effects; however, certain others are known to promote HCV replication in vitro, such as ISG15 and USP18 [109, 110]. The induction of ISGs in the liver is observed early following HCV infection regardless of the outcome of infection, implying that most HCV strains are resistant to antiviral effects of this primitive innate response [111-114]. Type III IFNs also generate ISGs similar to those induced by Type I IFNs in addition to distinct ISGs [115, 116].

1.4.1.1. Natural Killer (NK) Cells

NK cells constitute one of the earliest defenses of the innate immune response. Their main function consists of killing virally infected cells through the secretion of cytotoxic molecules such as granzymes and perforin, or via TNF-related apoptosis-inducing ligand (TRAIL)-mediated killing. In addition, NK cells can also secrete cytokines regulating innate and adaptive immunity such as IFN-γ, TNFα, IL-10 and IL-21. The activation of NK cells is controlled by the calibration of inhibitory and activating signals. The strength of the interactions between inhibitory KIRs expressed on NK cells and their MHC class I ligands expressed on target cells is the main determinant of NK activity [117].

NK cells are ubiquitous within the liver and execute an important role in defending against hepatotropic infections [118, 119]. The early activation of NK cells following an accidental exposure to HCV in healthcare workers was suggested to contribute to protection in 11/12 workers who remained aviremic [120]. In high risk PWIDs, the activation of NK cells and expression of the activating receptor NKp30 was associated with protection from HCV infection [121]. Furthermore, genetic studies revealed that KIR2DL3 expressing NK cells secreted more IFN-γ and that homozygosity for this allele correlated with spontaneous resolution of HCV infection [122]. Another report demonstrated that PWIDs resistant to HCV infection have an enrichment in KIR2DL3+NKG2A- NK cells. NKG2A is an inhibitory receptor and binds to HLA-E which is highly expressed during HCV infection. This suggests that low expression of NKG2A is advantageous in the presence of high HLA-E expression. Overall, KIR2DL3+NKG2A- NK cells are not susceptible to HLA-E-mediated inhibition and may explain the ‘natural resistance’ to HCV in PWIDs [123].

1.4.1.2. Dendritic Cells (DC)

Dendritic cells are the dominant antigen-presenting cells (APCs) in humans. They play a crucial role in bridging innate and adaptive immunity and also impact the priming of HCV-specific immune responses. DCs promptly differentiate into mature DCs following the sensing of danger signals through PAMPs, particularly TLR ligands, interactions with innate lymphocytes (NK and NKT cells), cytokines and other mediators of inflammation [124]. Myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) are the two major subsets of DCs and both play a role in HCV infection. mDCs account for the majority of DCs and are responsible for antigen processing and presentation, whereas pDCs sense viral infections and produce type I and type III IFNs. Additionally, pDCs can sense HCV RNA in a TLR-7 dependent manner when presented by an infected cell [102]. Therefore, DCs are regarded as a principal coordinator of the HCV innate and adaptive immunity.

The functional role of DCs in acute and chronic HCV continues to be controversial. Certain studies have demonstrated that the frequencies of mDCs and pDCs correlate with the outcome of infection whereby decreased frequencies were associated with chronic infection [125-128]. It was also reported that constant hyper-responsive DCs correlate with spontaneous clearance of HCV, implying a superior priming of HCV-specific T cells [129]. Moreover, DCs have been shown to be defective in chronic HCV particularly in response to TLR ligands [130-133] and may cause the proliferation of Tregs [134].

1.4.2. Adaptive immunity

Unlike the innate arm of the immune system which is induced within a short period of time (hours to days) after Hepatitis C infection, the adaptive arm requires 6-8 weeks for its induction, a phenomenon that is still poorly understood. The adaptive immune system comprises of two important components involved in viral clearance, namely T cell responses and humoral antibody responses [135].

1.4.2.1. Humoral Responses

Despite HCV RNA reaching high blood titres by 2 weeks post-infection, anti-HCV antibodies (seroconversion) are largely absent before week 8 [136, 137]. Initial reports showed that antibodies (Abs) directed at the HVR-1 region of the HCV E2 glycoprotein are neutralizing both *in vitro* and *in vivo* [138, 139]. Meanwhile, studies in chimpanzees demonstrated that Ab responses did not inherently correlate with viral clearance [140, 141]. In humans who spontaneously cleared HCV infection, Ab responses were reported to be of delayed onset, bearing low titers and fading rapidly [142-144]. One interesting study described how neutralizing Abs (nAbs) emerged in patients only once HCV had established a chronic infection, consequently failing to eradicate the virus and selecting for escape mutants [145]. Conversely, another study demonstrated that the early appearance of nAbs correlated with spontaneous clearance of a primary HCV infection [146].

A major obstacle in elucidating humoral immunity against HCV remains that lack of optimal tools to measure accurate levels of nAbs. The current approach determines neutralization of HCV pseudoparticles (HCVpp) displaying HCV E1-E2 envelope glycoproteins that correspond to small number of HCV reference sequences, and

unfortunately do not represent all the autologous E1-E2 sequences present in a patient [58, 59]. An HCVpp library consisting of 19 distinct sequences representing the natural variability of E1-E2 glycoprotein of genotype 1 strain was developed to show the evolution of the HVR-1 sequences in response to nAbs [147]. Applying this HCVpp library, a study demonstrated that clearance of HCV infection correlated with robust induction of nAb response mounted early in the infection [148].

There are three main hurdles in inducing a protective humoral response against HCV. Firstly, HCV envelope proteins (E1-E2) are not strongly immunogenic, leading to weak and late Ab response in primary infection [147]. Secondly, due to most Abs targeting the HVR of E2, a mutation-prone region, there is a high tendency for the selection of viral sequences bearing resistance to Ab neutralization [149]. Lastly, high glycosylation and association with host lipoproteins of epitopes targeted by nAbs, shield their visibility and restrict their efficacy *in vivo* [150].

1.4.2.2. Cell-Mediated Immunity (CMI)

The significance of CMI in the clearance of HCV is made evident by the correlation of specific HLA class I and class II alleles and spontaneous clearance and emphasized by depletion studies in the chimpanzee model demonstrating that both CD4+ and CD8+ T cells are necessary for viral clearance [151, 152]. CD8+ T cells mediate target cell killing by displaying viral antigens on the MHC class I molecules whereas CD4+ T cells mediate a helper role in priming and assisting cytotoxic CD8+ T cell (CTL) responses [152].

1.4.2.2.1. CD8+ T cell response in HCV infection

The number of epitopes targeted by CD8+ T cells, known as breadth, is an important factor in the spontaneous resolution of HCV infection. Up to nine unique epitopes were recognized by CD8+ T in the acute resolving HCV in humans as well as in chimpanzees, whereas few epitopes were targeted in chronic progressors [153, 154]. The NS proteins were shown to be immunodominant and correlated with clearance of the virus [155].

The magnitude of HCV-specific CD8+ T cells correlates with spontaneous resolution. Using MHC class I tetramers, it was reported that T cells specific for one epitope can attain up to 8% of total CD8+ T cells in spontaneous resolvers [153, 156]. *Ex vivo* phenotypic characterization of HCV-specific CD8+ T cells was also accomplished using MHC class I tetramers. It was demonstrated that the primitive expression of the IL-7 receptor alpha (CD127) on the surface of HCV-specific CD8+ T cells was a key predictor of viral clearance and the lack thereof was correlated with viral persistence [157, 158]. Meanwhile, during acute infection, PD-1 is variably expressed HCV-specific CD8+ T cells, indicating that it is an activation marker instead of an exhaustion marker [159, 160].

Additional exhaustion markers such as T cell immunoglobulin and mucin domain 3 (Tim-3), cytotoxic T lymphocyte associated antigen-4 (CTLA-4), CD160, KLRG-1 and 2B4 are expressed at varying levels in acute and chronic HCV indicating a range of exhaustion that is associated with chronic infection [161, 162].

The presence of HCV-specific CD8+ T cells in the liver and blood and presence of IFN-γ, CD3, CD4, and CD8 mRNA levels in the liver are kinetically associated to a decrease in viremia [113, 114]. However, they become challenging to detect in the peripheral blood in chronically progressing infections, albeit being easily detectable in the liver, displaying an exhausted and activated phenotype [163, 164]. Furthermore, peripheral blood HCV-specific CD8+ T cells are suggested to be somewhat defective in their proliferative and cytokine producing potential upon initial appearance in the blood [165]. Nevertheless, screening of simultaneous effector functions revealed the existence of a polyfunctional population of HCV-specific CD8+ T cells concurrently secreting IFN-γ, T cell growth factor, IL-2, and expressing the degranulation marker CD107a, a substitute marker of cytotoxicity [156]. Further experiments on sorted cells revealed that polyfunctionality was specific to CD127+ tetramer-positive CD8+ T cells, reiterating the significance of this T cell subset in resolving viremia [156]. Furthermore, it was reported that early administration and subsequent SVR to IFN therapy recovers CD127+ long-lived memory T cells [156, 166].

As HCV infection progresses, noteworthy differences in the CD8+ T cell functions are observed coupled with the loss of helper CD4+ T cell responses. In chronically progressing patients, continuous loss of function, diminished polyfunctionality and decreased proliferative capacity are manifest in CD8+ T cells [156, 167]. CD8+ T cell loss

of function is concomitant with their extent of exhaustion and is reversible upon *in vitro* blockade of inhibitory pathways such as PD-1- CTLA-4, and/or TIM-3 [168]. Nonetheless, there is a limited efficiency upon *in vivo* blockade of PD-1 in chronic humans and chimpanzees, indicating that a threshold of functional virus-specific T cells is necessary for the aforementioned approaches to be successful [169, 170]. This loss of function was suggested to be a result of antigen persistence, as demonstrated in the LCMV model whereby continuous exposure to viral antigens was the key source of diminished frequency and defective effector functions of LCMV-specific CD8+ T cells [171, 172].

1.4.2.2.2. CD4+ T cell response in HCV infection

The importance of HCV-specific CD4+T cells was first reported when patients with spontaneously clearing HCV mounted broad CD4+ T cell responses during acute infection with increased T cell proliferation and IL-2, IFN-γ, and TNF-α production compared to patients who developed a persistent infection [173-176]. The significance of CD4+ T cell help in sustaining a functional CD8+ T cell response was proven by *in vivo* chimpanzee studies whereby the depletion of CD4+T cells resulted in a gradual decline in the frequency of CD8+ T cells and their cytokine production capacity as well as an increase in escape mutation is targeted CD8+T cell epitopes [151]. Furthermore, it has been demonstrated that the specific expansion of $CD161^{hi}CCR6^{+}CD26^{+}$ CD4+ T cells expressing IL-17A, IL-21 and Th17 cell lineage-specific transcription factors have an important correlation with the course of the infection [161]. The plasma concentration of IL-17A are higher during the acute phase of infection in patients who spontaneously resolve their infection compared to their chronic counterparts [161]. A similar trend in IL-21 concentration is apparent a few weeks later and correlates with increased HCV-specific CD8+T cells, rescuing them from Tim-3/galectin-9 (Gal-9) -mediated apoptosis [161]. Furthermore, IL-21 is a signature cytokine of T follicular helper (Tfh) cells, a subtype of CD4+ T cells involved in providing maturation signals to B cells for antibody production [177]. Interestingly, HCV-specific-Tfh cells have been reported to accumulate in the liver and produce more IL-21 compared to their peripheral blood counterparts in HCV infected patients. However, the functional role of liver-resident HCV-specific Tfh cells in HCV immunity requires further investigation [177].

When broad CD4+ T cells are detectable in the acute phase in chronically progressing patients, these T cells suffer rapid exhaustion followed by consecutive loss of IL-2 production, proliferation and IFN-γ production [178, 179] as well as higher expression of TIM-3, PD-1 and CTLA-4 [180]. Lastly, it has been demonstrated that chronically evolving patients have an expansion of Gal-9 expressing regulatory T (Treg) cells as well as higher Gal-9 plasma concentrations, suggesting that binding of Gal-9 to Tim-3 blocks IL-21 production by HCV-specific Th17 cells [161].

1.4.2.2.3. Memory cell-mediated immunity and reinfection

A landmark study by Mehta et al. reported that patients who were previously infected with HCV and spontaneously cleared the virus were 12 times less prone to develop persistent viremia [181]. This may be attributable to the robust memory T cell population generated in subjects who successfully resolve their infection [182]. Another important study followed up a group of women many years after an accidental exposure to an identical strain of virus. Interestingly, HCV-specific CD4+ and CD8+ T cell responses were detectable up to 20 years after the successful clearance of primary infection in these women [183]. Among spontaneous resolvers, phenotypic characterization of HCV-specific CD4+ and CD8+ T cells identified the expression of CCR7+, a lymphoid homing marker, and CD45RO, both markers associated with memory T cells [184, 185]. Furthermore, in spontaneously resolving chimpanzees re-challenged with heterologous HCV isolates, duration of viremia was significantly decreased and associated with a high frequency IFN-γ secreting CD4+ and CD8+ T cells [185-188]. Additional investigation into the role of memory T cells was carried out using antibody-mediated depletion of either CD4+ or CD8+ T cells in chimpanzees. Depletion of CD4+ T cells led to low viremia levels where

CD8+ T cells were able to partially control the infection [151]. However, depletion of CD8+ T cells resulted in a considerable delay in the control of viremia, wherein re-appearance of CD8+ T cells coincided with viremia control [152]. In summary, the aforementioned studies highlight the significance of memory T cells in mediating protective immunity in reinfection. Importantly, this protective immunity acts to mainly reduce the duration and level of viremia in lieu of conferring 'sterilizing-immunity' [137].

1.4.3. Genetic Factors and Outcome of Acute HCV

In addition to the genetic influence of NK cell receptors mentioned earlier, three separate genome-wide association studies (GWAS) published reported an association between several single-nucleotide polymorphisms (SNPs) near the IFNλ3 (IL28B) gene locus and response to IFN therapy and spontaneous clearance of infection [189-192]. The C/C genotype at the SNP rs12979860 strongly correlates with spontaneous clearance of primary HCV infection among patients of both European and African backgrounds [189]. Furthermore, an RNA sequencing study performed in PHHs revealed a new gene variant upstream of IL28B called ss469415590 which creates a new gene called IFNλ4. The IFNλ4 was reported to be more predictive of HCV viral clearance compared to IFNλ3 in African subjects. [193].

The exact mechanism for how the IL28B SNPs impact HCV outcomes are unknown, however, it is well established that IL28A, IL28B and IL29, also known as type III interferons, are induced by viral infections and harbour antiviral activity [194]. Type III IFNs are suggested to have similar intracellular response as IFN-α but with greater specificity as their receptors have restricted expression. It is also reported that IL28B SNP may have an effect on NK cell functions or the interaction between the innate and adaptive

immune systems. For example, the KIR2DS3 and IL28B may be predictive of chronic progression of HCV infection [195]. Furthermore, it was reported that the IL28B polymorphism, HLA-C and KIRs additively predict HCV therapy outcomes [196]. Moreover, it was recently reported the CC genotype is associated with higher IFN-γ production by NK cells during acute HCV infection, although it does not prevent chronicity [197].

1.4.4. Viral evasion of Innate Immunity

As HCV infections can be spontaneously resolved in the acute phase, the innate immune response triggered by HCV PAMPs seems to be able to control the acute infection [106, 198]. However, 70% of acute infections progress to chronicity, implying that the virus has figured out strategies in order to escape or to counteract host defenses. Numerous studies have shown how several HCV proteins are capable of blocking host antiviral responses, ultimately leading to a chronic HCV infection. The HCV core, E2, NS3/4A, NS4B and the NS5A proteins all have unique evasion mechanisms to fight the host immune response [15].

Core protein: Expression of the core protein blocks IFN signaling by preventing STAT1 tyrosine phosphorylation, which in turn blocks STAT1 dimerization with STAT2, preventing its translocation to the nucleus and ultimately stopping IFN signal transduction and ISG expression [199]. Moreover, the core protein induces the expression of suppressor of cytokine signalling 3 (SOCS3) which is a repressor of the JAK-STAT pathway given its ability to block STAT1 phosphorylation [200, 201]. It has been described that that SOCS3 expression is increased in chronic HCV patients who are unresponsive to IFN therapy [201].

E2 : The HCV E2 protein utilizes the phenomenon of molecular mimicry as an evasion strategy to bypass host defense mechanisms [202]. Molecular mimicry is a mechanism whereby viral proteins structurally resemble host defense proteins and can behave as immune modulators [202]. The E2 protein contains a 12-amino acid sequence that is similar to eukaryotic initiation factor 2α (eIF2α) and PKR [203]. This identical domain blocks the phosphorylation of eIF2α ultimately repressing protein synthesis and conferring resistance to type I IFN treatment [204].

NS3/4A: In addition to its essential role in the maturation of NS proteins, in RNA replication and virus morphogenesis, the NS3/4A protease also possesses mechanisms to suppress the host antiviral system [106, 107, 198, 205, 206]. The protease contains the NS4A transmembrane domain and the amphipathic α-helix at the NS3 N-terminus which allow for the cleavage of their respective targets, MAVS and TRIF, which are fundamental proteins involved in the signaling cascade leading to type I IFN production [207, 208]. MAVS plays a critical role in the RLR pathway and is cleaved by NS3/4A at cysteine 508, causing the dislocation of the N-terminal part of MAVS from the mitochondria and therefore suppressing downstream IFN synthesis [209]. In chronically infected HCV patients, the cleavage of MAVS and therefore the decrease in IFN levels have been reported [210].

NS4B: Stimulator of interferon gene (STING) plays an important role in the initiation of transcription pathways that are crucial for innate immune signalling. When stimulated with dsDNA, STING polymerises and facilitates the connection of TBK1 with IRF3, phosphorylating IFR3 and therefore activating downstream transcription of type I IFNs

[211]. The HCV NS4B confines STING on the ER, preventing its association with TBK, thereby suppressing downstream IFN signalling [212, 213].

NS5A: This pleiotropic protein regulates the host environment such that it favours virus replication and persistence [214]. Moreover, NS5A binds to MyD88, a key player in the TLR pathway, and blocks the recruitment of IRAK1 to MyD88, thereby weakening TLR signalling and ultimately decreasing cytokine production [215].

1.4.5. Viral Evasion of Adaptive Immune Responses

Cellular immune responses are present during early HCV infection regardless of outcome and may even continue in chronic infection [216]. Several reports have elucidated that the immune response responses developed during early infection decline in chronically progressing patients [86, 153, 216, 217]. The majority of subjects with appreciable cellular immune responses during early infection exhibit loss of both breadth and magnitude of responses during the chronic phase of infection. The deterioration of T cell responses is not well understood and is believed to be a result of escape mutations. As pathogens replicate on the scale of hours and days and immune responses are generated over a period of weeks, it is suggested that escape mutations contribute to blunting the immune response efficacy [218]. Viral kinetic models show that up to 10^{12} virions are produced daily in persistent HCV infections [84]. This incredibly high rate of virion output, paired with the absence of proofreading activity of the HCV RNA polymerase amounts to common mutations in the viral genome. Consequently, mutations within the class I or II MHC restricted T cell epitopes lead to the delay in clearance of infected hepatocytes [219]. Escape mutations within CD8+ T cell epitope are correlated with chronicity and accounts for a key viral evasion mechanism. Mutated epitopes induce defective new T cell responses as they lose

binding and recognition capacity to their restricting MHC [220, 221]. Decreased recognition can result either from mutations in the epitope sequence itself or in flanking residues that are implicated in antigen processing [222]. Escape mutations are also influenced by the interaction between the host genetics and the virus. The host HLA alleles drive selective pressure on their corresponding epitopes. This is demonstrated when escape mutations convert to their wild type sequences when transferred to another person carrying a different HLA allele and therefore the epitope lacking selection pressure [223]. Importantly, the extent of epitope mutations is constrained by viral fitness cost [224-228]. Consequently, some HLA alleles such as HLA*B27 are regarded as protective since they prime responses to extremely constrained epitopes that due to high fitness cost, have rare tendency to mutate [226, 227].

1.5. HCV Treatment

1.5.1. Interferon Ribavirin Therapy

In the mid-1970's, following the identification of hepatitis A, non-A, non-B hepatitis (NANB) was acknowledged [2], and it was initially thought that it would have a negligible health impact. However, it was soon reported that NANB hepatitis mostly exhibited progressive disease leading to cirrhosis and potentially to liver cancer [229]. Therefore, coupled with efforts to identify the causative agent of NANB hepatitis, significant dedication was invested into discovering efficient drug therapies to hamper disease progression.

The noticeable success of IFN-α therapy for hepatitis B spurred a pilot study at the National Institutes of Health in 1986 where NANB hepatitis patients were treated with recombinant IFN-α**,** well before the discovery of the causative agent, the hepatitis C virus [230]. Promising results from this study encouraged further controlled trials with IFN for NANB hepatitis [231, 232], and partial efficacy was concluded. The first approved regimen consisted of IFN-α at three doses per week for 6 months, achieving sustained virological response (SVR) rates of approximately 6% [231, 232]. Prolongation of the treatment to 12 months only marginally improved SVR rates to 16%. The combination of IFN-α with a nucleoside-analogue antiviral, ribavirin (RBV), improved SVR rates to 34% after 6 months, and to 42% after 12 months of treatment [233, 234]. Further steps in improving the half-life of IFN-α consisted in covalently coupling it to polyethylene glycol (PEG) to generate PEG-IFN-α. In combination with RBV, PEG- IFN-α achieved SVR rates of up to 56% [235, 236].

Unfortunately, the majority of patients treated with combination IFN/RBV therapy experienced adverse side effects. In a trial of 1,000 patients treated with the standard dose, the following side effects were reported: fatigue (66%), headache (50%), nausea (42%), insomnia (41%), pyrexia (35%), anemia (34%), myalgia (27%), neutropenia (31%), depression (25%), irritability (25%) and rash (28%) [237]. Consequently, these adverse reactions resulted in the early interruption of therapy in 13% of participants and a dose decrease in 43% [237].

1.5.1.1. Patterns of Response to IFN/RBV Therapy

The outcomes of IFN-α based therapy can be divided into three categories: SVR, relapse and non-response (NR). The main goal of therapy is SVR, characterized as undetectable HCV RNA 6 months after completion of therapy. SVR seems to represent a cure of infection and is correlated with lack of intrahepatic RNA and histologic recovery [238, 239]. Conversely, in relapsers, viremia rebounds upon completion of therapy. Relapse patients can be re-treated and achieve SVR often with prolonged and increase dose of therapy. Lastly, non-responders fail to exhibit a decrease in viral load below detection levels throughout and after the course of therapy [240].

More comprehensive characterization of treatment outcomes include early virological responses (EVR) and early virological clearance (EVC) defined as negative or ≥ 2 logs decrease in HCV RNA after 12 weeks of treatment [241]. In patients who achieve SVR, the decline in viral load is biphasic: an early rapid reduction in HCV RNA within 2-24 weeks due to direct blocking of virus replication [242], followed by a delayed secondary phase with slower reduction due to death of infected cells by immune-mediated and other mechanisms [243, 244].

1.5.1.2. Factors Determining Outcomes of Therapy

The key viral factors influencing the outcome of therapy are the genotype and the delay in prescribing therapy [240]. In comparison to genotype 2 infection, genotype 1a and 1b progress to more severe liver disease and have low cure rates to IFN therapy. Among genotype 2 patients, 72% respond to IFN therapy, whereas only 28% of genotype 1a and 26% of genotype 1b exhibit a response [245]. Starting treatment early during the course of HCV infection remarkably increases SVR rate to 88% regardless of genotype [246, 247]. Additional factors associated with increased SVR rates are low baseline viral loads [240] and increased diversity of quasispecies before start of therapy [248].

Relevant host circumstances affecting the outcome of therapy include ethnicity and the lack of co-morbidities such as HIV infection, alcohol abuse, and renal diseases [240]. Accordingly, African Americans were reported to display fractionally lower SVR rates compared to Caucasians [249], and therapy was evidently less effective in patients suffering from HCV/HIV co-infections compared to infections with HCV alone. Additional factors associated with improved SVR rates include the female gender, young age, low body weight and better liver function [240].

1.5.1.2.1. IL28B SNP and outcome of therapy

It was long observed that treatment outcomes with the standard PEG-IFN and RBV therapy had distinct outcomes among different ethnic populations. For example, African Americans had approximately 50% reduction in SVR rates compared to non-Hispanic Europeans after accounting for socio-demographic characteristics and adherence to treatment [249, 250]. Several GWAS studies reported SNPs surrounding the IL28B region to be associated with treatment outcomes [189-192]. The rs12979860 SNP is the most clinically relevant locus as it was strongly associated with SVR among both individuals of European as well as African descent. The C/C genotype is considered the favourable allele as about 80% of patients with this allele achieved SVR compared to 30% with the T/T allele. This difference in allele frequency between African and European populations accounts for about half of the variation in response rates to therapy [251].

1.5.1.3. Mechanisms of Action of IFN/RBV Therapy

The mechanism of action of IFN-α is similar to endogenous IFN signaling as demonstrated by experiments wherein in exogenous IFN-α administration to cell-cultures lacking IFN signaling pathways resulted in induction of many ISGs [252-254].

The exact mechanisms of action of ribavirin remain to be understood. However, a proposed explanation is its direct inhibition of viral replication due to an increase in the error rate of RdRp, resulting in early chain termination [255]. Additional suggestions include its action as a viral mutagen, resulting in viruses with reduced infectious capacity [256], modifying the Th1/Th2 ration in favour of Th1 [257], and lastly, inhibiting IL-10 production [258].

Due to the considerable amount of side effects of PEG-IFN-α and ribavirin, and the varying efficacy of this treatment across genotypes, the success of this therapy was indeed limited. The combination of PEG-IFN-α and RBV was the standard therapy for HCV infection until the recent discovery of direct-acting antivirals (DAAs).

1.5.2. Direct-acting antivirals

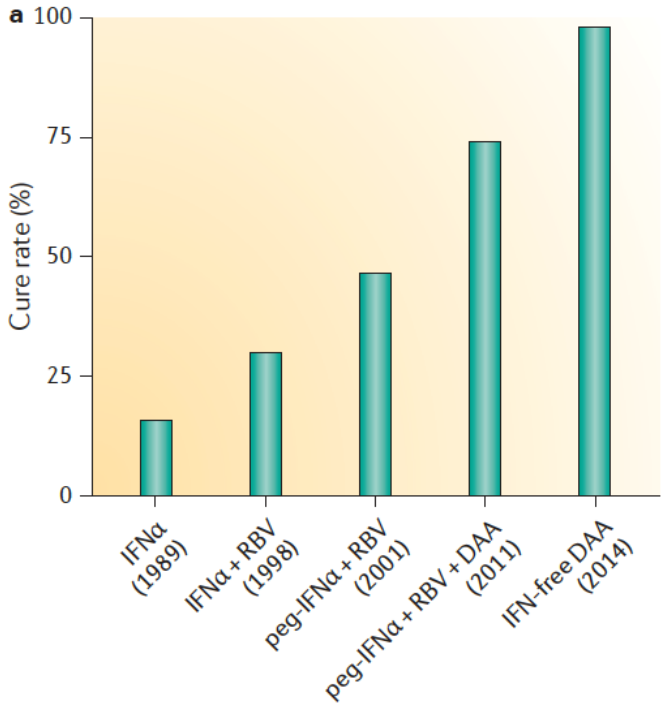

Figure 1.4: Increasing cure rates with newer antiviral therapies over the years. Graph indicating the percentage of cures among patients treated with different regiments from 1989 to 2014. (Adapted from Rehermann, 2016 [259])

Due to the remarkable advances in the understanding of the structure and molecular biology of the HCV viral life cycle, coupled with the suboptimal response rates to IFN/RBN therapy, intense efforts were undertaken towards the discovery of novel therapies targeted at the virus itself. In the past decade, new DAAs have been developed and are currently revolutionizing therapy standards for HCV. Initially, DAAs were used in combination with IFN and ribavirin to improve response rates, however this regimen was accompanied by toxic side effects [260]. Recently, the combination of DAAs that target different mechanisms in the viral life cycle have shown promising results without the need of IFN and ribavirin, and thereby reducing negative side effects. The cure rates achieved

with the new DAAs surpass 90% for most populations of treated patients (Figure 1.4.) [261, 262]. To date, the main three viral targets of DAAs are the NS3/4A protease, the NS5B RNA-dependent RNA polymerase and the NS5A protein [263].

1.5.2.1. NS3/4A Protease Inhibitors

The HCV NS3 is a protease with enzymatic activity, rendering it a suitable target for therapeutic intervention approaches. NS3 functions closely with its noncovalently bound cofactor NS4A that improves its protease activity. Recent crystal structures of NS3 and NS4A have exposed a shallow cleft between N- and C-terminal domains that accepts the substrate [264]. The NS3/4A complex processes the HCV polyprotein at four locations, separating the non-structural proteins that are essential for replication.

The first proof-of-concept study that pioneered the notion of targeting the HCV protease was the development of the protease inhibitor (PI), ciluprevir (BILN-2061), which went into phase II clinical trials [265] but was interrupted due to toxic effects in animals. The availability of crystal structures of NS3 in association with candidate molecules expedited the process of chemical synthesis, biological and structural evaluations that facilitated the creation of enhanced NS3 inhibitors. The structurally similar HCV PIs telaprevir and boceprivir were put on the market in 2011 and were the first successful class of DAAs to be used clinically [263].

The first-generation PIs, despite being a breakthrough in the treatment of HCV, exhibit certain limitations, namely their marginal genotype specificity and their low barrier to resistance [263]. The appearance of mutations in positions Arg155, Ala156, and Asp168 of the NS3 gene are the signature mutations for NS3/4A PIs, and give clear evidence for

the protease as a target. These resistance-conferring mutations impact the binding capacity of the inhibitor without affecting the binding of the natural substrate [266-268].

1.5.2.2. NS5B Inhibitors

Since NS5B is the RdRp of HCV, and uses the genome as a template for RNA synthesis, it is another logical target for therapeutic intervention. Crystal structures of NS5B describe a resemblance to the human right hand [269] with 'thumb', 'finger' and 'palm' subdomains. NS5B inhibitors can be divided into non-nucleotide inhibitors (NNI) and nucleotide inhibitors (NI) that function at different steps of RNA synthesis [270].

The various NNIs, despite being chemically distinct, act prior to or at the level of initiation and interrupt the elongation of the RNA transcript. Drugs available on the market target the three sites of the NS5B. Beclabuvir targets the thumb site I, whereas Dasabuvir is a potent palm site inhibitor [263].

Contrary to NNIs, NIs compete with entering nucleoside triphosphates for binding. NIs are inactive prodrugs that are activated metabolically to the triphosphate form that will ultimately attach to the nucleotide-binding site of NS5B [270]. Sofosbuvir, a uridine equivalent, is the only approved drug within this category, and acts as a robust chain terminator. Unlike NNIs, NIs display pangenotypic activity with an elevated barrier to resistance [263].

1.5.2.3. NS5A Inhibitors

The multifunctional protein, NS5A, responsible for HCV replication and assembly is another major target for therapy. NS5A does not contain enzymatic activity unlike NS3 and NS5B; therefore it was challenging to create assays for drugs targeting its functions. The crystal structure for NS5A is mostly unavailable; therefore cell-based replicon assays were instrumental in identifying its inhibitors [271]. NS5A inhibitors possess remarkable potencies, with EC_{50} amounts in the pM range. The first NS5A inhibitor on the market was daclatasvir, followed by the arrival of ledispavir and ombitasvir, which are used clinically [272]. The phenomenal potency of this class of inhibitors is attributable to the implication of the NS5A protein at many stages of the viral life cycle, notably, in replication and in assembly. The interference with the assembly of the virus-induced membranous web by these compounds may explain their potent effects [263].

1.5.2.4. Success and Remaining Challenges of DAAs

The achievement of DAAs since their arrival has indeed been phenomenal with admirable results in clinical trials as well as in real-world studies. Future agents in late phase clinical trials are believed to cover the efficiency gaps in present DAAs. Pangenotypic NS5A blockers in combination with sofosbuvir have potential for patients with genotype 3 infections with cirrhosis. Moreover, patients with HIV co-infection respond positively to DAAs and patients with co-morbidities such as renal disease will be able to benefit from future regimens. For example, the future DAA grazoprevir-elbasvir has been tested in a dialysis population achieving SVR rates of 95% with negligible adverse effects [263].

Evidently, the future goals of DAAs will not be to increase SVR rates necessarily, but rather to broaden the distribution of these drugs such that this exceptional progress can translate to global public health prosperity [273]. This calls for the expansion of HCV treatment outside specialty clinics. Moreover, an important goal is the development of pangenotypic therapies with a considerably shorter duration. Initiatives addressing treatment failures such as resistance to NS5A inhibitors is another major goal. Accordingly, upgraded and cheaper assays with resistance interpreting standards are essential in this endeavour [263]. Lastly, efforts in pricing reforms to result in more affordable regimens coupled with harm reduction strategies to curb new and reinfection in high-risk populations are essential for DAA therapy to truly cause global waves [273].

1.5.3. Vaccine against HCV: Need and Challenges

There is a major need for a prophylactic vaccine against HCV to prevent transmission and to reduce the global burden of disease. The abundance of research supporting cell mediated immunity as the major facet enabling spontaneous clearance of primary HCV infections gives reason to pursue this avenue for vaccine development. Moreover, the recent data suggesting the contribution of the role of neutralizing antibodies against HCV is also worth investigating and implementing in vaccine efforts [274].

There are many hurdles facing the development of an effective HCV vaccine. First of all, the genetic diversity of HCV and its high rate of mutations, even within the same individual is an important challenge on its own. The capacity of HCV proteins to evade host immune responses is another challenge. Lastly, assuming that an effective vaccine is developed, significant public health efforts must be planned in order to deliver and vaccinate all populations infected or at risk of infection. For example, there is already an

effective vaccine against hepatitis B, however the prevalence of HBV infection worldwide is astounding as close to 380 million people are still infected with HBV due to poor vaccination campaigns in endemic countries [275].

1.5.3.1. Vaccine Trials

There have been three major strategies for designing a vaccine against HCV. The more traditional approach aims to induce neutralizing antibodies by using recombinant envelope proteins, an endeavour that is gaining more potential with the recent discovery of broadly neutralizing antibodies. Immunization with recombinant E1-E2 proteins was reported to generate cross-reactive nAbs implying a degree of protection upon HCV exposure [276-278]. Another approach involved the use of virus-like particles (VLPs) that contain HCV structural antigens to generate humoral and cellular responses, although they lacked to show humoral responses in chimpanzees [279].

The most encouraging strategy is engineering an HCV vaccine to induce strong T cell responses, as the correlates of protection in HCV are T cell dependent. The proof of principle surrounding the idea that a prophylactic T cell vaccine against HCV may be an attainable goal was first demonstrated in a chimpanzee model. The method used adenoviral (Ad) and DNA vectors encoding for the non-structural (NS) HCV protein [280]. Following a heterologous viral challenge, four out of five vaccinated chimpanzees developed low-level viremia and went on to clear the virus rapidly [280].

To address the issue of pre-existing anti-Ad immunity in humans which can restrict the potency of the vaccine, a panel of replication-defective chimpanzee and human adenoviruses that exhibit low seroprevalence were developed to be used as vaccine vectors

[281, 282]. Adenovirus vectors derived from human (Ad6) as well as from chimpanzees (ChAd3) that encoded for the HCV NS3-NS5B polyprotein with a genetically inactivated NS5B polymerase were used in a human vaccine trial in healthy individuals [283]. After a single priming vaccination with either vector, an impressive immune response was achieved. The magnitude, breadth, and polyfunctionality of HCV-specific T cells were the most potent reported to date [284].

Unfortunately, there were two main caveats in this trial. Firstly, when boosted with a heterologous Ad, T cell responses failed to reach the same levels attained after priming and was suggested to be a result of the induction of cross-reactive anti-Ad antibodies. Secondly, the major subset activated by vaccination were CD8+ T cells whereas both CD4+ and CD8+ T cells are crucial for viral control, as evidenced by natural history, genetic and depletion studies [151-153, 175, 179, 183, 274]. In order to overcome these obstacles, the team developed another prophylactic T cell vaccination approach based on heterologous virus vectors ChAd3 and modified vaccinia Ankara (MVA) [285]. This strategy proved to be highly immunogenic following priming and boosting, and induced both CD4+ and CD8+ T cells that target various HCV antigens [285]. Remarkably, the phenotype of CD8+ T cells induced by Ad/MVA vaccination is closely comparable to that induced by the very potent yellow fever and smallpox vaccines, which both confer long-life protection [286]. This vaccine is currently in Phase II trial in a cohort of intravenous drug users in the United States and is registered in the ClinicalTrial.gov database (ID: NCT01436357) and is expected to finish in October 2017 [285].

1.6. Hypothesis and Objectives

Only 30% of individuals acutely infected with hepatitis C virus (HCV) clear the virus spontaneously, whereas the majority become persistently infected and develop chronic liver disease. Many individuals do not know that they are infected and continue to transmit the virus. Hence, there is an urgent need for a prophylactic vaccine to stop HCV transmission. Vaccine development is hindered by our limited knowledge of the correlates of protective immunity in real life settings. CD8+ and CD4+ HCV-specific T cell responses are essential for spontaneous resolution of acute HCV. The polyfunctionality of virus-specific CD8+ T cells characterized by the capacity to produce cytokines like IFN-γ and IL-2, and cytotoxicity correlated with spontaneous resolution of acute HCV and can be predictive of effective vaccines. Moreover, the presence of neutralizing antibodies (nAb) correlates with the spontaneous clearance of primary HCV infections in some individuals.

PWIDs are at high risk of HCV exposure, infection and reinfection. We intend to define the correlates of protective immunity against HCV in PWIDs who have been exposed to the virus repeatedly but who are resistant to subsequent infections. We hypothesize that HCV immune PWIDs who are resistant to reinfection develop an immune response of high protective capacity where the magnitude (frequency), breadth (number of epitopes targeted) and quality (number of functions) of the HCV-specific memory T cells measured following resolution of primary infection will likely determine susceptibility to reinfection. Furthermore, the neutralizing response of nAbs will be more robust in those PWIDs resistant to reinfection.

The aim of this project is to compare the protective immune responses against HCV in PWID who cleared a previous HCV infection spontaneously or following antiviral

treatment (PEG-IFN and Ribavirin) and achieved a sustained virological response (SVR) and remain protected from reinfection despite high risk exposure. We used peripheral blood mononuclear cell (PBMC) samples to perform IFN-γ enzyme-linked immunospot (ELISPOT) assay in re-infected versus exposed non re-infected individuals to compare the magnitude of the HCV-specific T cell responses. We also used flow cytometry to determine polyfunctionality of HCV-specific T cells in a subset of patients. Finally, we performed a neutralization assay using an HCV pseudoparticle assay.

Chapter 2: Article
Correlates of Long-term Immune Protection in Hepatitis C Virus (HCV) Exposed Non-reinfected Individuals

Manuscript in preparation for submission to Journal of Virology

Correlates of Long-term Immune Protection in Hepatitis C Virus (HCV) Exposed Non-reinfected Individuals

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2.1. Abbreviations

Abs	Antibodies
DAAs	Direct-acting antivirals
ELISPOT	Enzyme-linked Immunospot
FACS	Fluorescence-activated cell sorting
HCV	Hepatitis C virus
HCVpp	HCV pseudoparticles
IFN	Interferon
Ig	Immunoglobulins
IL	Interleukin
nAb	Neutralizing antibodies
PBMCs	Peripheral blood mononuclear cells
PEG	Polyethylene glycol
PSP	Percent specific production
PWIDs	People who inject drugs
PY	Person-years
RNA	Ribonucleic acid
SFCs	Spot forming cells
SR	Spontaneous resolver
SVR	Sustained virological response
TNF-α	Tumour necrosis factor-α

2.2. Abstract

Approximately 25% of individuals acutely infected with hepatitis C virus (HCV) clear the infection spontaneously and develop long-lived memory T cells. Yet, these individuals remain susceptible to reinfections and correlates of long-term protective immunity remain poorly defined. Generation and maintenance of high frequency, broad, polyfunctional CD4+ and CD8+ HCV-specific T cell responses correlate with spontaneous resolution of acute primary HCV and can be predictive of long-term protection but whether they are key determinants of long-term protective immunity is unknown. The protective role of neutralizing antibodies (nAb) is also unclear. Here, we examined the magnitude, breadth and polyfunctionality of virus-specific T cells and the efficacy of neutralizing antibodies in a cohort of HCV-resolved high-risk people who inject drugs (PWIDs) repeatedly exposed to but resistant to reinfections. We demonstrate a correlation between long-lived HCV-specific T cell responses and protection from reinfection in spontaneously resolved and treatment resolved PWIDs. We observed long-lived HCV-specific T cell responses in spontaneously resolved and treatment resolved PWIDs who are protected against reinfection. We did not observe a difference in the nAb responses nor in the functionality of HCV-specific T cells. Our results suggest that protection from reinfection in PWIDs is associated with a sustained high frequency of HCV-specific cellular immune responses.

2.3. Introduction

The hepatitis C virus (HCV) infects approximately 185 million people worldwide and is the leading cause of severe liver disease and hepatocellular carcinoma [1]. Despite the arrival of potent direct-acting antivirals (DAAs) with impressive cure rates, there remain substantial challenges in eradicating HCV [2]. Due to the asymptomatic nature of both the acute and chronic phases, infection incidences are increasing in North America [3] and extensive screening programs are almost non-existent in endemic countries. Consequently, less than 5% of the global HCV-infected population are conscious of the fact that they are infected [4].

The global HCV dilemma comprises numerous challenging factors including the identification of infected individuals before the onset of liver disease, the occurrence of repeated infections, the high cost and possible resistance to therapy, and the lack of therapeutic strategies to cure extensively damaged livers. Moreover, reinfection following successful sustained virological response (SVR) is a major problem among at-risk individuals, especially PWIDs. It was reported that reinfection rates after SVR range from 1 to 5 cases per 100 person-years (PY) among patients with a previous intravenous drug use, and augmenting to 3 to 33 cases per 100 PY in PWIDs with frequent risk behaviour [5]. Moreover, a recent meta-analysis demonstrated that the risk of reinfection 5 years post-SVR is 8.2% among PWIDs and prisoners [6]. Appropriately, the prevention of chronic HCV may be the most effective method of reducing HCV-related morbidity and therefore, the development of an effective prophylactic vaccine is crucial [7]. Studies modelling the impact of a hypothetical HCV vaccine with 80% efficacy have predicted a significant impact on the incidence of persistent HCV infection and suggest it to be highly cost-

effective [8, 9]. Unfortunately, vaccine development is hampered by our limited knowledge of the correlates of protective immunity in real life settings.

There is substantial evidence of protective immunity and long-lived memory immune responses in people infected with HCV. A landmark study by Mehta et al. reported that PWIDs who were previously infected with HCV and spontaneously cleared the virus were 12 times less prone to develop persistent viremia as compared to HCV-naïve PWIDs with similar risk exposures [10]. Another study that followed a group of German women after an accidental common source outbreak of HCV, demonstrated that HCV-specific CD4+ and CD8+ T cell responses were detectable up to 20 years after the successful clearance of primary acute HCV while antibody responses waned [11]. Moreover, despite the fact that spontaneous resolvers (SR) can be subject to recurrent HCV infection upon subsequent exposures, the resolution of the secondary infection occurs 80% rather than 25% of the time [10, 12-13].

Previous research has highlighted that CD8+ and CD4+ HCV-specific T cell responses are essential for spontaneous resolution of acute HCV [14, 15] and are expanded and correlate with resolution of reinfection [13, 16]. The polyfunctionality of virus-specific CD8+ T cells characterized by the capacity to produce cytokines like interferon-γ (IFN-γ) and interleukin-2 (IL-2), tumour necrosis factor-α (TNF-α) and cytotoxicity correlated with spontaneous resolution of acute HCV and may be predictive of effective vaccines [17]. Also, polyfunctionality of CD4+ and CD8+ T cells are associated with spontaneous resolution of reinfection [16]. Moreover, the presence of neutralizing antibodies (nAb) correlates with the spontaneous clearance of primary HCV infections in some individuals

[18], and the presence of cross-reactive nAb correlates with reduced magnitude and duration of viremia upon reinfection [13].

The aim of this study was to define the correlates of protective immunity that can be used to evaluate the efficacy of novel vaccines against HCV. Determining the correlates of protective immunity in real-life exposures such as among PWIDs is a crucial first step [19]. We thus examined the protective immune responses against HCV in PWID who cleared a previous HCV infection spontaneously or following antiviral treatment (PEG-IFN and Ribavirin) and achieved a SVR and remained protected from reinfection despite high-risk exposure. We hypothesized that PWIDs who are resistant to reinfection develop an immune response of high protective capacity. We demonstrate that a high frequency of HCV-specific T cells but not nAbs are associated with long-term protection against HCV reinfection.

2.4. Materials and Methods

Study Subjects

High-risk PWIDs who had previously resolved an HCV infection were recruited via the Montreal Hepatitis C Cohort (HEPCO) [20]. This study was approved by the institutional ethics committee (protocol SL05.014) and conducted according to the Declaration of Helsinki. All participants signed informed consent upon enrolment. HCV resolvers were identified as HCV-RNA negative using the COBAS Ampliprep/COBAS Amplicor HCV Test, version 2.0 (Roche Molecular Systems, Inc.) (Sensitivity 50 IU/mL), anti-HCV antibody positive (AxSym HCV Assay [Abbott GMBH & CO, Saint Laurent, Quebec, Canada]). Subjects' clinical history including history of previous infections and/or antiviral treatment was registered to define the date of clearance of primary infection. For subjects where the exact dates of primary infection and clearance were unknown, we estimated the time of their first infection using an epidemiological paradigm that predicts the date of HCV infection based on time since the beginning of injection drug use. According to Hagan et al. (2008) [21], it takes approximately 5 years to become infected with HCV since the initiation of injection drug activity. We obtained the age at initiation of drug use of our participants through questionnaires, and predicted the age of their primary infection accordingly. All subjects were followed for incidence of reinfection by retesting for HCV RNA every 3 months. HCV exposure and risk behaviour was assessed through comprehensive questionnaires. HCV reinfection was defined by an HCV RNA positive test after 2 negative tests at least 60 days apart. Exposed non-reinfected participants were defined as never having an HCV RNA positive test after a resolved primary infection (HCV

Ab positive or HCV RNA positive test). HCV genotyping was performed as described previously [22]. IFN-λ3 genotype was determined as previously described [23].

Enzyme-linked immunospot (ELISPOT) assay

Interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assay was performed against 11 pools of overlapping peptides representing the genotype 1a (H77) polyprotein or the genotype 3a (K3) as previously described [24]. Peptides were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA, USA). Briefly, all assays were performed in duplicates directly *ex vivo* on 2×10^5 cryopreserved peripheral blood mononuclear cells (PBMCs). Specific spot forming cells (SFCs) were calculated as mean number of spots in test wells minus the number of spots in negative control wells and normalized to SFC/10^6 PBMCs. Breadth was defined as the number of regions of the HCV polyprotein (Core, E1, E2, etc) targeted. In many of the exposed non-reinfected subjects who were recruited having already resolved a primary HCV infection, the genotype of the primary viral strain was unavailable. In this case, we chose to perform the IFN-γ ELISPOT using the 1a polyprotein as this subtype is the most prevalent in our cohort.

Antibodies

The following conjugated anti-human monoclonal antibodies (Abs) were used: CD3-Pacific Blue (clone UCHT1), CD4-BV605 (clone RPA-T4), CD8-APC-H7 (clone SK1), IL-2-PE (clone MQ1-17H12), IFN-γ-PECy7 (clone B27), TNFα-Alexa Fluor 700 (clone MAb11), CD107a-BV786 (clone H4A3). All Abs were obtained from BD Biosciences.

Polyfunctionality assays

Polyfunctionality assays were performed as previously described [16] using cryopreserved PBMCs. Briefly, 2×10^6 PBMCs were incubated with anti-CD107a and either dimethyl sulfoxide (0.1%) as a negative control or HCV peptide pools (10 μg/mL) at 37°C in R-10 medium (RPMI medium [Invitrogen, Carlsbad, CA] supplemented with 10% FBS). Following 1 h of stimulation, 10 μg/mL of brefeldin A (Sigma-Aldrich) and 6 μg/mL of monensin sodium salt (Sigma-Aldrich) were added, and cells were incubated for a total of 12 h. Cells were washed with fluorescence-activated cell sorting (FACS) buffer (PBS 13 [Wisent], 1% FBS [FBS; Sigma], 0.01% sodium azide [Thermo Fisher Scientific, Burlington, ON]) , stained for viability using LIVE/DEAD fixable aqua dead cell stain kit (Molecular Probes; Thermo Fisher Scientific) and cell surface antigens (CD3, CD4, CD8), and permeabilized using BD Cytofix/Cytoperm solution (BD Bioscience). Cells were then stained with anti-IL-2, anti-IFN-γ, and anti-TNF-α antibodies for 30 min, washed twice in BD Perm/Wash buffer (BD Biosciences), and fixed in FACS fix buffer. For analysis, cells were gated on viable CD3+, CD4+ or CD8+ T cells. Percent specific production (PSP) is calculated as the background-adjusted function in the presence or absence of cognate peptide stimulation. Cells were analysed using a standard BD LSR II instrument with FACSDiva software version 6.1.3 (BD Biosciences, San Jose, CA). Data files were analyzed using FlowJo software version 9.5 for Mac (Tree Star, Inc., Ashland, OR). Functionality data was analyzed with FlowJo and Boolean Gates were applied to generate combination gates.

Neutralizing antibodies assays

HCV pseudotyped viruses (HCVpp) expressing a luciferase reporter were generated as previously described [25]. Two different HCV glycoproteins derived from either genotype 1 (H77c) or genotype 5 (SA13) were used for pseudotyping. For neutralization assays, human hepatoma Huh7.5 cells were plated on collagen coated 96 well plates, 1 day prior to infection. HCVpp were diluted and premixed with purified immunoglobulins (IgG) from sera. IgG were purified using Melon-gel kit (ThermoFisher) according to manufacturer's suggestion. The final purity of IgG from sera was monitored by SDS-electrophoresis and coomassie blue staining. Four descending concentrations of antibody were used: 50 µg, 16.6 µg, 5.6 µg and 1.9 µg. Only the level of neutralization at 50 µg was shown in this study. The antibodies and HCVpp were preincubated at 37°C for 1 hour followed by addition to Huh 7.5 cells. Cells were then spinoculated at 500g, 1 hour at 37°C, followed by incubation another 5 hours at 37°C. The antibody-virus inoculum was then replaced with fresh culture media. Cells were processed 48 hours post-infection using Bright-glo luciferase assay system (Promega). Luminescence was measured using an Enspire plate reader (Perkin Elmer). The neutralization activity was calculated using the following formula: % neutralization = (HCVpp-HCVpp with antibody)/HCVpp ×100, where HCVpp represents the luciferase activity of transduced cells in absence of any antibody and HCVpp with antibody represents the luciferase activity of transduced cells in the presence of antibody isolated from volunteer's sera.

Statistical analysis

Statistical differences between groups were calculated using a two-tailed Mann–Whitney test. Correlations were analyzed by the Pearson test using GraphPad Prism version 5.0 (La Jolla, CA).

2.5. Results and Discussion

Long-lived, high frequency HCV-specific T cells in exposed non-reinfected compared to reinfected patients (≥ 2 years)

We sought to identify correlates of protective immunity in a cohort of HCV resolvers and treated PWIDs at risk of HCV reinfection identified and followed for incidence of reinfection as described in Materials and Methods. Subjects' demographics and characteristics are listed in Tables 1 and 2. First, we compared the magnitude of the HCV-specific T cell responses in the exposed non-reinfected group (n=27) versus the exposed reinfected group (n=14) using an IFN-γ ELISPOT assay as described in Materials and Methods. The time point studied in this cross-sectional analysis was > 6 months post-spontaneous clearance of the primary infection or post-SVR with PEG-IFN and ribavirin therapy. Depending on the availability of PBMC samples, the time range varied from >6 months to nearly 20 years post resolution of infection in some patients. We observed no significant difference in the magnitude of the T cell response (Figure 1A). Due to the fact that there may be viral rebound in the first two years after infection [12], we stratified the patients into two groups, <2 or ≥ 2 years post-resolution of primary infection. When these patients were separated into two groups, < 2 (n=8) or ≥ 2 (n=19) years post-resolution of primary infection, significant difference (p=0.0003) was observed (Figure 1B). The non-reinfected individuals that had been exposed for more than 2 years had significantly higher HCV-specific T cell responses than individuals that had been exposed for less than 2 years. Moreover, a significant difference was observed between the non-reinfected and reinfected patients exposed for more than two years (p=0.0224) (Figure 1B). Interestingly, within the non-reinfected group, HCV-specific T cell responses were very long lived, ranging from

<2 years all the way to 24 years post resolution of primary infection (Figure 1C). This is consistent with a previous report wherein HCV-specific T cell responses were detectable up to 20 years after a common source outbreak of HCV infection [11]. Overall, this indicates that protection from reinfection is associated with a high frequency of long-lived HCV-specific T cells.

Increased breadth of the HCV-specific T cell response in non-reinfected PWIDs

Next, we examined the breadth (the number of HCV regions targeted) of the HCV-specific T cell responses. We observed a significantly higher ($p=0.0039$) T cell breadth in individuals exposed non-reinfected (≥ 2 years) compared to those who became reinfected (≥ 2 years) (Figure 2A, Supplementary Figure S1A). Taking a closer look at the HCV-specific T cell responses to specific HCV region we noticed that all six reinfected patients failed to mount an immune response to the NS4A/NS4B, NS5A, as well as the NS5B proteins in comparison to the exposed non-reinfected patients (Figure 2C). Furthermore, in the exposed non-reinfected group, a similar dichotomy was observed as mentioned above. Within this group, those individuals protected from reinfection for ≥ 2 years exhibited significantly higher ($p=0.0008$) T cell breadth compared to their < 2 years exposed counterparts (Figure 2B, Supplementary Figure S1B), confirming that expanded T cell breadth is a crucial determinant of protective HCV immunity [26] and underscoring the immune dominance of the non-structural proteins [27].

Increased frequency HCV-specific T cell response in SVR non-reinfected PWIDs

We also compared the protective HCV-specific immune responses among patients who cleared their primary infection following antiviral therapy with PEG-IFN and ribavirin and attained SVR. We compared the HCV-specific T cell responses using IFN-γ ELISPOT in exposed non-reinfected (n=19) vs. exposed reinfected (n=10) subjects. We observed a significantly higher (p=0.0161) HCV-specific T cell responses in the SVR exposed non-reinfected patients compared to SVR reinfected participants (Figure 3A). Within the SVR non-reinfected, there was a striking dichotomy with some patients clustering together in the high SFC range. It is plausible that these patients were treated earlier, as we previously demonstrated that early IFN therapy rescues polyfunctional memory T cells [17]. Interestingly, there was no difference in the frequency of HCV-specific T cell responses when comparing the non-reinfected subjects in SVR and SR groups (Figure 3B) suggesting that treatment-induced protection is comparable to that of spontaneous clearance. Finally, there was no difference in the number of regions targeted (breadth) between the SVR non-reinfected and SR non-reinfected patients (Figure 3C). This suggests that IFN therapy can rescue the breadth of HCV-specific T cells, as many regions of the HCV polyprotein were targeted by SVR patients (Supplementary Figure S1C).

Risk exposure does not correlate with HCV-specific T cell responses

Next, we sought to determine whether the differences observed in immune responses were related to differences in risk exposure where increased exposure might have resulted in subclinical infection(s) that boosted the immune response. We evaluated this parameter by

assessing the number of injections administered in the month preceding the time point tested. A previous study in our cohort demonstrated that the incidence of HCV infection increases with ≥ 30 drug injections per month [28], therefore we hypothesized that subjects with higher IFN-γ ELISPOT responses have higher risk exposure through increased number of intravenous drug injections. However, there was no apparent correlation between the number of injections and the HCV-specific T cell responses (Supplementary Figure S2). We cannot exclude inaccurate disclosure of the number or frequency of injections by the PWIDs in response to the questionnaire. It is also plausible that there is no correlation between the number of injections and the frequency of HCV-specific T cells for this cohort.

Polyfunctionality does not correlate with protection from reinfection

Our group has previously demonstrated that polyfunctionality of HCV-specific T cells was a determinant of spontaneous resolution of HCV and protection upon reinfection [16-17]. We thus tested the polyfunctionality of HCV-specific CD4+ and CD8+ T cells by analyzing the production of IFN-γ, IL-2, and TNF-α, as well as expression of CD107a, a surrogate marker of cytotoxicity, which correlates with granzyme B, and perforin secretion as described in Materials and Methods. Representative flow cytometry data are depicted in Supplementary Figure S3. We stimulated the PBMCs from the same time point as tested in the IFN-γ ELISPOT. We tested four patients from each category (reinfected and non-reinfected). These patients were selected based on the highest response observed in the ELISPOT assay and we used the same peptide pool(s) generating the highest ELISPOT response. Unfortunately, we were not able to observe substantial polyfunctionality in both groups (data not shown). However, when examining the individual levels of cytokine

production, we noticed a trend in the CD107a expression between the two groups. Non-reinfected participants tend to have higher levels of CD107a expression in both CD8+ and CD4+ T cells, with higher frequency in CD4+ T cells (Figure 4A-B). Although there is no significant difference, this suggests that for this specific group of patients, the increased level of cytotoxicity may mediate protection from reinfection. Testing additional patients is necessary to confirm these findings, although cytotoxic CD4+ T cells have been reported previously in viral hepatitis and HIV-1 infection [29].

Increased nAb response in non-reinfected PWIDs over time (< 2 years vs. ≥ 2 years)

Next, we examined the presence of neutralizing Ab responses in non-reinfected versus reinfected PWIDs using two different HCVpp as described in Materials and Methods. Consistent with our ELISPOT data, we observed significantly higher percent neutralization in PWIDs non-reinfected ≥ 2 years (Figure 5A) compared to those non-reinfected for < 2 years. Interestingly, this difference was much more prominent when neutralization was tested against the heterologous genotype 5 (SA13) strain, rarely seen in North America (Figure 5B). This may suggest a cross-reactive nAb response among those non-reinfected, as there is approximately 30-35% divergence in the nucleotide sequences between the two different genotypes. These results suggest that nAbs that develop upon clearance of primary infection, tend to increase in titer or neutralizing efficacy with time as PWIDs continue to be exposed to HCV and maybe to subclinical infections. Given that both groups exhibited similar risk exposures, it was not surprising that there was no statistically significant difference in the nAb response between reinfected and non-reinfected individuals. This preliminary data suggest that nAbs are not associated with

protection from reinfection in this cohort but require further validation with a larger cohort. Nevertheless, there may be other benefits of these nAbs. Perhaps these long-lived humoral responses may play a role in the outcome of a subsequent infection, just as reported in a previous study wherein the presence of nAb response during reinfection was associated with lower duration and magnitude of viremia [13]. Comparing our results to the study by Takaki et al. where women who spontaneously cleared a common source outbreak of HCV had undetectable levels of HCV-specific Abs 18-20 years after resolution [11], suggest that in our cohort of PWIDs, long-term frequent risk behavior may be associated with exposure to subclinical HCV infection and stimulating the generation of long-lived HCV-specific nAbs. This suggests that among these PWIDs, there may have been HCV exposures that did not lead to productive infections or may have been blunted rapidly. Additional analyses on the viral load and infection outcomes with a closer follow-up interval need to be examined to confirm these possibilities. Moreover, it is important to note that there are much fewer participants in the reinfected group, thus, recruiting additional reinfected patients is crucial in further validating our results.

No correlation between nAbs and T cell responses

Next, we examined whether nAb responses correlated with HCV-specific T cell responses using the Pearson R correlation. Our results demonstrate no significant correlation in neither non-reinfected ($p=0.5493$) nor reinfected ($p=0.7902$) individuals (Supplementary Figure S4). The same is true when all participants were combined together ($p=0.6274$) (Supplementary Figure S4). This suggests PWIDs with high HCV-specific T cell responses do not necessarily have high nAb responses as well.

Concluding Remarks

Overall, we observed a correlation between long-lived HCV-specific T cell responses in exposed non-reinfected for ≥ 2 years and protection from reinfection in spontaneously resolved individuals. A trend in higher CD4+ and CD8+ T cell cytotoxic activity as characterized by the expression of CD107a is observed among the non-reinfected, however it is not-significant, most likely due to very few patients tested. Furthermore, increased nAb responses with time (< vs ≥ 2 years) among non-reinfected PWIDs suggest that humoral responses may be enhanced by subclinical exposure to HCV via drug use. However, the non-significant difference in the nAb response between the non-reinfected and reinfected patients suggest that nAb are not key determinants of protection but may play a complementary role to T cell responses, such as in the reduction in magnitude and duration of viremia upon reinfection, as reported previously [13]. SVR patients protected from reinfection have significantly higher HCV-specific T cells responses compared to SVR reinfected patients suggesting that immune responses restored by antiviral therapy can be protective. Altogether, our results suggest that protection from reinfection in PWIDs is associated with a good HCV-specific T cell response but the role of nAbs remains unclear. Further testing with additional patients from other cohorts is important to broaden our understanding of the protective immunity in PWIDs exposed but protected from reinfection.

2.6. Acknowledgements

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2.8. Tables

Table 1. Clinical Characteristics of Spontaneously Resolved Subjects

	Non-reinfected n=27	**Reinfected** n=14
Sex (M/F)	22/5	12/2
Mean Age (years)	45	42
HCV Genotype (1/3/NA)	8/1/18	5/2/7
IL28B (CC/CT/TT/NA)	15/7/1/4	6/6/2/0
Number of Injections during Past Month (≤30/>30/NA)	14/13/0	4/10/0

NA: Not Available

Table 2. Clinical Characteristics of Treated (PEG-IFN & RBV) Subjects

	Non-reinfected n=19	**Reinfected** n=10
Sex (M/F)	18/1	9/1
Mean Age (years)	46	45
HCV Genotype (1/3/2b/NA)	7/4/1/7	5/5/0/0
IL28B (CC/CT/TT/NA)	4/10/1/4	7/3/0/0
Number of Injections during Past Month (≤30/>30/NA)	13/6/0	6/4/0

NA: Not Available

2.9. Figures

Figure 1

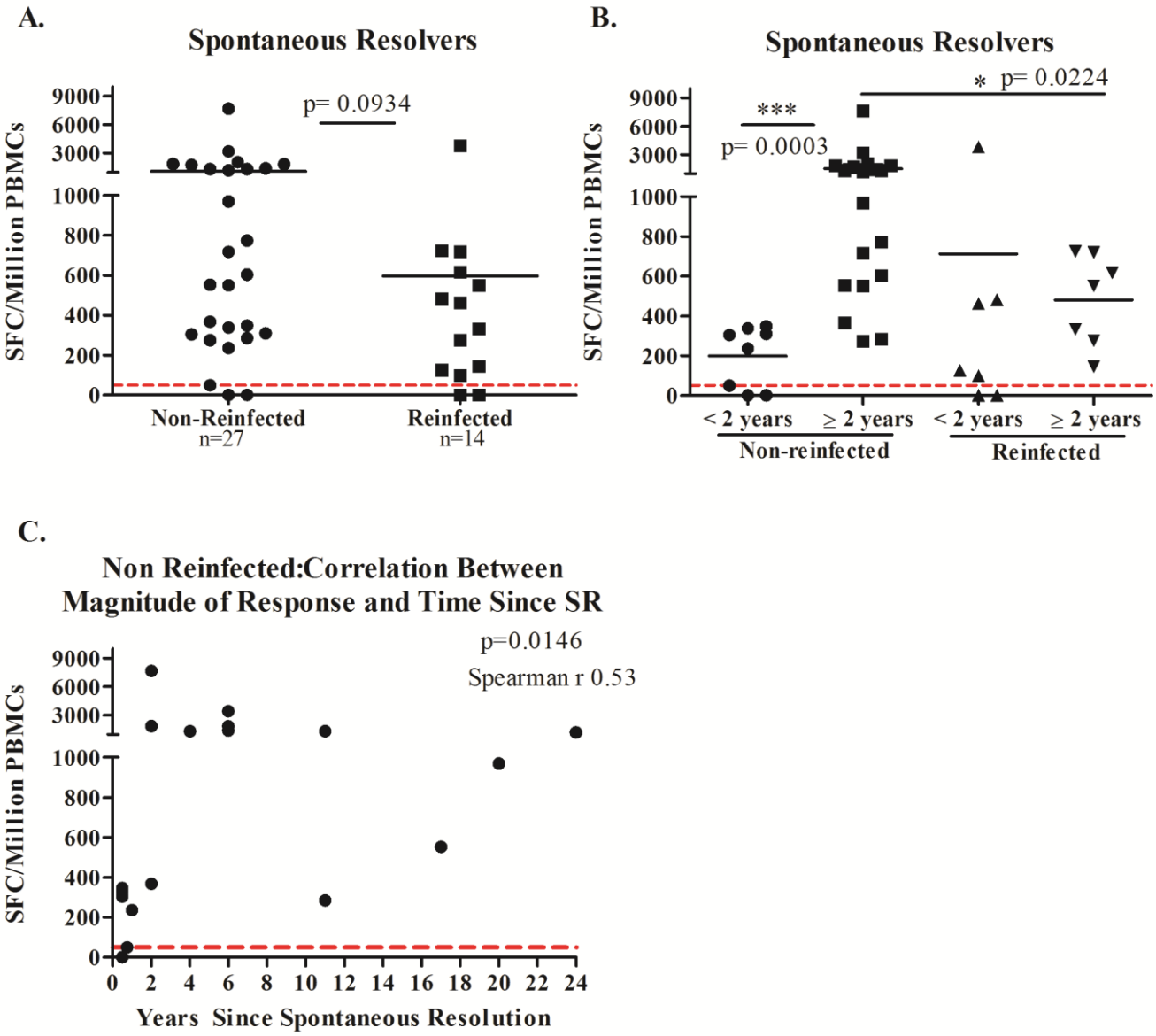


Figure 1. Long-lived HCV-specific T cell responses correlate with protection from reinfection. PBMCs from indicated time points (before reinfection or after resolution of primary infection) were tested in an IFN-γ ELISPOT assay against overlapping peptide pools representing the HCV genotype 1a (H77) or genotype 3a (K3) polyprotein sequence, depending on primary infection. Patients with unknown viral genotypes were tested with genotype 1a. The frequencies of IFN-γ spot forming cell (SFC) per million PBMCs from spontaneously resolved and/or reinfected patients are shown. The dashed line indicates the minimum cut-off at 50 SFC/million PBMCs. **A)** All patients, both reinfected and non-reinfected following spontaneous resolution. **B)** Patients who are non-reinfected separated by time point tested (<2 or ≥2 years). **C)** Correlation of magnitude of HCV-specific T-cell response and years since spontaneous resolution. P values were calculated using two-tailed Mann-Whitney test. (*p<0.05, **<p0.01, ***<0.0001) SR=Spontaneous resolver

Figure 2

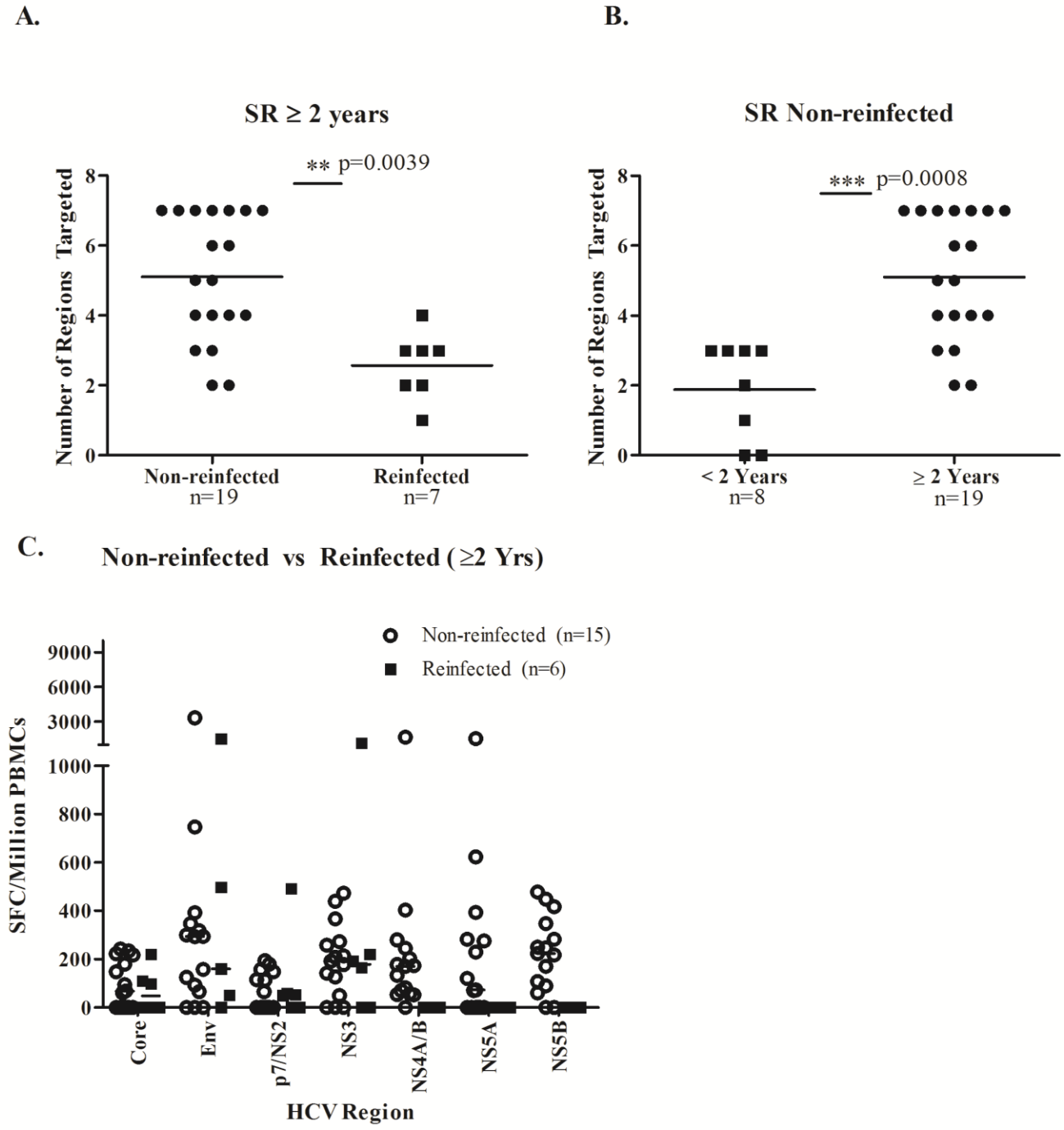


Figure 2. Increased breadth (number of regions targeted) of long-lived HCV-specific T cell responses in non-reinfected (≥2 years) individuals compared to reinfected ≥ 2 years as well as non-reinfected < 2 years. PBMCs from indicated time points (before reinfection or after resolution of primary infection) were tested in an IFN-γ ELISPOT assay against overlapping peptide pools (Core, Env, p7/NS2, NS3, NS4A/B, NS5A, NS5B) representing the HCV genotype 1a (H77) or genotype 3a (K3) polyprotein sequence, depending on primary infection. Patients with unknown viral genotypes were tested with genotype 1a. The frequencies of IFN-γ spot forming cell (SFC) per million PBMCs from spontaneously resolved and/or reinfected patients are shown. The dashed line indicates the minimum cut-off at 50 SFC/million PBMCs. **A-C)** IFN-γ ELISPOT results for indicated patient groups. HCV regions targeted include Core, Env, p7/NS2, NS3, NS4A/B, NS5A, NS5B. P values were calculated using a two-tailed Mann-Whitney test. (*$p<0.05$, **$p<0.01$) SR= Spontaneous resolver

Figure 3

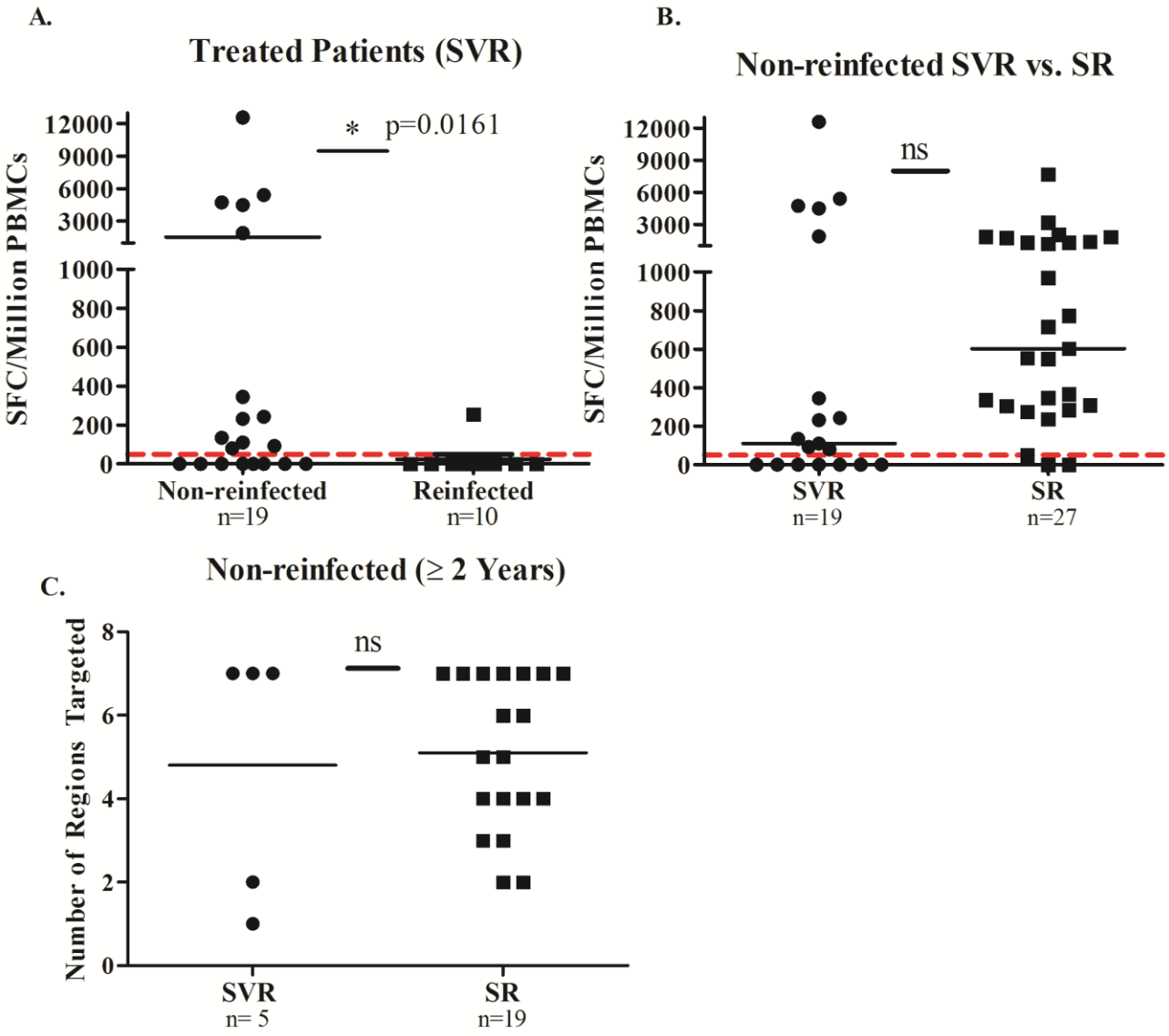


Figure 3. Increased magnitude and breadth (number of regions targeted) of HCV-Specific T cell responses in SVR non-reinfected individuals compared to SVR reinfected. PBMCs from indicated time points (before reinfection or after resolution of primary infection) were tested in an IFN-γ ELISPOT assay against overlapping peptide pools (Core, Env, p7/NS2, NS3, NS4A/B, NS5A, NS5B) representing the HCV genotype 1a (H77) or genotype 3a (K3) polyprotein sequence, depending on primary infection. Patients with unknown viral genotypes were tested with genotype 1a. The frequencies of IFN-γ spot forming cell (SFC) per million PBMCs from spontaneously resolved and/or reinfected patients are shown. The dashed line indicates the minimum cut-off at 50 SFC/million PBMCs. **A-C)** IFN-γ ELISPOT data for indicated patient groups. HCV regions targeted include Core, Env, p7/NS2, NS3, NS4A/B, NS5A, NS5B. P values were calculated using a two-tailed Mann-Whitney test. (*$p<0.05$) SR= Spontaneous resolver, SVR= Sustained virological response

Figure 4

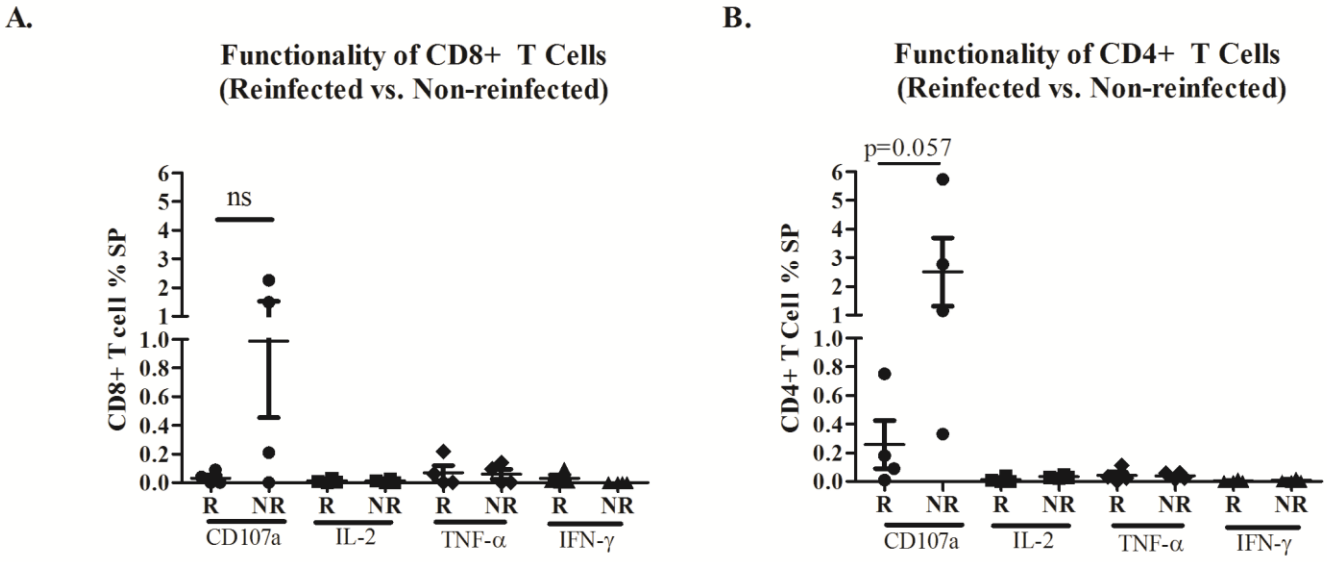


Figure 4. Increased cytotoxic profile in CD4+ T cells in non-reinfected PWIDs. A,B) Comparative graphs showing individual functionality analysis in non-reinfected versus reinfected PWIDs. Intracellular cytokine straining for functionality was performed on PBMCs from the same time point as tested in ELISPOT. The PBMCs were stimulated for 18 hours with peptide pools having the highest IFN-γ response in ELISPOT. P values were calculated using a two-tailed Mann Whitney test. **SP**=Specific Production.

Figure 5

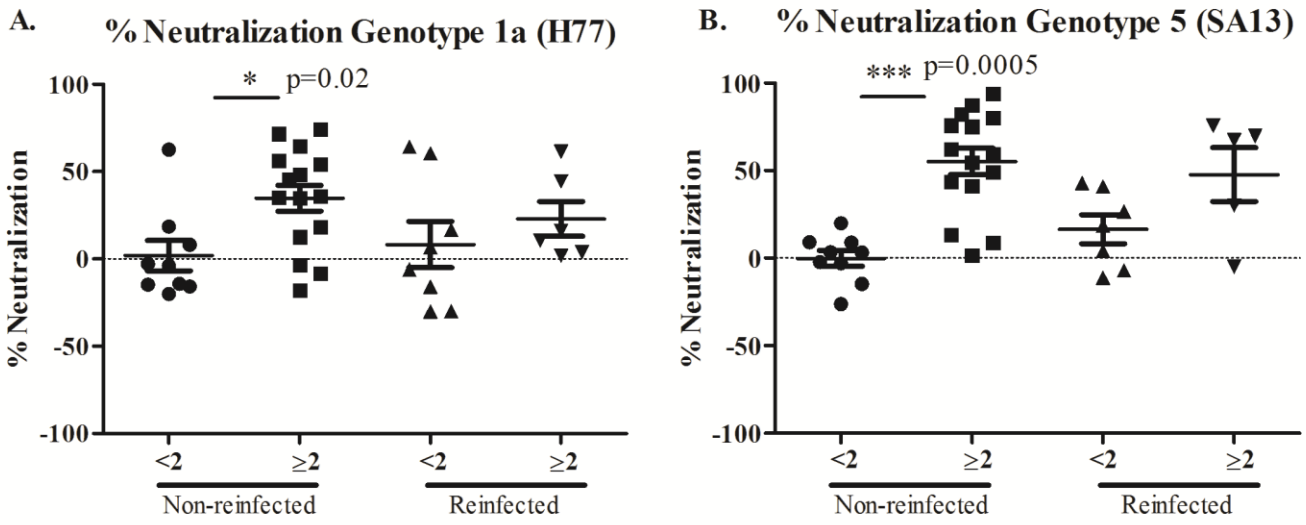
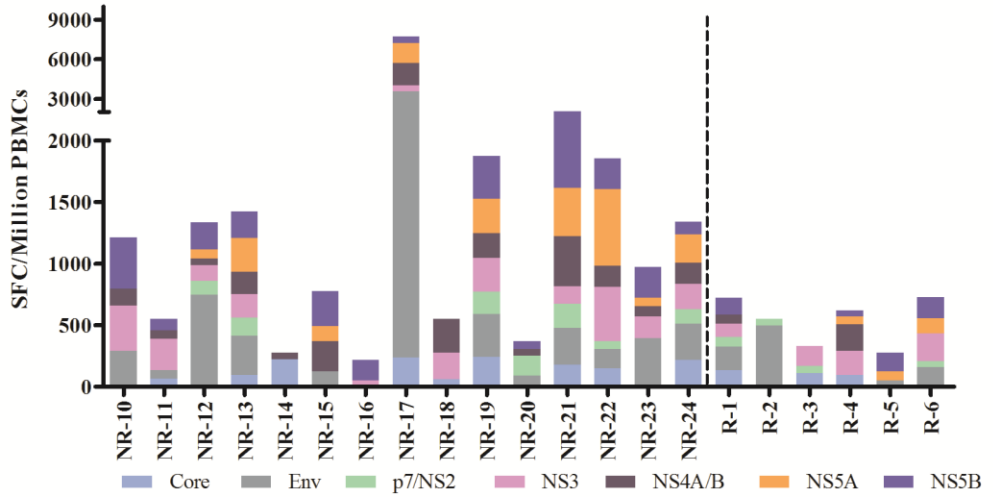


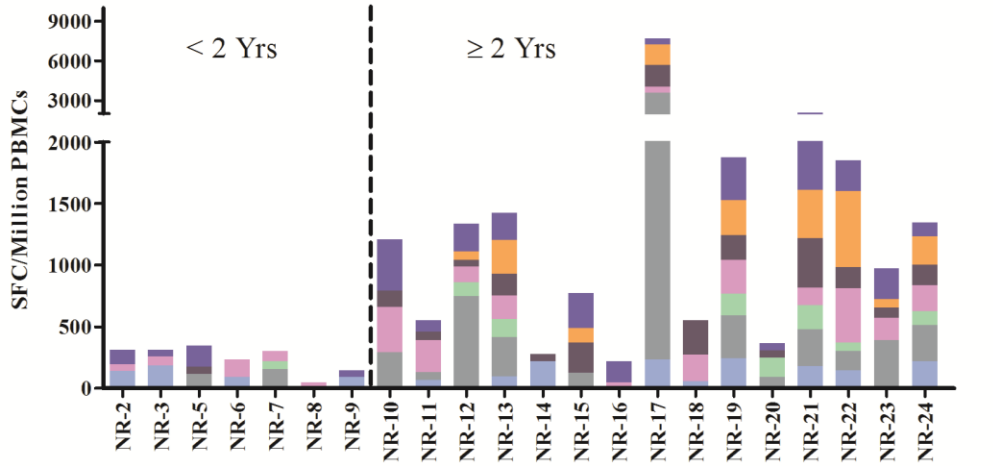
Figure 5. Increased percentage of neutralization in individuals non-reinfected for ≥ 2 years.
A) Neutralization assay done with HCVpp displaying E1-E2 glycoproteins of the H77 HCVgenotype 1 strain.
B) Neutralization assay done with HCVpp displaying E1-E2 glycoproteins of the SA13 HCVgenotype 5 strain.
P values were calculated using two-tailed Mann-Whitney test. (*$p<0.05$, ***$p<0.0001$)

Supplementary Figure S1

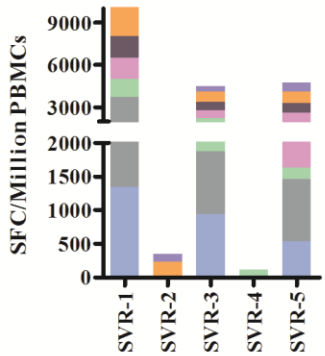
A. Magnitude and Regions Targeted in SR Non-Reinfected (NR) vs. Reinfected (R) (≥2 Years)



B. Magnitude and Regions Targeted in SR Non-reinfected (≥ 2 vs < 2 Years)

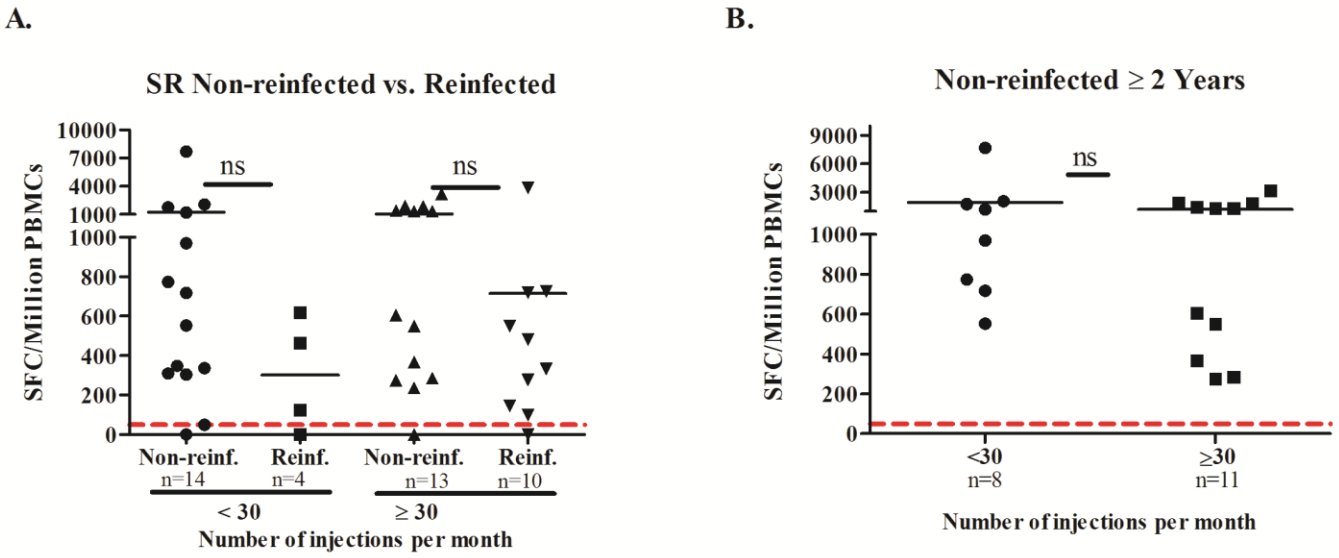


C. Magnitude and Regions Targetedin SVR Non-reinfected (≥ 2Years)



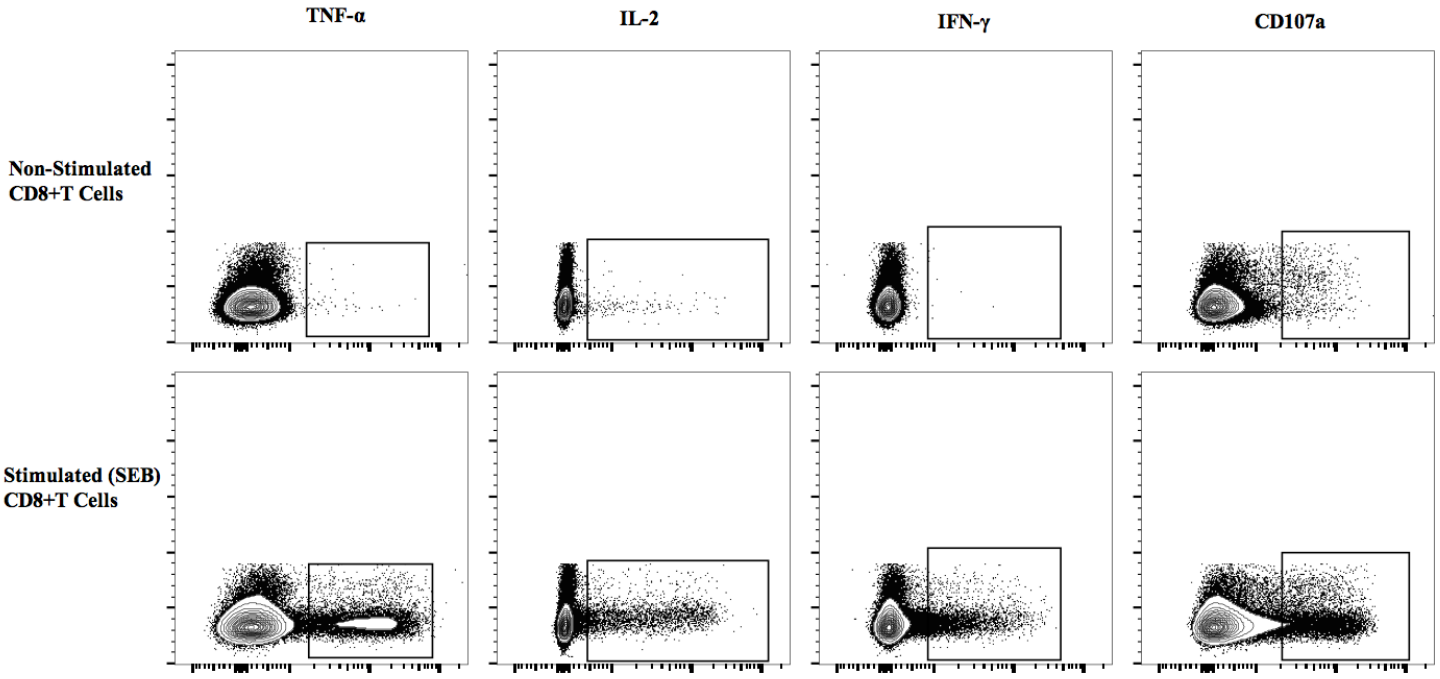
Supplementary Figure S1. A-C) Number of regions targeted (breadth) and magnitude of HCV-specific T cells. A) Comparison between non-reinfected (=2 years) and reinfected (=2 years). **B)** Comparison between non-reinfected (=2 years) and non-reinfected (<2 years). **C)** Regions targeted by HCV-specific T cells in SVR non-reinfected (=2 years) individuals.

Supplementary Figure S2



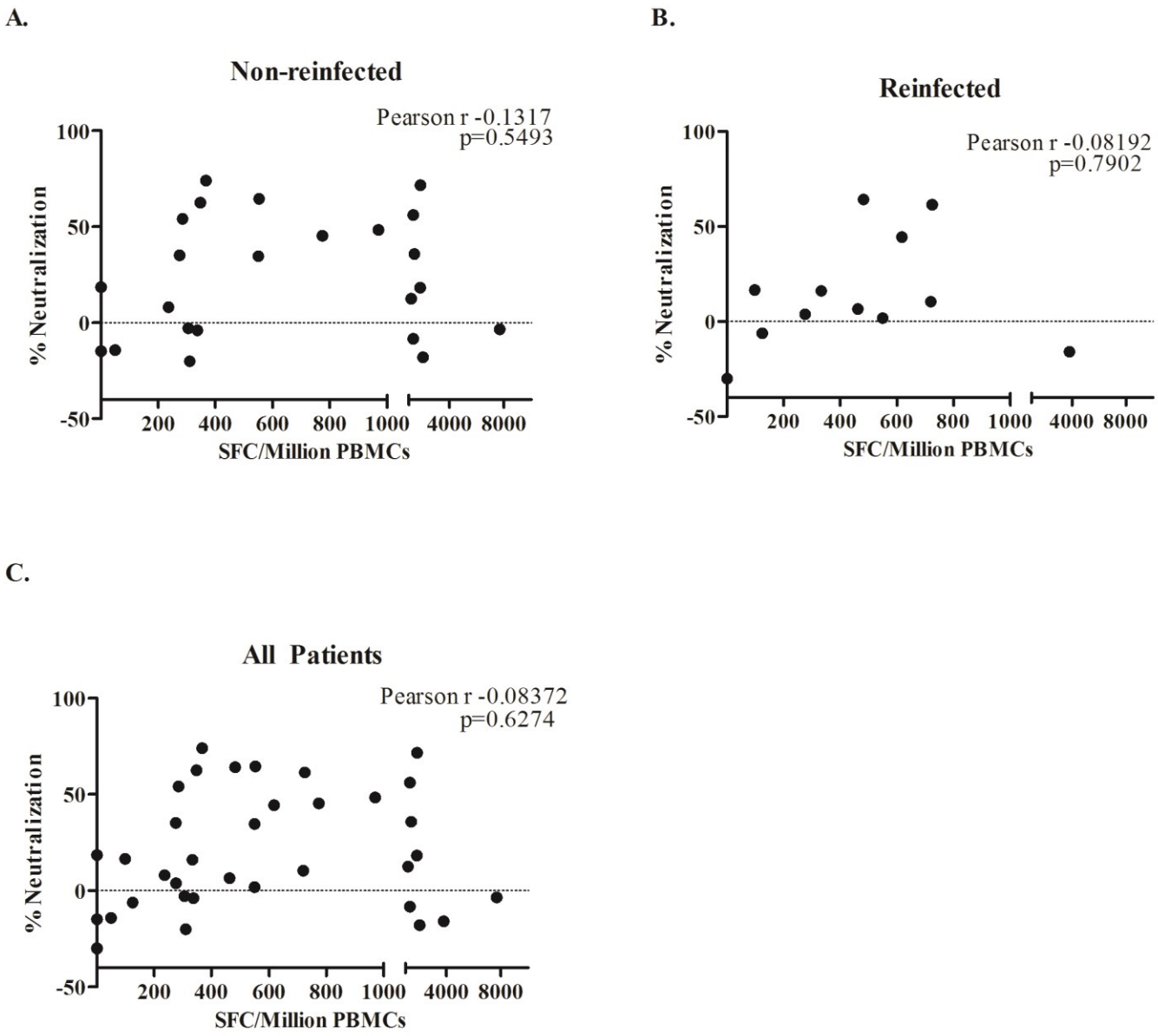
Supplementary Figure S2. Comparison of HCV-specific T cell response and number of injections in the past month before time point tested. A) Comparison between non-reinfected and reinfected and either < 30 or ≥30 injections per month. **B)** Comparison between non-reinfected (≥2 years) and number of injections in the past month. P values were calculated using two-tailed Mann-Whitney test. SR= Spontaneous Resolver

Supplementary Figure S3. Representative Flow Cytometry Plot of Stimulated and Non-Stimualted CD8+ T Cells



Supplementary Figure S3. Representative Flow cytometry gating for intracellular cytokine staining on stimulated (SEB) and non-stimulated CD8+ T cells. Experiments were performed directly *ex vivo* on cryopreserved PBMC samples. Gating was done on live, single, CD3+ and CD8+ T cells. The above gates were applied to determine the percentage of TNF-α, IL-2, IFN-γ, and CD107a-producing cells.

Supplementary Figure S4



Supplementary Figure S4. Correlation between HCV-Specific T cell responses and % Neutralization (against Genotype 1 H77 strain) PBMCs were tested in an IFN-γ ELISPOT as well as in an HCVpp assay displaying E1-E2 glycoproteins of the HCV H77 genotype 1 strain. **A-B)** Correlation between ELISPOT and % neutralization in Non-reinfected (A) and Reinfected (B) individuals. Pearson r values and two-tailed P values are shown. **C)** Pearson r correlation was applied to compare HCV-specific T cell responses and % Neutralization against HCV H77 genotype 1 in all patients (Non-reinfected and Reinfected combined).

Chapter 3: Discussion

Discussion

Results from our cross-sectional study comparing PWIDs exposed to HCV but non-reinfected to those who are reinfected allowed us to better understand the correlates of protective immunity against HCV. Despite the limited availability of samples and confounding factors associated with studies in PWIDs, we were able to organize our data to see interesting trends that will help in understanding the dynamic nature of HCV immunity in a "real-life" setting.

Overall, we see an association between long-lived HCV-specific T cell responses and protection from reinfection in spontaneously resolved individuals. Individuals resistant to a reinfection for more than 2 years post-clearance of a primary infection have significantly higher frequency ($p=0.0224$) and breadth ($p=0.0039$) of HCV-specific T cell responses as compared to individuals who are reinfected (Figure 1B and 2A). Although not significant, a trend in higher CD4+ and CD8+ T cell cytotoxic activity as defined by the expression of CD107a was observed among non-reinfected individuals. SVR patients protected from reinfection have significantly higher ($p=0.0161$) HCV-specific T cells responses compared to SVR reinfected patients suggesting that immune responses restored by antiviral therapy can be protective (Figure 3A). Overall, our results suggest that protection from reinfection in PWIDs is associated with a good HCV-specific T cell response.

3.1. Long-lived, high frequency HCV-specific T cells in exposed non-reinfected patients

A noteworthy observation in our exposed non-reinfected subjects is that HCV-specific T cell responses were detectable up to 20 years post-clearance of primary infection. This is similar to a previous study where a group of women infected through a common source outbreak also had

detectable HCV-specific T cell responses up to 20 years after the clearance of primary infection [183].

We examined the association between the frequencies of injection use with the magnitude of HCV-specific T cell responses. We did not observe any correlation, suggesting that the frequency of drug use is not directly influencing the T cell responses. Coupling this data with a previous report of the group of women accidentally infected during a common source outbreak [183], suggest that HCV-specific T cell responses are long lived independent of drug use activity. However, we cannot exclude the fact that despite our regular follow-up every 3 months, we could have missed short episodes of rapidly clearing viremia.

One interesting phenomenon that was observed in our non-reinfected subjects, whereby those exposed and resistant to reinfection for $\geq$ 2 years had significantly ($p=0.0003$) higher magnitude of HCV-specific T cell responses compared to those non-reinfected (< 2 years) is intriguing. It would be interesting to follow these individuals longitudinally to determine the dynamics of their T cell response to determine when exactly the T cell response surges. A plausible explanation that we cannot rule out is an undetected incident of reinfection that cleared rapidly. However, even if a reinfection occurred and was cleared rapidly, it suggests that the presence of robust memory HCV-specific T cell response was responsible for clearance. It is also possible that memory T cells in the context of HCV take more than 2 years to stabilize beyond the contraction phase of the immune response and during the first two years, individuals may be more susceptible to reinfection.

3.2. Increased frequency of protective IL28B genotype in non-reinfected subjects

When considering the IL28B SNP among non-reinfected versus reinfected individuals we see an interesting observation. Among non-reinfected subjects, 56% (15/27) have the protective CC genotype, whereas among the reinfected only 43% (6/14) have this genotype. It is true that there are fewer participants in the reinfected group, however this observation is in line with previous research that shows that the favourable CC allele is associated with spontaneous resolution of primary HCV infection, response to therapy and re-clearance upon reinfection [189-192, 287]. No doubt, many more participants need to be recruited to make a correlation between the CC genotype and protection from reinfection, however our results show an interesting trend that deserves further investigation.

3.3. Increased cytotoxic profile of HCV-specific T cells in non-reinfected subjects

An interesting observation in our study is the increased expression of the surrogate marker of cytotoxicity, CD107a, in CD4+ T cells as compared to CD8+ T cells. This may seem unusual, however several studies have reported the existence of cytotoxic CD4+ T cells in chronic viral infections. For example, cytotoxic CD4+ T cells as characterized by the secretion of perforin have been reported in HIV-1, Hepatitis D Virus (HDV), HBV and HCV infections [288]. Interestingly, it was reported that perforin-positive CD4+ T cells decreased upon spontaneous clearance of HCV, and remained stable in chronically evolving HCV. A longitudinal follow-up for our patients would be necessary to further elucidate the role of cytotoxic CD4+ T cells in protection against HCV reinfection.

Additionally, it would be interesting to consider the individual secretion of granzyme B and perforin to see if it correlates with CD107a expression. Furthermore, to validate that CD107a+

CD4+ T cells are indeed cytotoxic, standard ^{51}Cr release or similar direct cytotoxicity assays can be performed whereby target cells labelled with ^{51}Cr are incubated with CD4+ effector T cells at varying effector to target ratios [289]. A correlation between CD107a expression and cytotoxicity would further validate our findings.

3.4. SVR-associated protection from reinfection

Reinfection following successful SVR is a major problem among at-risk individuals, especially PWIDs. Due to the high rates of reinfection after SVR, we were interested in examining if there was a difference in the HCV-specific memory response in reinfected versus non-reinfected patients after SVR.

According to our findings, the increased magnitude ($p=0.0161$) of HCV-specific T cell responses post-SVR is an important factor in protection from reinfection against HCV (Figure 3A). When comparing the magnitude of HCV-specific T cell responses in treated (SVR) versus spontaneously cleared participants we observe no statistical significance (Figure 3B) indicating that there is similar protection. In other words, spontaneously resolving an HCV infection seems to be equally as effective in protection from reinfection as getting treated using IFN-α and RBV therapy. However, we did not normalize for risk factors and our sample sizes were small. It would be important to recruit more participants to get a more accurate representation.

Previous research by our group demonstrated the early therapeutic intervention using the standard PEG-IFN and RBV therapy rescued polyfunctional HCV-specific T cells. Importantly, these polyfunctional T cells were phenotypically and functionally comparable to memory T cells in spontaneously resolving patients [156]. We suggest that the non-reinfected patients with higher

magnitude of HCV-specific T cell responses had an early therapeutic intervention that rescued polyfunctional memory T cells. Unfortunately, we were not able to verify the exact times of treatment, as this information was not available as treatment was done before recruitment into the cohort.

Our results suggest that the old-generation antiviral therapy, PEG-IFN and RBV, may play an important role in conferring protection from intravenous drug use-associated reinfection. We hypothesize that those PWIDs with an early therapeutic intervention are the ones with the highest HCV-specific T cell responses that are responsible for this protection.

With the arrival of newer, more potent DAAs, it will be interesting to follow-up patients treated with these new drugs and assess the rate of reinfection, and potentially compare them with the older generation IFN-based therapy. Indeed, novel data suggest that DAAs are able to restore certain aspects of HCV-specific immunity [290]. Furthermore, it would be interesting to investigate the role of nAb in this group of patients to assess if they confer a protective role.

3.5. Increased antibody neutralization among non-reinfected subjects over time

Our results from the antibody neutralization pseudoparticle assay demonstrate that individuals resistant to a reinfection for a long time (≥ 2 years) have significantly higher ($p=0.02$ and $p=0.0005$) level of antibody-mediated neutralization compared to those exposed for less than 2 years against both genotype 1 (H77) and the genotype 5 (SA13) strains, respectively. However, no significant difference was observed between the reinfected and non-reinfected subjects. We also observed that there was a greater neutralization response against the genotype 5 strain compared to the genotype 1 strain which is the predominant strain in our cohort. This data seems

to suggest that the presence of nAb response does not protect against reinfection, however it may play a complementary role, perhaps in reducing the magnitude and duration of a secondary infection as reported previously [274]. However, further experiments examining the viral load and duration of the secondary infection is necessary to confirm this. More importantly, recruiting additional reinfected patients and carrying out nAb assays is crucial in adding more strength to the data as the reinfected group is smaller compared to the non-reinfected group.

Looking more closely at the types of antibodies and their specificities would enable further understanding of the differences between reinfected versus non-reinfected participants. The literature on the role of Fc-mediated antibody activity in controlling HCV is limited and only a single paper reports that ADCC can be mediated by antibodies targeting the E2 protein [291]. Finally, as there are more non-reinfected individuals than reinfected individual in our cohort, recruiting additional patients to the reinfected category will provide more conclusive data.

Strengths and Limitations

The greatest strength of this project is the access to rare and unique documented cases of HCV infection and reinfection among a high-risk cohort. Obtaining access to these patient samples allowed us to study the correlates of HCV immune protection in a real-life setting. Since this cohort is comprised of many marginalized and, often, homeless individuals, there are a number of challenges associated with working with this population. Firstly, it is sometimes difficult to contact the participants if they miss an appointment to donate blood, thereby making it challenging to have a longitudinal approach to the study. Ideally, a longitudinal approach would have been more informative, however, due to unavailability of blood samples at exactly the same time-points in the infection for each participant and the loss to follow-up in this cohort, this was not feasible.

Secondly, working with patient samples, we are restricted to the number of parameters we can test, as there is a limited supply of PBMCs, requiring resourcefulness and careful planning.

The ELISPOT technique is a very powerful and sensitive technique that allows for the detection of antigen-specific cells. It also allows for the quantification of the number of regions targeted as overlapping peptides can be added into separate pools. This technique is even more sensitive than flow cytometry as we were able to detect IFN-γ producing cells in ELISPOT, and not with flow cytometry.

The HCVpp neutralization assay is based on lentiviral particles expressing the HCV Env proteins, and is therefore not a real infectious virion. In our study, the Env glycoproteins were derived from only 2 different viral genotypes (Genotype 1a and 5). Optimally, it would have been more relevant to our cohort to have an HCVpp expressing the Env glycoproteins of genotype 3 since it is prevalent in our cohort. A more authentic system is the HCV cell culture (HCVcc) particles which are derived from the JFH-1 strain that can generate infectious particles. In the future it would be interesting to use this HCVcc system to study in-depth the neutralizing activity in patient serum against genetically diverse viruses.

Conclusions

We were able to demonstrate a correlation between long-lived HCV-specific T cell responses and protection from reinfection in spontaneously resolved individuals. Individuals resistant to a reinfection for more than 2 years post-clearance of a primary infection have significantly higher frequency and breadth of HCV-specific T cell responses as compared to reinfected individuals. We demonstrate that there is an increase in the nAb responses over time (<2 years vs ≥ 2 years) among the non-reinfected individuals, however this difference is not significant when compared to the reinfected individuals. This may be due to the very low number of participants in the reinfected category, and further participants should be recruited to validate these findings. Moreover, there is a trend of higher CD4+ and CD8+ T cell cytotoxic activity as defined by the expression of CD107a among the non-reinfected subjects, however this difference was not significant. Furthermore, our results suggest that the old-generation antiviral therapy, PEG-IFN and RBV, may conferring protection from intravenous drug use-associated reinfection. We hypothesize that those PWIDs with an early therapeutic intervention are the ones with the highest HCV-specific T cell responses that are responsible for this protection.

Future Directions

The unique group of non-reinfected patients with extremely robust T cell responses as well as substantial neutralizing antibody responses are worthy of further experimentation. Further tests to determine other important parameters that influence antibody responses such as Tfh cells are worth investigating. Moreover, as some reinfected individuals also displayed high HCV-specific T cell responses, examining markers of T cell exhaustion, such as TIGIT, PD-1, CTLA-4 will perhaps help elucidate the differences in the quality of the T cell response.

As we were unable to observe substantial polyfunctionality in our patients, further optimization of protocols should be undertaken to obtain more representative results. Moreover, as we noticed increased cytotoxicity as characterized by the expression of CD107a, it would be interesting to look at individual perforin and granzyme secretion by intracellular cytokine staining. In addition, direct cytotoxicity assays (ex. ^{51}Cr release) would further confirm our observations. Furthermore, an in depth longitudinal follow-up of the reinfected and non-reinfected patients will also be beneficial in elucidating the dynamics to the T cell and B cell response since we observed a significantly higher HCV-specific T cell response after ≥ 2 years compared to < 2 years post-resolution of primary infection. Lastly, as we had limited numbers of participants in our cohort, collaborations with similar cohorts would allow us to expand the number of subjects to ultimately have a better understanding of protective immunity against HCV.

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Appendix I: The candidate's contribution to the article

The candidate, Asiyah Siddique, performed 90% of the ELISPOT experiments and analysis, and all of the flow cytometry work and analysis. She also participated in writing the manuscript and prepared all the figures included.