Université de Montréal

Virus–Host Interaction during Therapy against Hepatitis C Virus

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Résumé

Le virus de l'hépatite C (VHC) est un problème mondial. La majorité des personnes infectées (70-85%) développent une infection chronique qui cause des complications hépatiques. Le seul régime thérapeutique approuvé pour le VHC est l'interféron alpha (IFN-α). Ce traitement a un taux de réussite de 50-80% selon le génotype de virus et le moment de l'initiation de la thérapie. Les facteurs régissant la réponse au traitement ne sont pas bien définis. Des études antérieures ont suggéré un rôle potentiel de la réponse immunitaire de l'hôte au succès de la thérapie, toutefois, ces résultats sont controversés.

Nous avons émis l'hypothèse que la réponse immunitaire de l'hôte sera plus efficace chez les patients qui commencent la thérapie tôt pendant la phase aiguë de l'infection. En revanche, la réponse immunitaire sera épuisée lorsque le traitement est initié pendant la phase chronique. L'objectif principal de ce mémoire est d'étudier les facteurs immunologiques qui régissent la réponse à la thérapie, et de déterminer si la contribution de la réponse immunitaire de l'hôte peut être influencée par la période de l'infection.

Nos résultats démontrent l'efficacité de la restauration de la réponse immunitaire spécifique au VHC lorsque la thérapie par l'interféron est initiée tôt. Ceci est démontré par le sauvetage des cellules T efficaces spécifiques au VHC efficace similaires à celles observées chez les individus qui ont résolu spontanément, suggérant ainsi qu'elles jouent un rôle actif dans la réponse au traitement. Toutefois, cette réponse n'a pas été restaurée chez les patients traités au cours de la phase chronique. Ces résultats ont des implications importantes dans la compréhension des mécanismes sous-jacents à la réponse aux traitements actuels et au développement des nouvelles thérapies.

Mots-clés : virus de l'hépatite C (VHC), l'interféron alpha (IFN-α), immunité adaptative, infection aiguë, infection chronique.

Abstract

Hepatitis C virus (HCV) is a major public health problem worldwide. Only 15-30% of infected individuals clear the virus spontaneously, while the majority develops chronic infection that causes liver complications. The only approved therapy for HCV is interferon alpha (IFN-α) based. This therapy has a 50-80% success rate depending on the infecting virus genotype and the timing of initiation of therapy. Factors governing the response to therapy are not well defined. Previous studies have suggested a role for the host immune response in the success of therapy. However, these results were controversial.

We hypothesized that host immunity has an effective role in the success of IFN-α therapy when initiated early during the acute phase of HCV infection, while late initiation during the chronic phase minimizes this role. The main objective of this thesis was to dissect the immunological factors governing the differential response to IFN-α therapy, and to determine if the contribution of the immune response to success of therapy might be influenced by the period of infection.

Our results demonstrate restoration of efficient HCV-specific immune responses when therapy is initiated early during the acute phase. This is demonstrated by the rescue of functional HCV-specific T cells similar to those observed in spontaneously resolved individuals, suggesting that they may play an active role in response to therapy. However, such responses were not restored following late therapy suggesting irreversible damage to the host's defence system with chronicity. These findings have important implications in understanding the mechanisms underlying response to current treatments and development of novel therapies.

Keywords: Hepatitis C virus (HCV), Interferon alpha (IFN-α), adaptive immunity, acute infection, chronic infection.

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List of abbreviations

APC	Antigen presenting cell
ARFP	Alternative reading frame protein
CCR	Chemokine receptor
CFSE	Carboxyfluorescein succinimidyl ester
CLDN1	Claudin-1
CMI	Cell-mediated immunity
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte associated antigen-4
DC	Dendritic cell
dsRNA	Double-stranded RNA
E1-E2	Envelope glycoprotein 1- Envelope glycoprotein 2
eIF	Eukaryotic initiation factor
ELISpot	Enzyme-linked immuno spot
EVC	Early virological clearance
EVR	Early virological response
F protein	Frameshift protein
FasL	Fas ligand
FOXP3	Forkhead transcription factor 3
GTP	Guanosine triphosphate
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVpp	Hepatitis C virus pseudoparticles
HLA	Human leukocyte antigen
HVR1	Hypervariable region-1
ICTV	International committee for the taxonomy of viruses
IDU	Intravenous drug user
IFN	Interferon

IL	Interleukin
IMPDH	Inosine-monophosphate dehydrogenase
IP-10	Interferon-inducible protein-10
IRES	Internal ribosome binding site
ISG	Interferon stimulated genes
IU	International unit
JFH1	Japanese fulminant hepatitis 1
Kb	Kilo bases
KIR	Killer cell immunoglobulin-like receptors
MAP	Multi-analyte profiling
mDC	Myeloid dendritic cell
Met	Methionine
MHC-I,II	Major histocompitability complex class I, class II
nAb	Neutralizing antibody
NANB	Non-A non-B
nm	Nanometre
NCR	Non-coding region
NK	Natural Killer
NR	Non-responder
NS	Non-structural
OAS	Oligoadenylate synthetase
OCLN	Occludin
ORF	Open reading frame
PAMP	Pathogen associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PD-1	Programmed death 1
pDC	Plasmacytoid dendritic cell
PEG	Polyethylene glycol

RdRp	RNA-dependant RNA-polymerase
RIG-I	Retinoic-acid-inducible gene I
RNAi	RNA interference
SCID	Severe combined immunodeficiency
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SR-BI	Scavenger receptor class B type I
+SS RNA	Positive single-stranded RNA
SVR	Sustained virological response
$t_{1/2}$	Half-life
T_{CM}	Central memory T cells
T_{EM}	Effector memory T cells
Th1,2	Helper T-cell subtype 1, 2
TJ	Tight junction
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
T_{TEM}	Transitional effector memory T cells
tRNA	Transfer RNA
uPA	Urokinase-type plasminogen activator
US	United States
UTR	Un-translated region
WHO	World Health Organization

To the three most important ladies in my life;
my mother, who taught me how to be a real man,
my grandmother, who inspired my love for knowledge,
and my wife, who supported me through the most challenging of times.

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Chapter 1: Literature Review

1.1. Historical preface

Hepatitis C virus (HCV) emerged as a major health problem worldwide since the seventies when it was recognized as a causative agent of transfusion–associated liver inflammation (hepatitis) other than hepatitis A and B viruses, leading to its primary classification as non-A non-B (NANB) [1, 2]. It was characterized as a small enveloped virus being able to pass through 50 nanometre (nm) filters and inactivated by lipid solvents [3, 4]. In 1989 a team led by Michael Hughton at Chiron corporation cloned the genome of HCV from a large volume of plasma obtained from a chronically infected chimpanzee [5]. They demonstrated that it was a positive single stranded RNA (+ssRNA) molecule, nearly 10,000 nucleotides long [6]. The earliest epidemiological studies reported that over 130 million people worldwide were infected with this newly discovered virus [7].

1.2. The virus

1.2.1. General characteristics and structure

HCV is a non-cytopathic virus primarily infecting hepatocytes [8], yet the high rate of extra-hepatic manifestations accompanying HCV infection suggested that peripheral blood mononuclear cells (PBMCs) were infected. It is already reported that B cells, T cells and dendritic cells contained HCV RNA [9].

Despite the huge advances in the study of the HCV genome and proteins, viral particles have only been extensively studied through culture systems since 2005 [10]. Early data form electron microscopy and imaging demonstrated that the HCV nucleocapsid is approximately 40-70 nm in size and built from units of Core protein together with the RNA genome surrounded by a human-cell derived membrane with embedded envelope glycoproteins (E1/E2) heterodimers [10, 11].

1.2.2. HCV genome and proteins

The HCV genome is an uncapped +ssRNA of approximately 9.6 kilo bases (kb) [11]. It contains an uninterrupted open reading frame (ORF) encoding a polyprotein precursor of approximately 3000 amino acids [5] which is further processed by cellular and viral proteases into ten proteins: four structural proteins (Core, E1, E2, p7) and six non–structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Fig. 1.1.), all of which are transmembrane proteins associated with intracellular membranes [12]. The genome also contains a 5'untranslated region (5'UTR) and a 3'UTR [13]. An alternate reading frame protein (ARFP), also known as frameshift protein (F protein), was identified in the HCV Core region due to a ribosomal frame-shift [14]. Yet, it is not required for HCV replication [15] and its function(s) remain to be elucidated.

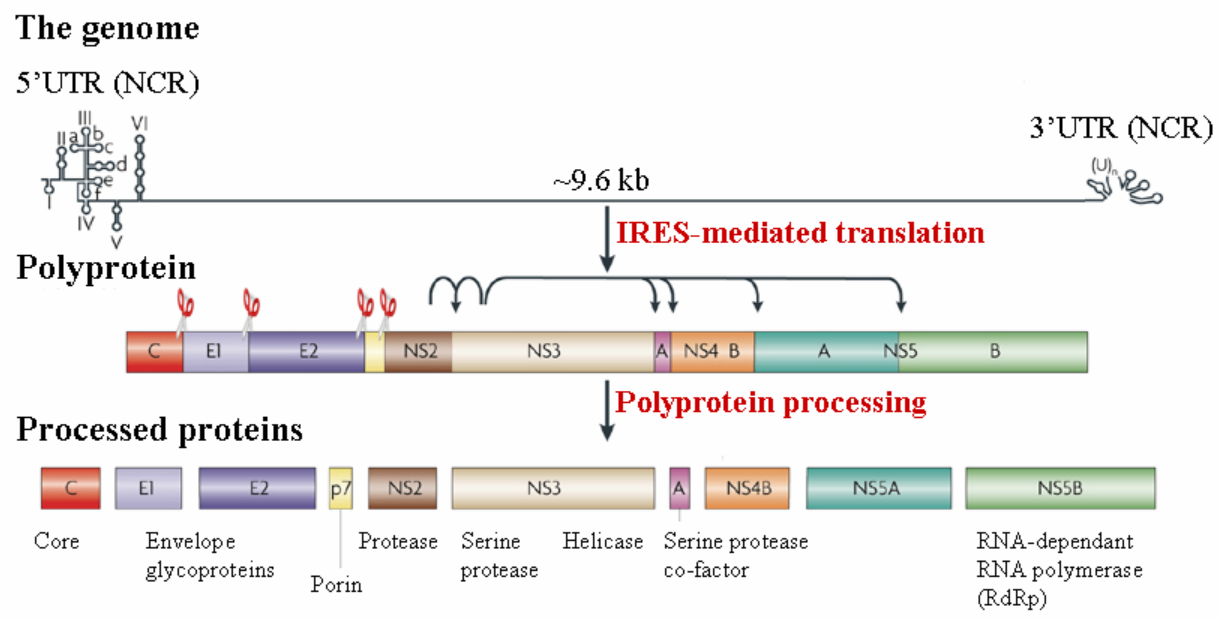

Figure 1.1: HCV genomic organization and encoded proteins. HCV RNA is translated into a single polyprotein molecule which is further processed into the HCV proteins and enzymes. The scissors show the early processing steps that take place by cellular enzymes, followed by auto-processing by the virus's own enzymes shown by arrows.
(Adapted from Moradpour DF, et al., *Nat. Rev. Microbiol.*, 2007) [12]

1.2.2.1. The non-coding regions (NCRs)

Most of the 5'UTR is occupied by the internal ribosome binding site (IRES) that can direct translation of the downstream reading frame in a cap independent manner [16-18]. It forms 4 domains (I-IV), domains I and II are essential for HCV RNA replication [19]. 5'UTRs from different genotypes direct translation with different efficiencies [20, 21]. The 3'UTR is formed of a short variable region of approximately 30 nucleotides, a poly (U/UC) stretch and a highly conserved 98-base sequence designated the 'x-tail' [22, 23]. Current evidence suggest the importance of 3'UTR in viral replication [24]

1.2.2.2. The viral proteins

The Core protein amino acid sequence is highly conserved among different HCV strains [25]. The Core is involved in the formation of the HCV nucleocapsid [12] and was shown to modulate the host's intracellular signalling pathways [26]. The two envelope glycoproteins (E1 and E2) form complexes which are the building unit of the envelope [27], and are necessary for viral entry [28, 29]. The P7 protein is a small hydrophobic polypeptide suggested by many reports to function as a viroporin or ion channel [30, 31].

NS2 is essential for viral replication *in vivo* [32]. NS2/3 possesses a cysteine autoprotease activity necessary for the polyprotein processing. This activity resides in the C-terminal half of NS2 and the N-terminal third of NS3 [33, 34].

NS3 is a complex multi-functional molecule essential for both viral protein processing and RNA replication [34], with a serine protease located in the N-terminal third and RNA helicase/NTPase residing in the C-terminal two thirds of the protein [35]. The helicase domain unwinds RNA-RNA substrates in a 3' to 5' direction [36]. The serine protease catalyses the processing of the polyprotein downstream of the NS3 region [25]. NS4A is the co-factor for the NS3 serine protease [37].

NS4B and NS5A are poorly characterized with functions not fully elucidated. However, studies show that NS4B induces the formation of a membranous-web compartment where viral replication takes place [38, 39], and cell-culture adaptive mutations mapped to the NS5A enhance RNA replication suggesting its importance for viral replication [40-42].

NS5B is the viral RNA-dependant RNA-polymerase (RdRp) responsible for HCV-RNA replication [12] with the hallmark GDD motif [43] and a typical right-hand structure [44, 45]. As with other RNA viruses this enzyme lacks proof-reading activity and is highly error prone, leading to the emergence of different viral populations circulating in the blood of an individual patient as a 'mosaic' of highly related sequences termed 'quasispecies'. These quasispecies despite phylogenetic proximity and limited sequence differences differ in replication fitness and response to therapy [46].

1.2.3. Classification

Given that HCV has genomic organization similar to flaviviruses [47] the International Committee for the Taxonomy of Viruses (ICTV) classified HCV as a member of the hepacivirus genus in the *Flaviviridae* family since 1991 [48]. Despite variability of the HCV genome, certain highly conserved regions were identified in the Core/E1 and NS5B that were used as the basis of HCV genotyping in 1994, thus classifying HCV into genotypes 1 to 6 which differ from each other by 31–33 % at the nucleotide level, and many subtypes (*e.g.* 1a, 1b, 1c) with 20–25 % differences [49-52]. HCV variants are classified based on partial sequencing of the highly conserved regions Core/E1, NS5B, 3'UTR and 5'UTR [53], 5'UTR being the region commonly used for genotyping [54, 55]. Despite the sequence diversity of HCV, all genotypes have an identical set of genes of nearly identical size [55]. Yet, the different genotypes show marked differences in geographic distribution and response to therapy [56].

1.2.4. Difficulties in studying HCV

The major obstacles facing HCV research include the difficulty of early diagnosis of acute cases, the absence of animal models other than the chimpanzee and the inability to produce complete infectious virus particles in an *in vitro* culture system.

Acute infection, empirically defined as the first six months after infection, is often undiagnosed because it is usually asymptomatic [57]. That is why most of the initial views about HCV were mainly based on studying chronically infected cases [46]. Studies of early events of acute infection relied on clinical parameters to identify patients with symptomatic HCV [58-60]. Recently there was a focus on prospective studies of human cohorts at high risk of HCV infection, as health care workers and intravenous drug users (IDU) [61].

Different animal models were developed for studying HCV, but these supported limited replication of the virus (e.g. SCID/uPA mice) [62]. Chimpanzees remain the only available model for HCV persistence [63]. Despite the high cost, different clinical course and ethical limitations, this model enabled assessment of the infectivity of HCV clones and provided insight into the early immune events and protective immunity [64].

The inability to produce infectious HCV particles in culture systems promoted the development of alternative *in vitro* models to study the HCV viral cycle, such as, using recombinant HCV envelope proteins to generate HCV pseudo-particles (HCVpp) [29, 65]. But these models were only useful for studying the entry steps of the viral life cycle. Replicon systems were the starting point for studying the virological characteristics of HCV and enabled the recapitulation of intracellular events of the viral cycle, but theses replicons were unable to produce infectious viral particles [66]. In 2005 Wakita *et al.* identified a genotype 2a HCV isolate from a Japanese patient with fulminant hepatitis (JFH-1) that replicated in tissue culture of a human hepatoma cell line producing high titres of infectious virus particles *in vitro* capable of infecting naïve culture cells and chimpanzees [10].

1.2.5. HCV life cycle

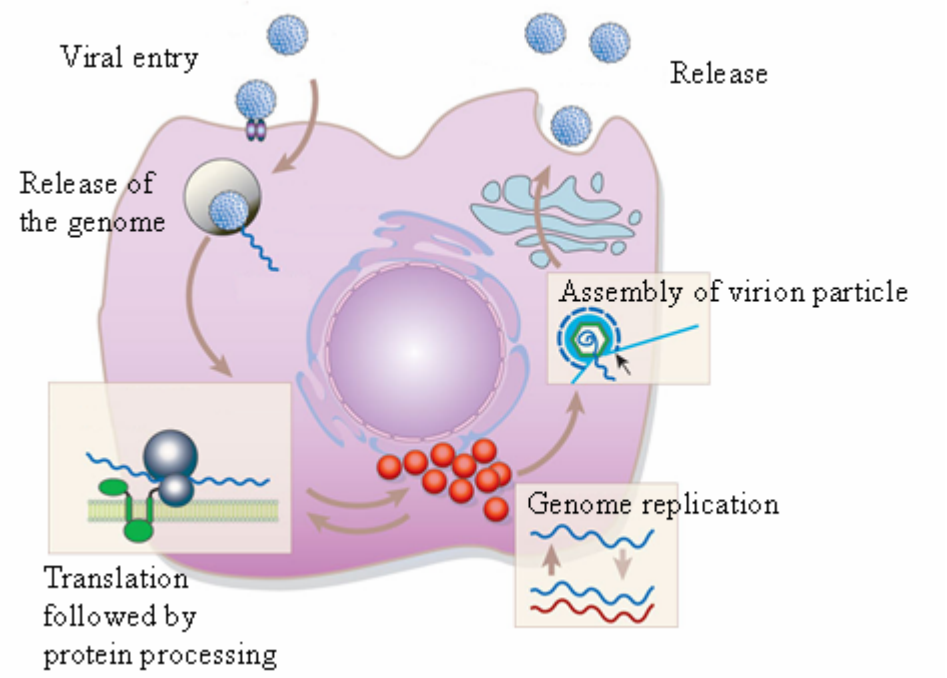

Figure 1.2: A schematic diagram of HCV's replication cycle. The HCV replication cycle steps: 1- Binding to specific receptors and entry, 2- Release of the genome, 3- Translation and processing of the produced polyprotein, 4- Replication producing numerous genomes, 5- Assembly of virus particles and 6- Release through the cell's secretary pathways. (Adapted from Lindenbach BD and Rice CM, *Nature*, 2005) [67]

1.2.5.1. Receptors and viral entry

Receptors involved in binding and entry of a given virus determine its tissue tropism and host range [25]. The first described receptors for HCV were CD81 [68] and scavenger receptor class B type I (SR-BI) [69], supported by the observation that antibodies or RNA interference (RNAi) against either CD81 or SR-BI potentially inhibit entry of HCVpp into hepatoma cell lines that are normally permissive [70-73]. Antibodies against CD81 do not affect HCVpp binding to cells, but inhibit viral entry after the initial attachment has taken place. Therefore it is suggested to be a co-receptor, while SR-BI is suggested to be a primary receptor [74, 75]. Tight junction (TJ) protein Claudin-1 (CLDN1) has also been identified as a receptor/co-receptor for HCV. Kinetics of inhibition suggest that the first encounter is with SR-BI before interacting with CD81, then CLDN1 might play a role in a late step (Fig. 1.3) [76]. However, cell lines expressing all three entry factors are resistant to HCV entry [77], suggesting the involvement of more receptors and co-receptors. The most recently reported factor essential for HCV cell entry is another TJ protein, occludin (OCLN), which conferred HCV permissiveness to mouse cell lines [78, 79].

Binding is followed by fusion of the viral envelope with the cellular membrane by clatherin mediated endocytosis [80] then fusion with the endosomal membrane and the viral genome is released into the cytosol [28].

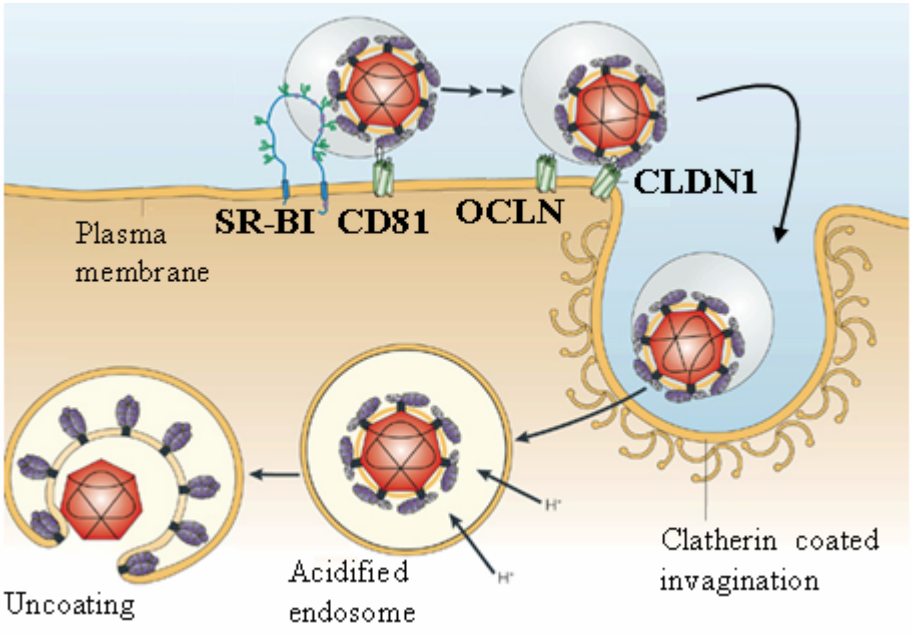

Figure 1.3: Early steps of HCV's replication cycle. Sequential binding to receptors and co-receptors as suggested by kinetics studies, followed by clatherin dependant endocytosis, entry and uncoating.

(Adapted from Moradpour DF, et al., *Nat. Rev. Microbiol.*, 2007) [12]

1.2.5.2. Translation, replication and virion release

Translation of the HCV genome (lacking a 5' cap) depends on the IRES [17], which binds the 40s ribosomal subunit directly without the need for pre-initiation factors [81] forming a binary complex that binds to eukaryotic initiation factor 3 (eIF3). This nascent complex recruits the ternary complex of Met-tRNA-eIF2-GTP to form non-canonical 48s intermediate that attaches to the 60s subunit to finally give the active 80s complex forming the first peptide bond [82, 83].

The major product of HCV ORF translation is a large polyprotein that is subsequently processed into mature structural and non-structural proteins (Fig. 1.1). Junctions between structural proteins are processed by host signal peptidases from the endoplasmic reticulum [12, 84]. The viral non-structural (NS) proteins are processed by two proteases encoded by HCV RNA: the NS2/3 autoprotease that accomplishes the processing between NS2 and NS3 [12, 25] and the NS3/4A serine protease that processes the remaining four junctions by sequential cleavage [85].

Replication starts by the NS5B RdRp synthesis of a complementary full-length negative strand RNA using the genome as a template then using the negative strand for the production of many copies of the genomic positive strand in a replication complex [86, 87] associated with lipid-raft based membranes [88, 89]. Using the reverse genetics approach, it has been shown that all viral enzymatic activities, the P7 gene and the 3'UTR are necessary for *in vivo* HCV-replication [32, 90, 91].

Data from expression systems show that once an HCV nucleocapsid is formed in the cytoplasm it acquires an envelope as it buds through an intracellular membrane [65, 92, 93] producing particles of heterogeneous size ranging from 30 to 80 nm [84] that are released from the cell through the secretary pathway (Fig. 1.2) [25].

1.3. The disease

1.3.1. Epidemiology

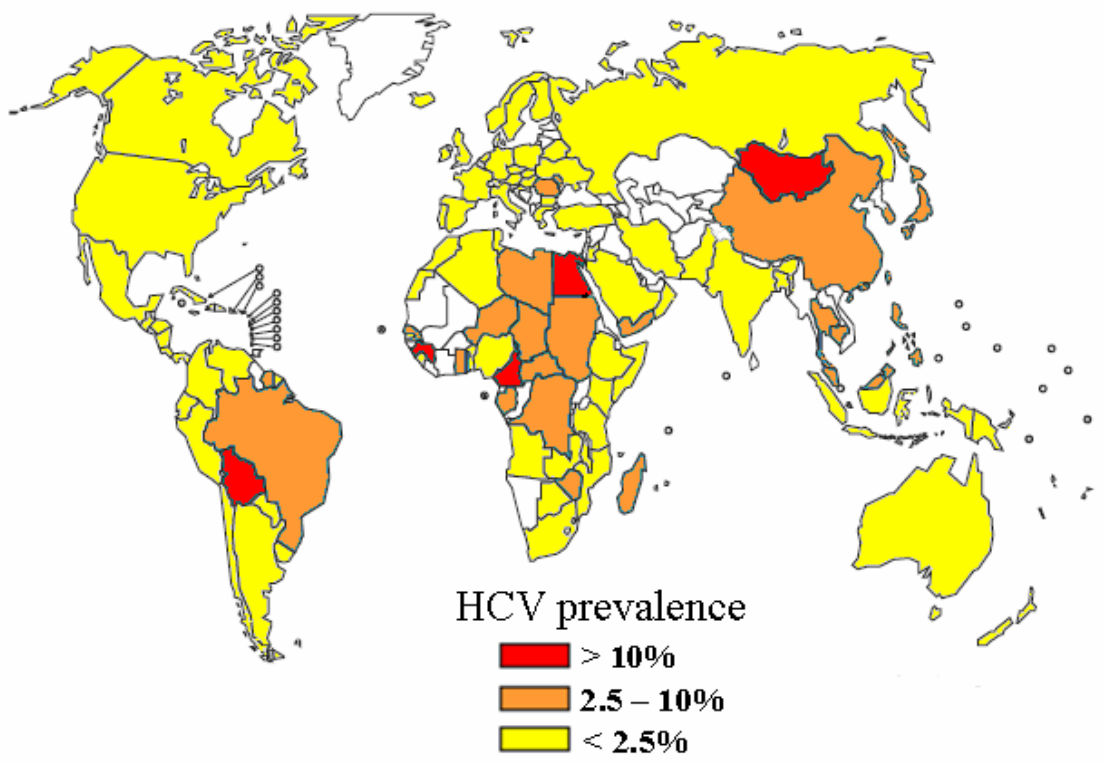

Figure 1.4: Prevalence of HCV infection worldwide. A world map showing the higher prevalence of HCV in many developing countries. The countries with the highest rate worldwide include Egypt, Bolivia and Mongolia.
(Adapted from the International travel and health report, WHO, 2008) [94]

The World Health Organization (WHO) estimates HCV prevalence at 3.1% worldwide, approximately 170 million people [95]. The prevalence is higher in developing countries posing both a public health problem and an economic burden (Figure 1.4), the highest being in Egypt (15-20%) [96, 97]. Moreover, new cases occur every year, approximately 38,000 in the US alone [98]. In Canada, the prevalence is 0.8% with continuous new incidence [99]. This is equal to approximately 275,000 individuals, of which at least 30% are intravenous drug users (IDUs) [100]. These numbers might not be representative as many HCV patients belong to marginalized groups or live in secluded areas in developing countries with limited access to health care, where the inclusion in epidemiological studies is very difficult and the rate of undiagnosed infection is high [46].

1.3.2. Transmission

The most efficient mode of transmission of HCV is direct exposure to infected blood (*e.g.* transfusion from infected donors, haemodialysis or injection drug usage) [101-103]. The former was the main etiological route for the spread of HCV before its cloning and the implementation of blood-donor screening since the early nineties. This lead to a vast reduction of transfusion-related transmission [104], which now stands at less than 1% in developed countries [105]. Other methods of transmission are less efficient. Perinatal transmission takes place at an average rate of 4-7%, and only with mothers who are HCV-RNA positive at delivery [106]. Occupational transmission [107] and the still controversial sexual transmission [108, 109] account for 1.8% and 1.5%, respectively. Increased risk was observed with intranasal cocaine use, tattooing, body piercing, ritual scarification and circumcision, but still none of these activities was consistently correlated with HCV transmission [110, 111].

The high HCV prevalence in Egypt was linked to the massive anti-schistosomal public health program that took place during the late sixties and the early seventies. During this program non-disposable syringes were used with insufficient sterilization between

individuals, thus contributing to HCV spread. This represents a perfect example of iatrogenic transmission [96].

Presently there seem to be two patterns for HCV transmission. In developed countries, the sharing of contaminated needles between IDUs accounts for ~68% of new cases, while in the developing world unsafe therapeutic injections and transfusions account for ~40% [97].

1.3.3. Natural history of HCV infection

An important characteristic of HCV is its high tendency to establish a chronic infection in the host. Approximately 70% (55-85%) of acutely infected patients progress to chronicity [57]. Another characteristic of HCV is its rapid turnover with a half-life ($t_{½}$)of 3 hours, producing up to 10^{12} viruses in a single patient per day [112], thus reaching high serum titres of the virus by week one into the infection [113, 114]. Nevertheless, only one third of the patients develop jaundice during the acute phase of the infection [6] while the rest remain undiagnosed for years before any symptoms of liver disease are evident, thereby leading to the spread of infection and missing the opportunity of early treatment [115, 116]. In chronically infected patients, it was observed that levels of viraemia remain stable over long periods of time [117].

Being a non-cytopathic virus, many studies focused on the contribution of immunopathogenesis to liver damage (fibrosis and cirrhosis) in HCV-infected individuals. Many mechanisms were proposed for immunopathogenesis, either directly due to enhanced CTL-mediated apoptosis of hepatocytes [118], or indirectly by persistent secretion of chemokines causing chronic inflammation of the liver, thus leading to tissue damage [119]. These studies were initially driven by the finding noting the temporal coincidence of acute hepatitis C symptoms –when present– with the expansion of virus-specific CTLs and the increase in their effector functions [120, 121].

Up to 20% of patients with chronic viral hepatitis C subsequently develop complications including end-stage cirrhosis, liver failure and hepatocellular carcinoma (HCC) [122]. These patients represent a quarter of the cirrhosis and HCC cases worldwide [123]. This makes hepatitis C the most common indication for orthotopic liver transplantation (40-50%) [124], but viral recurrence usually occurs after transplantation despite prophylaxis with immune globulin or other agents [125].

1.4. The immune system and HCV

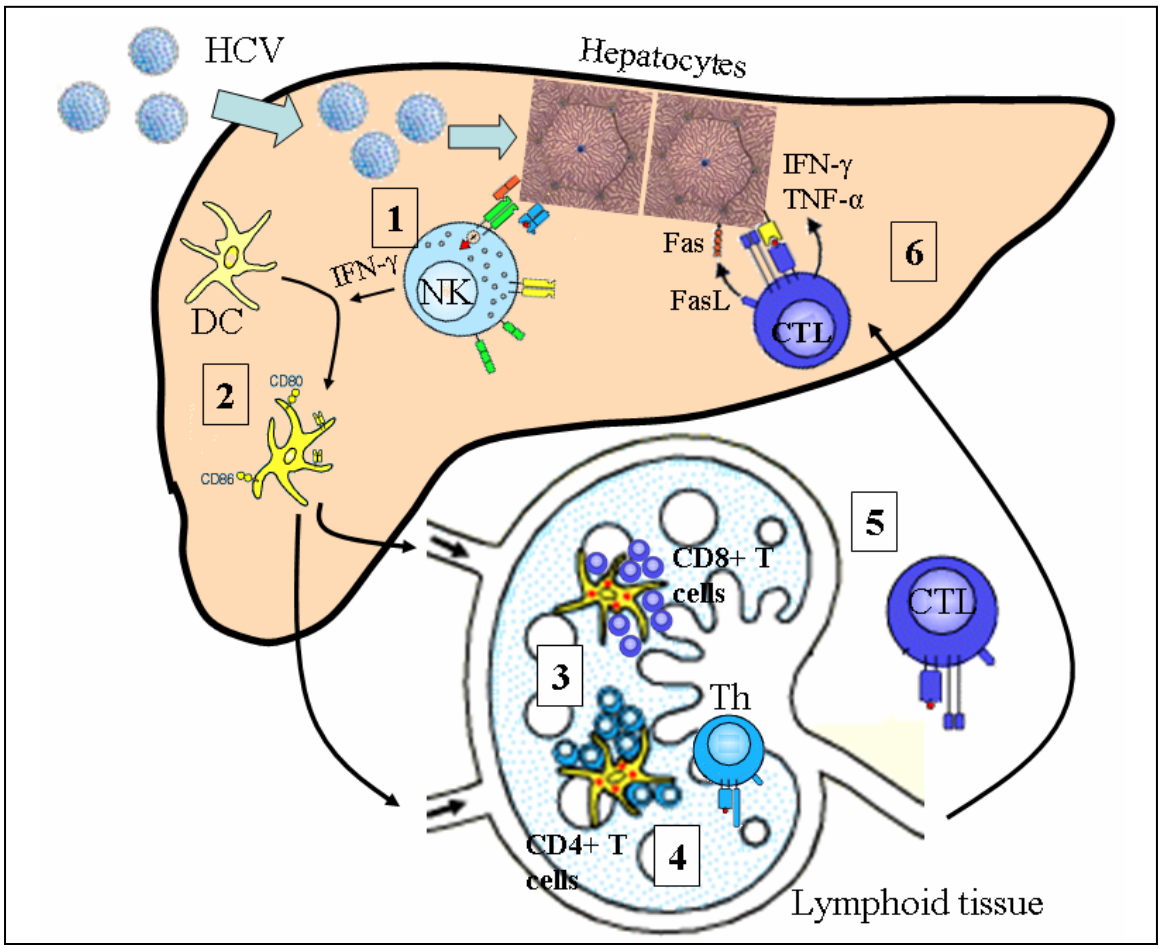

Figure 1.5: Major events in the immune response against HCV. 1, the first cells to act directly against the virus are NKs. 2, NKs also induce DC maturation. 3, DCs migrate to the lymphoid tissue where they stimulate both CD4+ T cells to become effector Th cells and CD8+ T cells to become effector CTLs, 4 and 5. 6, CTLs mediate cell death by direct cytotoxicity, through cytokines and/or Fas/FasL mediated apoptosis. CTL, cytotoxic T lymphocyte ; DC, dendritic cells; FasL, Fas ligand; IFN-γ, interferon-gamma; NK, natural killer cells; Th, helper T cell; TNF-α, tumour necrosis factor-alpha.
(Adapted from Kanto T and Hayashi N, *Internal Medicine*, 2008) [118]

1.4.1. Innate immunity

Innate immunity is the first line of defence triggered by foreign pathogens including viruses. The initial step is recognition of molecules shared by interrelated pathogens known as "Pathogen Associated Molecular Patterns" (PAMPs) by cell surface or intracellular receptors [126]. RNA viruses are recognized by Toll-like receptors (TLRs) or nucleic acid-binding proteins [127, 128]. In hepatocytes, pathways downstream of TLR-3 and retinoic-acid-inducible gene I (RIG-I) are the two major signalling pathways triggered by foreign dsRNAs, including HCV RNA which has secondary dsRNA structures and a dsRNA replication intermediate [46, 129-131] culminating in the secretion of IFN-β which binds to the cell-surface IFN receptor initiating a positive feedback loop within infected cells [132, 133]. This induces the expression of hundreds of type I interferon-stimulated genes (ISGs) in the infected cells and sends a danger signal to neighbouring uninfected cells inducing ISGs expression in them as well, creating a general antiviral state in the liver that limits HCV RNA replication and cell-to-cell spread [126, 134].

Induction of intracellular signalling pathways was detected as early as day two after HCV infection in microarray studies of liver biopsy samples obtained from HCV-infected chimpanzees. This activation was detected as an upregulation of ISGs in liver cells. However, this upregulation is detected irrespective of the final outcome of the infection, suggesting that most HCV isolates are resistant to the antiviral effects of this first line of defence [135, 136]. Nevertheless, a high level of ISGs expression was a major characteristic of a strong host response associated with spontaneously resolving infection, suggesting that the level of upregulation has an impact on HCV-outcome [137].

As determinants of innate immunity, natural killer cells (NKs) and dendritic cells (DCs) are the first cells to interact with foreign pathogens non-specifically. NKs have a direct cytotoxic action and antiviral action through secretion of IFN-γ and tumor necrosis factor-alpha (TNF-α) [138], as well as having the ability to stimulate DC maturation

representing the link between innate and adaptive immunity (Fig. 1.5) [139]. NKs are constitutively inhibited from reacting against autologous-MHC class I (MHC-I) molecules and to be activated they have to receive activating signals surpassing the inhibitory ones [140-142]. This is under the control of a variety of stimulatory, co-stimulatory and inhibitory receptors that either bind to pathogen components or host cell ligands, one of the most important inhibitory receptors are members of the killer cell immunoglobulin-like receptors (KIRs) family [143, 144].

NKs are abundant in the liver and have been shown to influence the outcome of HCV infection [145]. One of the determinants correlating to HCV outcome being the threshold of NK activation which is governed by the form of the alleles of both the KIRs expressed on NKs and the HLA-A, -B, or -C molecules expressed on other host cells. Khakoo *et al.*, reported in a large genetic study that individuals homozygous for KIR2DL3 and HLA-C1 alleles were more likely to clear HCV infection than individuals with other KIR2DL:HLA-C combinations [146]. It is known that KIR2DL3 has a lower affinity for HLA-C1 than other KIR2DL alleles [147], so HLA-C1-mediated inhibition of NK cells is thought to be weaker (*i.e*. easier NK activation) in individuals homozygous for KIR2DL3 and HLA-C1.

Dendritic cells (DCs) are the most potent professional antigen presenting cells (APCs) able to activate naïve T cells [148]. The two major populations of DCs are the myeloid DCs (mDCs) and the plasmacytoid DCs (pDCs). mDCs are the major APCs inducing the primary T-cell response while pDCs mainly produce IFN-α thus initially termed interferon producing cells (IPCs) as well as amplifying the antiviral response and expanding memory T-cell population [149].

1.4.2. Adaptive immunity

1.4.2.1. Humoral immunity

The humoral immune response is mediated by antibodies secreted by B cells following their activation by helper T-cell subtype 1 (Th2) CD4+ T cells to differentiate into plasma cells. Humoral immunity against viral infections is effective through its ability to produce neutralizing antibodies (nAb) targeting surface epitopes, thus preventing viral binding to and/or entry into target cells [148].

Although, HCV RNA is detectable in the serum by day two post infection [46] reaching high serum titres by week 1-2, anti-HCV antibody response(s) (seroconversion) are usually detected by weeks 8-12 post-infection [64, 133]. Early studies showed that antibodies targeting the hypervariable region-1 (HVR1) of the E2 glycoprotien of HCV are neutralizing *in vitro* and *in vivo* [150, 151], and might select for mutations in the envelope region [152]. However, a chimpanzee study showed that clearance was not associated with the generation of anti-envelope antibody response in 28 animals [153] and persistence could not be correlated with viral escape by mutations in the E1-E2 regions (especially HVR1 region) [153, 154]. Moreover, another chimpanzee study demonstrated that the presence of antibodies targeting HVR1 during acute infection was associated with the development of chronic infection suggesting that they do not mediate protection *in vivo* [113]. On the other hand, Law *et al.* [155] reported the identification of monoclonal neutralizing antibodies that could protect against heterologous viral infection, and the results obtained by Lake-Bakaar *et al.* [156] through using reversible B-lymphocyte depletion suggested a protective role of B cells. These inconclusive *in vitro* and *in vivo* data suggest that the role of antibodies in HCV infection needs further investigation.

1.4.2.2. Cell Mediated Immunity (CMI)

CMI is mediated through CD8+ and CD4+ T cells. CD8+ T cells kill target cells displaying viral antigens within the context of their MHC class I molecules [148]. CD4+ T cells are known to play an essential role in priming and sustaining cytotoxic $CD8^+$ T-cell (CTL) responses [157]. CMI is of special importance in immunity against non-cytopathic viruses like HCV [6].

1.4.2.2.1. CD4+ T cells in HCV infection

HLA class II restricted T-cell responses were shown to be of direct impact on the outcome of acute HCV infection [158, 159] and some HLA class II alleles were either associated with resolution or chronicicty (*e.g.* HLA-DRβ1*0101 was associated with clearance, whereas HLA-DRβ1*0701 was associated with HCV persistence) [160-162]. Also, there is an association between the prevalence of either helper T-cell subtype 1 (Th1) or Th2 cytokine profiles and resolving acute HCV infection or developing persistent disease, respectively [163], all of which suggests the existence of a correlation between the quality of CD4+ T-cell responses and the outcome of acute infection.

The three major characteristics of a successful helper T-cell response is being early, vigorous and multi-specific, and in patients that proceed to chronicity either one or more of theses were lacking [46]. Early CD4+ responses were temporally and kinetically correlated with control of viraemia and patients who were able to clear the infection spontaneously during the acute phase demonstrated $CD4^+$ T cells able to proliferate efficiently following stimulation by total recombinant protein and had a robust IFN-γ production (Fig. 1.6 c) [120, 164].

Another key factor is the breadth of the CD4+ helper T cell targeting immunodominant epitopes. Patients who spontaneously resolved the infection were shown to target multiple HCV epitopes (up to 14) mapped to Core and non-structural regions.

Several MHC-II restricted epitopes were recognized by CD4+ T cells from resolved patients even years after clearing the virus [164]. Some subdominant CD4+ T-cell activities may be observed, but this could occur even after the viraemia has already been controlled [165]. The hierarchy of CD4+ T-cell responses was preserved even during secondary infection years later [165].

In conclusion, an early functional CD4+ T-cell response directed against multiple immunodominant epitopes predicts control of viraemia, while in cases that develop chronic disease CD4+ T cells are either absent or if present are functionally altered or target fewer epitopes [158, 166-168]. This indicates that contraction of the CD4+ T-cell population and its failure in sustaining a robust CD8+ T-cell effector response is a hallmark of HCV persistence [46].

1.4.2.2.2. CD8+ T cells in HCV infection

Several studies in humans and chimpanzees demonstrated that the three success factors for CD8+ T-cells are the same as for CD4+ T-cells; the onset, the strength and the breadth of the response, are all correlated with the ability to clear the virus during acute infection [120].

Several studies have more precisely defined the role of CD8+ T cells as compared to helper T cells. In a chimpanzee study all the animals that were unable to mount a functional CTL response developed chronic infection, while in spontaneously resolved animals the onset of the cytotoxic T lymphocyte (CTL) response coincided exactly with the termination of infection [169]. Using the MHC-I tetramer staining technique which enables the detection of cells targeting an individual epitope, strong correlation of outcome could be established with functional CD8+ T cells targeting a specific HCV epitope, as their detection at high frequencies in the blood and liver of humans and chimpanzees is temporally and kinetically associated with the control of the viraemia [46]. As much as 8% of the total CD8+ cells in the blood targeting a single immunodominant HCV epitope could

be detected in spontaneously resolved patients [120] and several fold higher detected in the liver [170]. These tetramer positive cells displayed markers of T-cell activation (*e.g.* CD38, CD69 and MHC II) [114, 171]. Furthermore, multi-specificity is more pronounced in humans and chimpanzees able to clear the infection, with eight or nine different epitopes recognized simultaneously by CD8+ form the blood and liver [120, 169]. Genetic restriction was shown to have an impact on HCV-specific T-cell responses, with some HLA-B alleles (*e.g.* HLA-B*27) demonstrating a strong association with clearance, while chronically infected individuals expressing HLA-B*27 demonstrated epitope polymorphisms [172].

1.4.2.2.3. Memory CMI and recurrent infection

Mehta *et al.* [173] demonstrated that individuals who were previously exposed to HCV and successfully cleared the infection were 12 times less likely than their naïve counterparts to develop persistent viraemia. This may be due to the fact that individuals able to spontaneously clear HCV acute infection are able to generate a robust memory T-cell population [174]. A group of women infected from a common source were followed up several years after the initial infection. It was found that those who spontaneously resolved the infection possessed HCV-specific CD4+ and CD8+ T cells that could be detected in their peripheral blood even after 20 years post-clearance [164]. Phenotypic characterization of HCV-specific CD4+ and CD8+ T cells in spontaneously resolved cases showed they were expressing the lymphoid homing marker CCR7+ and CD45RO, both characteristic of memory T cells [175, 176]. Upon rechallenging spontaneously resolved chimpanzees with HCV isolates even from a different genotype the duration of infection was significantly shorter, and was associated with the detection of IFN-γ producing CD4+ and CD8+ T cells at a higher frequency and as early as day 14 into the second infection compared to 8 weeks into the primary infection [176-179]. The role of memory T cells was further verified by using antibody-mediated depletion of either CD4+ or CD8+ T cells. CD4+ T-cell depletion was associated with low level viraemia but memory $CD8^+$ T cells were able to partially

control the viraemia [157], whereas memory CD8+ T cell depletion led to a significant delay of viraemia control, and this control coincided with the re-detection of the cells [121]. Taken together these data underscore the importance of memory T cells in conveying protective immunity upon repeated infection, and show that it mainly reduces the level of viraemia and the duration of infection rather than conferring 'sterilizing-immunity' [133].

1.4.3. Failure of the immune response against HCV

Several factors contribute to the failure of the HCV-specific immune response to contain HCV infection leading to the high rate of HCV persistence. These factors are due to either direct failure of the HCV-specific immune response or indirect effects of HCV proteins on components of the immune system. Inadequate HCV-specific immune responses may be due to the primary failure of one or more of the components of the immune system from the start of the infection or their exhaustion over time. Nevertheless, even an initially functional HCV immune response could be subverted by viral evasion strategies.

1.4.3.1. Primary failure of the HCV-specific immune response

Defects in antiviral signalling pathways or in functions of innate immune cells were both reported in persistently infected HCV patients. NK numbers and functions, especially cytotoxic activity, were shown to be lower in chronic hepatitis C patients compared to healthy donors [180-182]. Some reports demonstrated a reduction in number of mDCs and pDCs, but these studies reported them to be fully functional on the individual-cell basis [183-185]. In contrast, other reports demonstrated impaired antigen presentation in DCs from chronic HCV patients due to defects in the ability of natural killer cells (NKs) to derive DC maturation as shown in an *in vitro* culture system [186]. DCs from chronic HCV patients were also reported to have impaired allostimulatory functions for HCV-specific CD4+ T cells [187-189], but evidence confirming this dysfunctionality against other

viruses in the same patients are lacking. On the other hand, multianalyte profiling (MAP) of the key chemokines and cytokines involved in pDC activity suggested/indicated that they are fully functional in chronic hepatitis C patients [190].

Primary defects in adaptive immune cells' frequency and/or functions were consistently reported from chronically infected patients; CD4+ T-cell responses are very weak or even absent in individuals with chronic HCV [175]. When present, they were either non-functional or transiently controlled the infection then decreased in frequency and their function waned just before the viraemia rebounded (Fig. 1.6 a, b) [135]. In some of these chronic cases even if HCV-specific CTL responses could still be detected –albeit at minimal level– CD4+ helper T-cells were functionally absent [164, 166, 174, 191], as indicated by decreased cytokines' production, especially failure to secrete interleukin-2 (IL-2) [192]. CD8+ T cells in chronic patients were also reported to be anergic or of an arrested developmental phenotype ($CD27^{+}CD28^{+}$) with less potent cytotoxic functions than the more differentiated ones [193, 194], which were also impaired in cytotoxic function and proliferative capacity [171, 194], described as a 'stunned' phenotype [114, 120]. Furthermore, CD8+ T HCV-specific cells from chronic individuals were shown to be exhausted, as demonstrated by the high expression of programmed cell death 1 (PD-1) receptor [195], and their functions restored by its blockade [196]. Other studies argued that PD-1 levels of expression did not correlate with the outcome of acute HCV infection [197, 198]. Recently, it was proposed that PD-1 and cytotoxic T lymphocyte associated antigen-4 (CTLA-4) contribute synergistically to CTL exhaustion [199]. Nevertheless, the defects in HCV-specific CTLs in patients with persistent infection are not due to generalized immunosuppression, as the CTL responses against other viruses (as influenza, Epstein-Barr and cytomegalo viruses) are normal [17, 200].

Suppression of HCV-specific immunity can contribute to chronicity. CD4+ T-cells producing the anti-inflammatory cytokines IL-4 and IL-10 were detected in chronic patients [163, 201] and the percentage of CD4+ CD25+ regulatory T cells (Tregs) expressing the

forkhead transcription factor 3 (FoxP3+) was shown to be elevated in chronic versus spontaneously resolved or infection naïve individuals [202]. A study also showed an unexpectedly high proportion of CD4+ cells in liver biopsies from chronic patients, and their role in HCV persistence was supported by the increase in populations of functional CD8+ T cells upon their depletion [203, 204]. Moreover, Tregs derived from chronically infected patients were significantly more suppressive than those from spontaneously resolved ones [205].

Finally, the liver as an immunological milieu is associated with the induction of tolerance despite its ability to sustain effective responses [206]. This suggested that primary contact of T cells with sinusoidal endothelial cells in the liver rather than lymph nodes typically results in tolerance [207, 208], and this ‘abortive’ activation could be the reason why HCV-specific T cells with the highest avidity undergo functional ‘silencing’ [209].

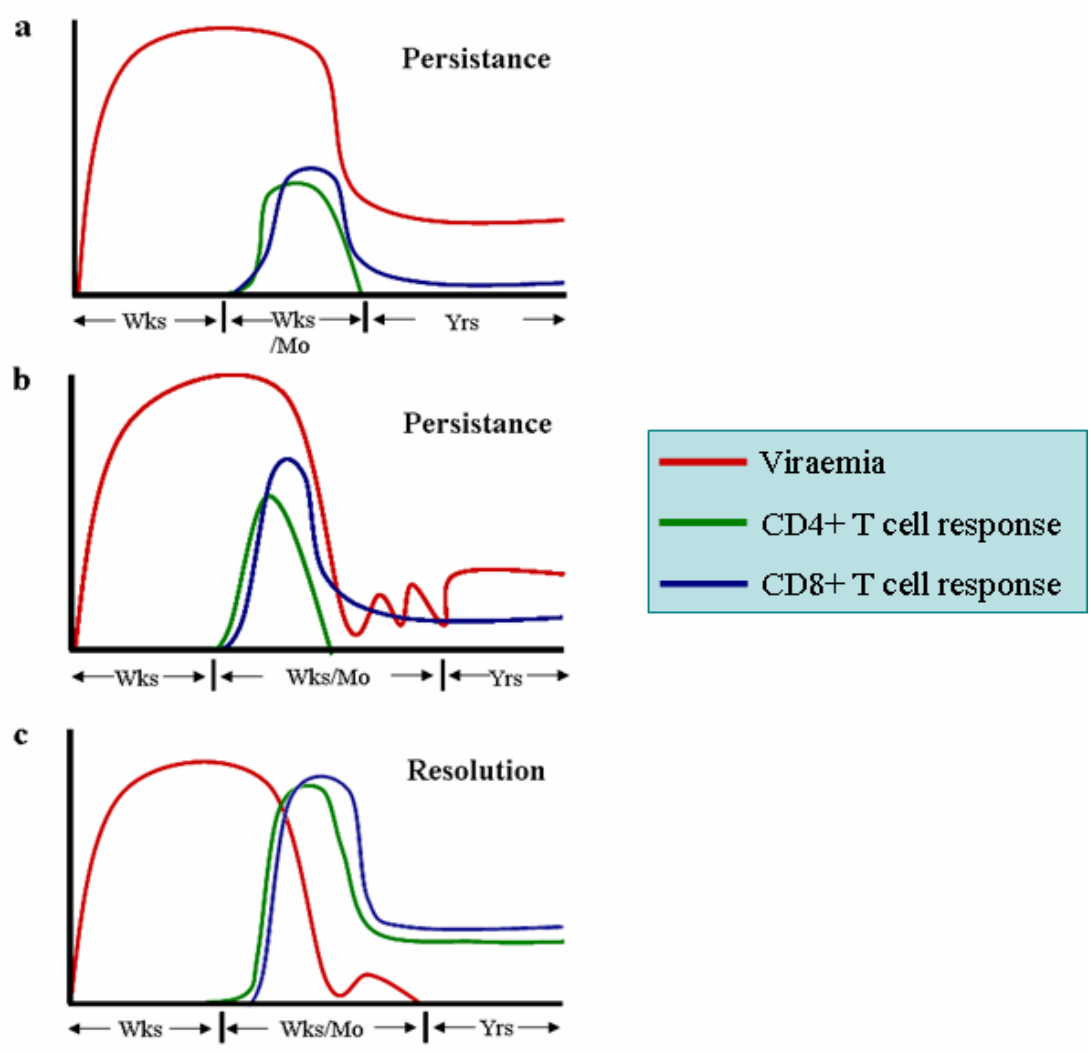

Figure 1.6: Successful versus unsuccessful CMI response during acute HCV infection. Viraemia falls from the high initial level to a lower stable level irrespective of the outcome. a, In case of weak CD4+ and CD8+ T cell responses from the beginning, no control of viraemia is observed. b, Initial CD4+ and CD8+ T cell responses could be able to transiently control the viraemia, but as the CD4+ T cell response contracts and functions wane the viraemia rebounds. c, Early, strong and sustained CD4+ and CD8+ T cell responses are able to control and clear the viraemia.

(Adapted from Bowen DG and Walker CM, *Nature Insight Review*, 2005) [117]

1.4.3.2. Viral evasion strategies

Several HCV proteins inhibit specific steps in the intracellular antiviral signalling pathways *e.g.* E2 and NS5A bind protein kinase R (PKR) [210], NS5A inhibits 2',5'-oligoadenylate synthetase (OAS) which triggers RNAse L [211] and the NS3/4A encoded serine protease blocks RIG-I signalling [212].

HCV-E2 recombinant glycoprotein engagement to CD81 receptor was reported to directly inhibit NK cytotoxicity and IFN-γ production in HCV chronic patients [213, 214]. However, a recent report showed that envelope glycoproteins when expressed as a part of HCV virus particles do not modulate NK function [215]. The hindrance of DC functions by uptake of HCV proteins has been reported, but is still controversial [216-218]. Impaired antigen presentation in DCs from chronic HCV patients due to defects in the antiviral signalling pathways within DCs was reported, and Rodrigue-Gervais *et al.* [219] correlated this to the viral load within DCs. Whether these defects were restricted to liver-DCs remains to be carefully studied [133]. The NS3/4A encoded serine protease alters the function of the secretary apparatus in infected cells inhibiting transfer of glycoproteins, which suggests interference with MHC-I presentation of viral peptides [220].

Mutational escape within targeted epitopes is the most commonly observed evasion strategy in RNA viruses. HCV possesses an error-prone RNA dependant RNA polymerase (RdRp) causing mutation at a high rate of $\sim 2 \times 10^{-3}$ base substitution per HCV-genome site per year [49]. In cases that develop chronic disease, mutations were observed in the E2 region that lie within or outside the CD81-binding site, thus preventing recognition by nAbs [221, 222]. No escape mutations were noticed in MHC II restricted epitopes. This may be due to the lower selection pressure exerted by CD4+ T cells [223]. In contrast, lack of sufficient CD4+ T-cell help combined with the fact that functional CTL response is inherently delayed for up to 8 weeks after the commencement of infection [121, 224] causes CTLs to exert sub-optimal immune pressure on their corresponding viral epitopes

not enough to clear the virus, yet selects for the resistant variants with the highest replication-fitness. These resistant variants revert if transmitted to another individual lacking the restricting MHC allele, thus removing the selection pressure [225]. This does not merely have a micro-effect on the selection of quasispecies of certain viruses that become dominantly fixed in the niche of an individual, but also a macro-effect on the viral variant(s) pre-dominating the population as a whole [226]. Large scale, full-length HCV sequencing revealed certain patterns of HCV sequence evolution correlated to HLA allele of the host, providing indirect evidence of selection pressure by HLA-restricted CD8+ T-cell responses [227]. In a chimpanzee study and human studies, the rate of non-synonymous mutations in MHC I-restricted epitopes was significantly higher (13-fold more) compared to other regions outside T-cell epitopes, indicating that these mutations were in response to a CD8+ T-cell imposed pressure [228, 229]. Antibody-mediated depletion of CD4+ T cells in chimpanzees was associated with low level viraemia but memory $CD8^+$ T cells were able to partially control the viraemia associated with the emergence and selection for escape mutants [157]. With escape mutations, even a multi-specific T-cell response can be overcome [157], and in some cases specific mutations in one epitope were associated with the persistence [230] suggesting that mutations in a fraction of the targeted epitopes is enough to achieve an optimized immune-escape/replication-fitness balance needed by the virus to establish a persistent infection [229, 231, 232].

1.5. HCV treatment

Interferon-α (IFN-α) was shown to have a positive effect on patients with NANB hepatitis in 1986, before HCV was cloned [233]. The first regimen introduced was IFN-α monotherapy for six months with a success rate of 6-12%. Prolonging the treatment duration to 12 months increased the rate only slightly to 16-20% [234]. The addition of the nucleoside-analogue antiviral ribavirin increased the rate to 35-40% [235]. By covalently binding IFN-α to polyethylene glycol (PEG), PEG-IFN molecule was generated with better pharmacokinetics and a longer half-life boosting the success rate to 56% [236-238].

1.5.1. Treatment regimens

The only approved therapy for HCV at present is pegylated-IFN-α (PEG-IFN) based therapy. The recommended regimen for chronic hepatitis C is a combination of PEG-IFN weekly injection and oral ribavirin twice daily [239, 240] for 48 weeks in patients infected with genotypes 1 and 4, and 24 weeks for genotypes 2 and 3, and sometimes with a lower ribavirin dose [237]. Current clinical guidelines suggest that patients diagnosed with acute hepatitis C be observed for 12 weeks into the infection; if spontaneous clearance does not occur treatment should be offered [241]. Treatment during the acute phase is usually for 24 weeks although some protocols use shorter periods with genotypes other than genotype 1. It was demonstrated that combining PEG-IFN with ribavirin does not improve the response in acute treatment, so it is dispensable [242, 243]. Interestingly, the treatment duration and sustained virological response (SVR) rates during the acute phase are not correlated with genotype [244].

The guidelines recommend stopping the treatment if the patient remained positive after 24 weeks of treatment or HCV RNA became detectable between weeks 24-48 [245]. Also, “The week 12 early-stopping rule” recommends stopping treatment if the patient does not show at least a 2-log decrease in HCV-RNA at week 12, because several studies report

94-100% therapy failure in patients who did not [246, 247]. More aggressive induction of therapy at higher doses to improve the early virologic response did not improve SVR rates [248, 249]. Early stopping of treatment when success is not expected is mainly due to the accompanying side effects that range from rash, malaise, hair loss to lymphopenia, marked depression and anxiety that might develop into suicidal tendencies [250].

1.5.2. Patterns of response to therapy

Responses to IFN-α based therapy falls into three main categories; SVR, relapse and non-response (NR). SVR is defined as the lack of HCV detection during treatment that continues for six months after the end of treatment. In relapsers there is no detection of viraemia during the course of therapy, but the viraemia rebounds after the end of treatment. These relapsers could achieve SVR when re-treated but usually with a longer course or a higher dose. Non-responders are individuals in whom the viral load does not decrease below the level of detection throughout the duration of therapy nor thereafter [251].

Further classification of treatment responses offers an earlier insight into the expected response to therapy, early virological response (EVR) and early virological clearance (EVC) define ≥ 2 log decline in HCV-RNA or negative HCV-RNA at week 12 of therapy [241]. Also, in 10% of patients who have attained undetectable HCV RNA during treatment RNA reappears during therapy, a phenomenon termed ‘breakthrough’ [250]. In patients with SVR the reduction in the viral load is biphasic; a short primary phase where there is a rapid decline in HCV RNA and it becomes undetectable within 4–24 weeks due to direct inhibition of virus replication [250], and a long secondary phase with a slower rate of decline attributed to death of infected cells that is either immune-mediated or mediated by other mechanisms [252, 253].

Taking into consideration the high cost and the side effects of IFN-α therapy, defining reliable prognostic markers that can predict the response to therapy before its

initiation is a critical objective in the HCV field. Some reports demonstrated that elevated baseline levels of ISGs in the liver [254], as well as high serum levels of IFN-induced mediators (as IP-10) [255, 256] can be predictive of a low response to therapy. Other studies argue that the initial decline of HCV RNA is a better predictor of SVR than other baseline parameters [257].

1.5.3. Factors affecting response to therapy

Several viral and host related factors are correlated with SVR to therapy. The two most important viral factors are the genotype and the timing of commencement of therapy [251]. Genotype 1a is the most resistant genotype with an SVR rate of 42-46% [236, 238]; in contrast genotypes 2 and 3 have an SVR rate of ~80% even with a shorter course of therapy [237]. Early initiation of therapy during the acute phase markedly increases the SVR rate to 88%, regardless of the genotype [258, 259]. Other viral factors associated with a higher SVR rate are lower baseline viral load [251] and greater quasispecies diversity pre-commencement of treatment [260].

Ethnicity and absence of accompanying co-morbidities (*e.g.* human immune-deficiency virus (HIV) infection, alcohol abuse, renal diseases) are considered the most relevant host factors [251], for example African Americans were shown to have a response rate of one-half to one-third that of Caucasians [261] and therapy was reported to be less effective in HCV/HIV co-infected individuals than in those with HCV alone [262]. Other factors correlated with a better SVR rate are female gender, younger age, lower body weight and less liver fibrosis [251].

1. 5.4. Mechanisms of action of therapy

Exogenous IFN-α administration to cell-cultures defective in IFN signalling pathways, led to the stimulation of many ISGs, suggesting that its mechanism of action is similar to endogenous IFN-α [40, 263-265]. Also, it has modulatory effect on the immune response (discussed later).

Ribavirin is a nucleoside (guanosine) analogue that was already available since the seventies. When tested in the early nineties against HCV, it had no effect on decreasing viral titres in patients' sera [266]. Nevertheless, when used in combination with IFN-α it markedly increased the percentage of SVR and decreased the rate of relapse [235]. Mechanisms of action of ribavirin are not well understood. One proposed mechanism is direct inhibition of HCV replication due to erroneous incorporation by RdRp leading to early chain termination. Another mechanism is competitive inhibition of inosine-monophosphate dehydrogenase (IMPDH) causing GTP depletion. However, neither is the main mechanism of action [267, 268]. Other proposed mechanisms include acting as a viral mutagen driving the virus towards an 'error catastrophe' producing viruses with reduced infection ability [269], altering the Th1/Th2 balance in favour of Th1 that potentially improves response to therapy [270] and suppressing interleukin 10 (IL-10) production [271].

1.5.5. Novel therapies and vaccine trials

Novel therapies have various approaches targeting different steps in the HCV replication cycle. A novel class of viral entry inhibitors based on cyclic D,L-α-peptides is currently under investigation [272]. Being essential for viral protein processing, replication and a major determinant of HCV resistance, NS3/4A serine protease is the most attractive target for therapy trials. The most promising protease inhibitor was BILN 2061, but research was stopped at Phase II of the clinical trial due to potential cardiotoxity [273] and

the other protease inhibitor is VX-950 in Phase Ib of trials [274]. Other viral-enzyme inhibitor trials adopt the classical approach of inhibiting the NS5B (RdRp) function, being either nucleotide analogues [275] or non-nucleotide analogues [276, 277]. Most recently, short interfering RNA (siRNA) as well as short hairpin RNA (shRNA) were shown to block the replication of HCV in tissue culture [278] providing the basis for clinical trials on RNA-based therapeutic candidates for HCV infection [279].

Other therapies aim at restoring an effective immune response. Whether they have a potential is questionable, taking into consideration the limited responses detected in chronic patients [280]. Immunomodulatory agents as TLR agonists, especially TLRs 7 and 9, have started to show their potential in controlling HCV infection in patients with different genotypes, reducing the viral load by 90% [281]. A therapeutic vaccine based on seven HCV peptides is already in phase II clinical trial [282].

So far, no preventive vaccine exists for HCV and vaccine development remains a great challenge, taking into consideration that the immune correlates of protection are still under investigation, as well as the heterogeneity of the viral sequences. Vaccines based on peptides from E1-E2 or Core [283] and genetic vaccines encoding for Core-E1-E2, NS3/4A or the complete NS region [284-286] are in various phases of clinical trials. Some of these vaccines (especially the ones not containing E1-E2) induced only T-cell responses. This underscores the importance of studying the role of B-cell responses and neutralizing antibodies to vaccine efficacy.

1.5.6. Role of the immune system in the response to therapy

In addition to its direct antiviral actions, IFN-α has an important impact on the immune status by activating innate immunity through NK stimulation which aids DC maturation, as well as enhancing adaptive immunity through promoting survival of T cells –by preventing T-cell apoptosis– and enhancing T-cell proliferation [287], suggesting a role for the immune response in the success of therapy.

The few studies on the correlation between response to therapy and the HCV-specific immune response, especially T-cell responses, showed discrepancies. For studies on patients receiving IFN-α therapy during the acute phase, Kamal *et al.* [58] showed that multispecific HCV-specific CD4+ T-helper type 1 responses were enhanced compared to their baseline activity in patients who cleared HCV versus untreated patients or non-responders. However, Lauer *et al.* [59] showed that CD8+ T-cell responses decreased as the virus was controlled. These findings were similar to the ones reported by Rahman *et al.* [60] showing that HCV-specific T-cell responses gradually decreased in SVRs until they disappeared and the only patient with a sustained immune response was the one who developed a rebound in viraemia. For patients receiving therapy during the chronic phase, Kamal *et al.* [288] demonstrated that SVR may be due to the ability of PEG-IFN to induce and maintain significant multispecific HCV-specific CD4+ T-helper 1 responses. In contrast, Barnes *et al.* [289] found no significant enhancement in the T-cell responses whatever the response to therapy was. The latter reported only a transient enhancement in proliferative responses which they considered unreliable as a marker of immune activation, being often not associated with functions such as IFN-γ production.

1.6. Hypothesis and objectives

One of the most understudied areas in the field of HCV is the antiviral and immunomodulatory mechanisms of the current standard of care therapy, as well as host and viral factors that dictate the differential response to therapy. Understanding the mechanisms of action of current treatments would have important implications in the development of novel therapies. Multiple studies have clearly demonstrated that a successful immune response capable of spontaneously clearing HCV is mediated mainly by the adaptive arm of the immune response, particularly early, multispecific and sustained cell-mediated immunity (CMI). CD8+ T-cells are indispensable in clearing the virus, but not without the sustained support of CD4+ T-cells. Individuals who fail to mount or sustain such a response usually progress to a chronic infection with persistent viraemia [46, 120]. In contrast, studies focusing on elucidating the role of an effective CMI in the success of IFN-therapy are inconclusive and somewhat controversial [58-60, 288, 289].

Given the indispensable role of the immune system in clearing natural acute-HCV infection, and taking into consideration the immunomodulatory effects of therapy, we hypothesized that success of IFN therapy is likely related to restoration of a fully functional immune response in individuals who achieve a SVR to therapy, especially if initiated early during the acute phase of the infection.

The objective of this project was to understand the influence of the current therapy on the host's immune response and whether the success of therapy in achieving SVR relies on the restoration of an effective immune response depending on the timing of the initiation of therapy relative to the infection period.

The very early studies tackling the role of an effective CMI in the success of IFN therapy lacked some important determinants due to technical limitations at the time. These early studies focused on studying one or two parameters of T-cell function (*e.g.* IFN-γ

production) or relied on the proliferative capacity as evidence of effective immunity against HCV, and most used recombinant proteins to screen for functional cells [58, 60, 288]. With the technical advances in multiparametric flowcytometry and studies in other viral infections, it became obvious that a multiparametric approach examining several functions simultaneously *i.e.* poly-functionality is essential to study immunity against chronic viral infections including HCV and understand the role of the host immunity in determining the response to therapy [290, 291]. Also, the use of tetramer staining techniques allowed the dissection of responses targeting different specific epitopes [292]. Finally, results of earlier studies were difficult to interpret because of the different patient populations studied, different therapeutic regimens and unclear definition of acute HCV infection [58-60].

Hence, we planned the current study to perform extensive multiparametric analysis of immunity against HCV, immune failure and restoration after therapy in a well defined cohort of acute and chronic HCV infected patients. We were able to accurately define acute HCV infection by following-up HCV negative individuals from cohorts at high risk of infection (*e.g.* IDUs) at short intervals, enabling us to define the early immune factors that influence the outcome of acute HCV, and to identify immune markers that could predict spontaneous resolution. The specific aims of the study were to study both host and viral factors involved in the differential response to IFN therapy, as well as determine immune factors associated with SVR. To achieve this, we performed longitudinal analysis of the immune responses and host-virus interaction in acute and chronically infected individuals, before, during and after receiving IFN-α based therapy, thus enabling us to define possible immune factors that might contribute to enhanced response to IFN therapy, as well as identifying immune markers that could predict the differential response to therapy. A cross-sectional study at the time point right after clearing the virus or at the end of treatment (for patients with SVR) was performed to focus on the possible mechanisms involved in viral clearance in response to therapy. We used both peptide pools to screen for immune responses against HCV in general, as well as MHC class I tetramers to detect HCV-specific T cells targeting a specific epitope. We applied a multiparametric approach when defining a

successful HCV-specific T-cell response, through assessing individual functions, poly-functionality analysis, proliferative capacity and phenotype.

Chapter 2: Articles

Article 2.1.

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Early Interferon Therapy for HCV Rescues Poly-functional Long-lived CD8+ Memory T Cells

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2.1.1. Abstract

The majority of acute hepatitis C virus (HCV) infections progress to chronicity and progressive liver damage. Interferon-alpha (IFN-α) antiviral therapy achieves the highest rate of success when administered early during the acute phase but the underlying mechanisms are unknown. We used a panel of MHC class I tetramers to monitor the phenotypic and functional signatures of HCV-specific T cells during acute HCV with different infection outcome and during early interferon therapy. We demonstrate that spontaneous resolution correlates with the early development of poly-functional (IFN-γ+, IL-2+ and CD107a+) virus-specific CD8+ T cells. These poly-functional T cells are distinguished by the expression of CD127 and Bcl-2 and represent a transitional memory T cell subset that exhibits the phenotypic and functional signatures of both central and effector memory T cells. In contrast, HCV-specific CD8+ T cells in acute infections evolving to chronicity were $CD127^{lo}$, $Bcl\text{-}2^{lo}$, exhibited diminished proliferation and cytokine production and eventually disappeared from the periphery. Early therapeutic intervention with pegylated IFN-α (PEG-IFNα) rescued $CD127^{hi}$, $Bcl\text{-}2^{hi}$ poly-functional memory T cells. These cells were detectable for up to 1 year following discontinuation of therapy. Our results suggest that poly-functionality of HCV-specific T cells can be predictive of the outcome of acute HCV and that early therapeutic intervention can reconstitute the pool of long-lived poly-functional memory T cells.

2.1.2. Introduction

Hepatitis C virus (HCV) infection resolves spontaneously during the acute phase in a minority of infected individuals while the majority develops persistent viraemia and chronic hepatitis over a period of years or decades (28). Understanding the immune response leading to spontaneous resolution during acute HCV has been hampered by the asymptomatic nature of the disease. Studies in the chimpanzee model and high risk populations like intravenous drug users (IDUs) or healthcare workers following HCV exposure have demonstrated the absolute requirement for the CD8+ and CD4+ HCV-specific T cell responses to prevent viral persistence (reviewed in (12, 50)). A cellular immune response is induced in most infected individuals resulting in spontaneous resolution or transient control of viraemia. However, this response is not sustained in individuals who develop viral persistence. The inefficiency of this initial immune response and viral recurrence in individuals who progress to chronic infection is likely due to the loss of CD4+ T cell help (16, 20, 56, 57) and the rapid emergence of viral escape mutants in targeted CD8+ CTL epitopes (9). Individuals who spontaneously resolve HCV infection develop long-lived memory T cell responses (54) that can be protective upon re-exposure (51). In contrast, cellular immune responses are mostly undetectable in the blood of persistently infected individuals and mostly localized in the liver (19, 27). Visualization of HCV-specific T cells ex-vivo using MHC class I tetramers have demonstrated that HCV-specific CD8+ T cells are severely impaired in function in chronically infected individuals as demonstrated by the lack of proliferation (60) and cytokine production (22) in response to specific antigen stimulation. At present it is difficult to establish if such aberrant responses are the cause or the effect of persistent viraemia and T cell exhaustion.

Memory T cells in humans are classified based on the expression of the chemokine receptor CCR7 and CD45RA into 3 subsets: central memory (T_{CM}) defined as CCR7+CD45RA- with a high proliferative capacity and lower IFN-γ production, effector

memory (T_{EM}) defined as CCR7-CD45RA- with lower proliferative capacity and higher IFN-γ production and terminally differentiated effectors (T_{EMRA}) defined as CCR7-CD45RA+ having the lowest proliferative capacity and highest IFN-γ production (46, 47). Additional markers have been used to identify different subsets or maturation status of memory T cells like the co-stimulatory molecules CD27 and CD28, IL-7 receptor-alpha (CD127) as a marker of cells destined to become memory T cells (29) and programmed death receptor-1 (PD-1) as a marker for T cell exhaustion in several viral infections (14). Recent studies have associated spontaneous resolution of acute HCV with up-regulation of (CD127) (17, 58) and down-regulation of the PD-1 (18, 39, 42, 59). In contrast, other studies have demonstrated that $CD127^{+}$ HCV-specific CD8+ T cells are detectable in persistently infected individuals (4, 42) and that PD-1 does not correlate with outcome of acute HCV (8, 35), suggesting that PD-1 may not be a marker for T cell exhaustion but rather activation. In addition, virus-specific memory T cells in chronic HCV were shown to be arrested at an early maturation stage CD27+CD28+ (3). However, contribution of the different memory T cell markers to defining functionally competent HCV-specific T cell populations associated with viral clearance and the development of such subsets during clearly defined acute HCV infection remain elusive.

There is no vaccine for HCV and the current therapy, a combination of pegylated interferon-alpha (PEG-IFN-α) and ribavirin, is only effective in approximately 50% of chronically infected individuals (13). Nevertheless, response to therapy is enhanced if started early during the acute phase (32, 34, 62) suggesting progressive damage to the host defence system with prolonged infection. The role of the immune response in determining the outcome of HCV therapy has been controversial with some studies demonstrating an enhanced immune response in individuals who respond to therapy (31, 33) while others showing no correlation and even a decline in the immune response following therapeutic elimination of the virus (37, 43). Most importantly, comprehensive analysis of the

functional and phenotypic signatures of HCV-specific T cells when therapy is introduced early during the acute phase is unknown.

In this study, we used a panel of five MHC class I tetramers and 9 color multi-parametric flow cytometry to monitor longitudinally the phenotypic and functional changes in HCV-specific T cells in a unique cohort of IDUs at high risk of HCV infection, before and during acute HCV infections that progressed to spontaneous resolution or viral persistence. In addition, we followed a subset of these individuals during early IFN therapy. We demonstrate that acute resolving HCV infections are characterized by the early development of poly-functional CD127+, Bcl-2+ HCV-specific T cells consistent with a transitional effector memory T cell profile. Most importantly, early therapeutic intervention with PEG-IFN-α rescued $CD127^{hi}$, $Bcl\text{-}2^{hi}$ long-lived poly-functional memory T cells.

2.1.3. Materials and Methods

2.1.3.1. Study subjects and clinical follow-up

HCV acutely infected subjects were recruited among high risk IDUs participating in the St-Luc cohort study or methadone treatment program or presenting to the hepatology clinic at St-Luc hospital of the CHUM. This study was approved by the institutional ethics committee (SL05.014 and SL05.025) and conducted according to the Declaration of Helsinki. All participants signed informed consent upon enrolment. Acute HCV infection was defined as either i) detection of positive HCV-RNA in the absence of HCV antibodies at recruitment followed by seroconversion: patients R1, C2, T1, T4; ii) a positive HCV antibody test following a previous negative test in the presence of positive HCV RNA, time intervals ranging between 63 and 126 days: patients R2 (126 days), T2 (63 days), T3 (90 days); and iii) patient C1 presented to the hepatology clinic with symptomatic acute HCV including elevated serum ALT levels and HCV-RNA and antibody positive test following a high risk exposure. All patients tested negative for HIV and HBV. Duration of infection was defined as the time (in weeks) from the first positive HCV-RNA test. Spontaneous viral resolution or persistent infection was defined as the absence or presence of HCV RNA at 12 weeks post enrolment. In accordance with the clinical guidelines at the time, interferon therapy was offered to participants if they tested HCV RNA positive at 12 weeks after HCV detection (61). Patients who accepted treatment received 12-16 weeks of PEG-IFNα–2a (Pegasys) (Roche diagnostics, Welwyn Garden City, Hertfordshire, UK) (180 µg/week) and no ribavirin. HLA typing was performed by the Core facility of the FRSQ AIDS and Infectious Disease Network (SIDA-MI), Montreal, QC, Canada using standard SSP-PCR high resolution techniques as previously described (63).

2.1.3.2. HCV RNA testing and quantification

Qualitative HCV-RNA tests were performed using automated COBAS AmpliPrep/COBAS Amplicor HCV test, version 2.0 (sensitivity 50 IU/ml) (Roche Molecular Systems, Inc., Branchburg, NJ). HCV genotyping was done using standard sequencing for the NS5B region and HCV RNA was quantified by the COBAS Amplicor HCV Monitor test, Version 2.0 (sensitivity 600 IU/ml) (Roche Molecular Systems). Both tests were performed by the Laboratoire de santé publique du Québec (LSPQ) (Ste-Anne-de-Bellevue, QC) as part of the clinical follow-up of patients. Additional HCV RNA quantification was performed using an in-house quantitative real time PCR assay adapted from Takeuchi T *et al.* (55). Briefly, total viral RNA was extracted from 200 µl of plasma collected in EDTA using QIAmp Mini Elute Spin Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol and eluted in 25 µl. Quantitative PCR was performed in duplicate using 10 ul of extracted RNA in a 25 µl reaction volume of one step RT-PCR using QuantiTect Probe RT-PCR kit (Qiagen). The following primers were used: Forward primer: 5'-CGGGAGAGCCATAGTGG-3', Reverse primer: 5'-AGTACCACAAGGCCTTT-3', Probe: 5'-6-FAM-CTGCGGAACCGGTGAGTACAC-IBFQ-3'. Real time PCR was performed using Rotor Gene 3000 Instrument (Corbet Research, Sydney, Australia) as follows: reverse transcription for 30 min at 50ºC, PCR initial activation step 15 min at 95ºC followed by 50 cycles of 2-step PCR(15 sec at 95ºC and 60 sec at 58 ºC). Plasma RNA concentration was calculated using Rotor Gene 6 analysis software (Corbet Research) from a standard curve prepared using standard patient plasma of known HCV-RNA content.

2.1.3.3. Peptides and peptide-HLA class I tetramers.

Overlapping peptides corresponding to the HCV-H77 genotype 1a polyportien reference sequence used in the ELISpot screening assays were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources), Manassas, VA. All other peptides were synthesized by Eastern Quebec Proteomics Core Facility, Quebec, QC, Canada. MHC class I tetramers were synthesized by the National Immunomonitoring Laboratories (NIML), Montreal, QC, Canada and are as follows: HLA-A1 restricted HCV NS3 peptide 1436–1444 (ATDALMTGY), HLA-A2 restricted HCV NS3 peptide 1073–1081 (CINGVCWTV), HLA-A2 restricted HCV NS3 peptide 1406–1415 (KLVALGINAV), HLA-B7 restricted HCV Core peptide 41–49 (GPRLGVRAT) and HLA-B8 restricted HCV NS3 peptide 1395–1403 (HSKKKCDEL), and control tetramers as: HLA-A2 restricted CMV pp65 peptide 495–503 (NLVPMVATV), HLA-B7- restricted CMV pp65 peptide 1014–1023 (TPRVTGGGAM) and HLA-A2 restricted FLU peptide 1007–1015 (GILGFVFTL).

2.1.3.4. Flow-cytometry antibodies and reagents

Directly conjugated antibodies against the following surface molecules were used: CCR7-PE-Cy7 (clone 3D12), CD3-APC (clone UCHT1), CD3-Pacific Blue (clone SP34-2), CD4-Pacific Blue (clone RPA-T4), CD8-Alexa Fluor 700 or –Pacific Blue (clone RPA-T8), CD28-PerCP.Cy5.5 (clone L293), CD127-Alexa Fluor 647 (clone HIL-7R-M21) and PD-1-APC (clone MIH4), all from BD Biosciences, San Jose, CA; CD27-APC-Alexa Fluor 750 (clone 0323) (eBioscience, San Diego, CA); CD3-ECD (Clone UCHT1) and CD45RA-ECD (clone 2H4) (Beckman Coulter, Marseille, France); CD127-Alexa Fluor 488 (clone HCD127) (Biolegend, San Diego, CA). The following intracellular antibodies were used: IL-2-PE (clone MQ1-17H12), IFN-γ-Alexa 700 (clone B27), Bcl-2-FITC (clone 100), CD107a- PE-Cy5 (clone H4A3) all from BD Biosciences.

Live cells were identified using Aqua Live/Dead fixable dead cell Stain Kit (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Fluorescence minus one (FMO) control stains were used to determine background levels of staining. Multiparameter flow cytometry was performed using a standard BD LSR II instrument equipped with blue (488 nm), red (633 nm), and violet (405 nm) lasers (BD Biosciences,) to systematically perform 9 colors staining using FACSDiva software (BD Biosciences). Compensation was performed with single fluorochromes and BD CompBeads (BD Biosciences). Data files were analyzed using FlowJo software version 8.6.3 for Mac (Tree Star, Inc., Ashland, OR). Polyfunctional data was exported using Boolean Gates in FlowJo and further analysed using PESTLE software (version 1.5.4) and SPICE software (version 4.1.6) obtained from M. Roederer, National Institutes of Health (NIH), Bethesda, MD. Polyfunctional T cells were defined as viable CD3+, CD8+ cells that produce IFN-γ, IL-2 and express CD107a simultaneously. A cutoff of 0.02% of specific IFN-γ production was considered for any poly-functionality analysis.

2.1.3.5. Multiparametric phenotypic characterization of HCV-specific T cells.

All flowcytometric assays were performed on cryopreserved samples. For phenotype analysis, 2.5 x 10^6 PBMCs were stained with freshly prepared tetramer-PE for 30-min at room temperature, washed in FACS buffer (PBS 1X, 1% FBS, 0.02% NaN_3), then stained with surface antibodies for 30 min at 4°C, washed twice in FACS buffer and fixed in FACS Fix buffer (PBS 1X, 1% formaldehyde). For intracellular Bcl-2 detection, PBMCs were first stained with tetramers and surface markers, lysed with BD Lysing and Permeabilizing Kit (BD Biosciences) according to the manufacturer's instructions, stained with anti Bcl-2 antibody then washed twice and fixed in FACS Fix buffer.

2.1.3.6. Intracellular cytokine staining (ICS) and CD107a degranulation assay.

2 x 10^6 PBMCs were incubated with anti-CD107a and either DMSO (0.1%) as a negative control or HCV peptide (1 μg/ml) at 37°C° in R-10 media (RPMI medium (Invitrogen, Carlsbad, CA), 10% FBS). Following one hour 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 16 hrs. Cells were then washed with FACS buffer and stained for viability and cell surface antigens, permeabilized using BD Cytofix/Cytoperm solution (BD Bioscience) then stained with anti-IL-2 and anti-IFN-γ 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+, CD8+ T cells and percent specific expression is calculated as the background adjusted function in presence or absence of cognate peptide stimulation.

2.1.3.7. CFSE proliferation assays

PBMCs resuspended at 20x10^6/ml PBS were stained with 0.63 μM of Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) for 8 minutes at room temperature. Reaction was stopped with FBS, cells were washed three times in PBS, then re-suspended at 2x10^6 cells/ml in warm R-10. CFSE–labeled cells were stimulated for 6 days with or without HCV peptide (final concentration 1 μg/ml) at 37°C and 5% CO_2. Recombinant human IL-2 (20 IU/ml) (NIH AIDS Research and Reference Reagent Program, Germantown, MD) was added on day 3. On day 6, cells were stained with HCV tetramer(s) and surface antigens as described above.

2.1.3.8. Cell sorting experiments

PBMC were labelled and sorted into $CD3^+$,$CD127^{hi}$ and $CD3^+$,$CD127^{neg}$ populations using a FACS Aria Instrument (BD Biosciences, San Jose, CA) employing

FACS-Diva software. Sorted cells were collected, washed and suspended in warm R-10 for 30 minutes before being prepared for CFSE, ICS and phenotypic assays as described above. For ICS and CFSE proliferation assays, autologous irradiated PBMCs were added to each tube to act as antigen presenting cells at an E:T ratio of 2:1.

2.1.3.9. HCV epitope sequencing

HCV RNA was extracted from 140 µl of thawed EDTA plasma using QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's protocol. HCV RNA was reverse transcribed and PCR amplified by the sets of nested primers listed in Table S1. 5-30 ul of viral RNA were used for a single step reverse transcription and first round PCR with 20-100 ρmoles of outer PCR primers using the SuperScript III One-Step RT-PCR system with Platinum *Taq* (Invitrogen). Briefly, reverse transcription was performed at 55°C for 45 min, followed by heat inactivation at 94˚C for 2 min. First round PCR was performed directly for 40 cycles (denaturing at 94°C for 30 sec, annealing at 55°C for 1 min, extension at 68°C for 2 min) and a final elongation cycle at 68°C for 7 min. First round PCR products were purified using PCR Purification Kit (Qiagen) then 5 µl of the product was used in the second PCR using the nested set of primers listed in Table S1. Nested PCR was performed for 30 cycles (denaturing at 94°C for 30 sec, annealing at 55°C, extension at 72°C for 1 min) followed by a final elongation step at 72°C for 15 min. PCR products were purified and cloned in pCR4-Topo (Invitrogen) using the Topo TA cloning kit for sequencing (Invitrogen) and sequenced.

2.1.4. Results

2.1.4.1. Identification of acute HCV and longitudinal phenotypic analysis of HCV-specific CD8+ T cells.

Eight subjects acutely infected with HCV were identified among IDUs, the group at highest risk of new HCV infections, as described in Materials and Methods. Patients' demographics and characteristics are listed in Table 1. HLA typing and tetramers used are listed in Table 2. The overall breadth of the immune response was monitored at the earliest time point by an IFN-γ ELISpot assay using a panel of overlapping peptides corresponding to the HCV-H77 genotype 1a polyprotien reference sequence. As documented in the literature, the immune response was broader in the patients who resolved spontaneously (data not shown). A panel of 5 different HLA-class I tetramers corresponding to predefined dominant HCV responses were then used together with a comprehensive 9 color panel of phenotypic markers for T cell maturation (CD27, CD28), T cell memory (CCR7, CD45RA, CD127) and T cell exhaustion (PD-1). Control tetramers containing Cytomegalovirus (CMV) or Flu peptides were used in most patients (Table 2). Representative phenotyping results, using A2/NS3-1073 tetramer, from patient R1 with acute resolving HCV and patient T4 with chronic evolution are presented in Figures 1A and 1B, respectively. HCV-tetramer positive cells were not detectable in patient R1, 9 weeks prior to his first positive RNA test or in patient T4 at 2 weeks post detection of viraemia (PDV). Although NS3-1073 tetramer frequency was very high in patient R1 as compared to T4 (4.5% versus 0.16 %), there was no difference in the phenotype of HCV-specific cells in both patients at the early stages of infection as cells were primarily: CD27+, CD28+, CCR7-, CD45RA-, $CD127^{lo}$, $PD\text{-}1^{lo}$. Nevertheless, as patient R1 eliminated the virus and despite a decline in the frequency of tetramer positive cells they gradually up-regulated CD127 expression. Cells detectable at a late follow-up time point at 86 weeks PDV were 93% positive for CD127 (Figure 1A). In patient T4, as viraemia persisted, the tetramer positive cells lost

CD127 and up-regulated PD-1 expression (Figure 1B). Similar results were obtained with other patients studied, HCV-tetramer positive detected in all patients early were primarily: CD27+, CD28+, CCR7-, CD45RA-, $CD127^{lo}$, $PD\text{-}1^{lo}$ (data not shown) and did not change as infection progressed to chronicity or spontaneous resolution with the exception of CD127 and PD-1. Re-examining the phenotype of HCV-tetramer+ CD8+ T cells at the latest follow-up time point prior to commencement of therapy, demonstrated that CD127 was up-regulated on HCV-specific CD8+ T cells in patients R1 and R2 who spontaneously resolved acute HCV infection (Figure 1C) and slightly upregulated or unchanged in patients T1 and T2 who exhibited low viral loads 1,170 and 9,300 IU/ml plasma, respectively (Table 1). Similar to previous reports, PD-1 expression did not necessarily correlate with infection outcome (8, 35). Expression was generally low during the early phase of infection in all patients but then was up-regulated on HCV positive cells in patients T3 and T4 (Figure 1C), who also exhibited the highest viral loads around 10^7 IU/ml (Table 1).

2.1.4.2. Spontaneously resolved HCV infection is associated with the early development of poly-functional T cells.

Given that we could not establish an early phenotypic marker for T cells associated with spontaneously resolved HCV infection except CD127, we hypothesized that CD127 expression delineates a unique subset of long-lived memory T cells that have antiviral effector functions, similar to earlier observations in murine and human viral infections (29, 30). To evaluate longevity of the CD127+ cells, we monitored the longitudinal co-expression of the anti-apoptotic molecule Bcl-2 and CD127 in tetramer positive cells in patients R1 and R2. As illustrated in Figure 2A, A2/NS3-1073 tetramer positive cells in patient R1 and B8/NS3-1395 tetramer positive cells in patient R2 expressed low levels of CD127 and Bcl-2 at the first detection of viraemia although it was partially controlled (< 600 IU/ml). As virus was completely eliminated, tetramer+ cells gradually upregulated CD127 and Bcl-2 with distinct enrichment in the CD127+, Bcl-2+ tetramer population that represented 81% and 69% of tetramer positive cells at the latest follow-up time point in

patients R1 and R2, respectively (Figure 2A). Furthermore, up-regulation of CD127 and Bcl-2 was associated with increased proliferative capacity as observed in a CFSE dilution assay (Figure 2B). The proliferation of A2/NS3-1073 tetramer positive cells increased from 44% at the first detection of viraemia to nearly 97% by week 86 PDV despite a 4-fold decline in tetramer frequency from 4.5% to 0.95% (Figure 2A) and consistent with a memory T cell profile (24-26). Similarly, proliferation of B8/NS3-1395 specific T cells in patient R2 increased gradually from 15% to 95% at the latest follow-up. Finally, we evaluated effector functions including the production of the TH1 cytokine IFN-γ and the T cell growth factor IL-2 and cytotoxicity through a CD107a degranultion assay (5) following stimulation with the cognate peptide NS3-1073 or NS3-1395. Representative intracellular cytokine staining (ICS) data are demonstrated in supplementary Figure S1. As illustrated in Figure 2C, NS3-1073-specific cells in patient R1 and NS3-1395-specific cells in patient R2 produced significant levels of IFN-γ and IL-2 and degranulated as measured by CD107a in response to stimulation with the specific peptide. The major function observed was production of IFN-γ either alone or together with IL-2 or CD107a (Supplementary Figure S1) . Most importantly, the mean fluorescence intensity (MFI) of IFN-γ was high, suggesting high levels of IFN-γ production on a per cell basis (Figure 2C).

Given that poly-functionality has been associated with control of several viral infections, high IFN-γ MFI and with a central memory T cell phenotype (24-26), we defined the poly-functionality of IFN-γ producing cells to estimate the proportion of cells producing both IFNγ and IL-2; IFNγ and CD107a; or IFNγ, IL-2 and CD107a simultaneously. Poly-functional T cells (IFNγ+, IL-2+ and CD107a+) were detected at the earliest time point studied despite active viral replication. The proportion of poly-functional T cells (depicted in red) increased overtime, in particular in the long-term memory phase representing 15% and 26% of all IFN-γ producing cells in patients R1 and R2, respectively. Most importantly, the majority of HCV-specific cells exhibited at least 2 functions (Figure 2D).

2.1.4.3. Acute HCV with chronic evolution is associated with diminished function and rapid loss of HCV-specific T cells

We hypothesized that HCV acutely infected individuals who develop persistent infection fail to develop or sustain poly-functional virus-specific T cells. We thus monitored the phenotype and function of HCV-specific T cells longitudinally in patient C1, using two different HCV tetramers A2/NS3-1073 and A2/NS3-1406 (Figure 3A). Although the two populations of tetramer positive cells were detectable at a considerable frequency of (0.1-0.4%) up to 18 weeks PDV in patient C1, the tetramer positive cells remained $CD127^{lo}$, $Bcl\text{-}2^{lo}$ (Figure 3A, middle panel). Furthermore, CD8+ T cells interacting with both tetramers became undetectable in the peripheral blood by week 41 PDV (Figure 3A). Longitudinal analysis of the functionality of the cells demonstrated weak proliferative capacity against the NS3-1073 peptide during the early time points of the infection and diminished proliferation overtime (Figure 3A, middle panel). Furthermore, ICS analysis in response to stimulation with a mixture of the NS3-1073 and NS3-1406 peptides demonstrated very low or undetectable cytokine production or CD107a degranulation. Poly-functionality could not be assessed due to the very low level of IFN-γ production. Similar results were observed in patient C2 using B7/Core-41 tetramer with disappearance of tetramer positive cells by week 17 PDV, low level expression of CD127 and Bcl-2 on tetramer positive cells, diminished proliferation and cytokine production (Figure 3B).

2.1.4.4. CD127 distinguishes a unique poly-functional, memory T cell population

Given the observed correlation between upregulation of CD127 and increased proliferation and effector functions, in particular in spontaneously resolved HCV, we sought to confirm that CD127 distinguishes a unique T cell subset destined to become poly-functional long-lived memory T cells. We sorted CD3+, $CD127^{hi}$ or CD3+ $CD127^{neg}$ T cells form the PBMCs of patient R2 at week 17 PDV. Post-sort phenotypic

characterization using B8/NS3-1395 tetramer demonstrated that indeed CD127+ tetramer+ cells were all Bcl-2+ consistent with long-lived memory T cells profile (Figure 4A). Furthermore, $CD127^{hi}$ tetramer+ cells proliferated extensively in response to peptide stimulation demonstrating proliferation of 99% of tetramer+ cells (Figure 4B) versus 65% of tetramer positive cells in the pre-sort sample (Figure 2B). Proliferation was not performed on $CD127^{neg}$ cells due to the limited number of recovered cells. Finally, ICS and poly-functionality analysis demonstrated that the $CD127^{hi}$ population was poly-functional in response to stimulation with the cognate peptide with production of IFNγ, IL-2 and degranulation while $CD127^{neg}$ cells produced very little cytokines (Figure 4C), despite a higher frequency of B8 tetramer positive cells of 1.58% in the $CD127^{neg}$ versus 0.25% in $CD127^{hi}$ populations (Figure 4A). Similar results were observed in patient R1 using sorted cells from week 57 PDV (Supplementary Figure S2). These results confirm that CD127 indeed marks a distinct population of poly-functional HCV-specific T cells in these spontaneously resolved individuals.

2.1.4.5. Early therapeutic intervention rescues poly-functional long-lived memory T cells

Patients T1, T2 and T3 remained persistently infected for up to 15-22 weeks PDV and exhibited predominantly a $CD127^{lo}$ phenotype of HCV tetramer specific T cells since the early onset of viraemia (Figure 1C). In accordance with published data and clinical guidelines at the time, showing that most acute resolving HCV cases resolve within the first 12 weeks of infection and recommending IFN therapy for patients who remain HCV RNA+ after 12 weeks (61), the patients received IFN monotherapy with no ribavirin for 12-16 weeks. The duration of treatment was based on the reports of Kamal et al. demonstrating that treatment administered for 8 to 12 weeks was effective to achieve SVR in genotypes other than genotype 1, and among patients who present a rapid virologic response i.e. HCV-RNA negative at week 4, regardless of the genotype (32, 34).

We hypothesized that early interferon therapy will induce up-regulation of Bcl-2 and may rescue HCV-specific T cells from deletion as observed in patients C1 and C2 who declined therapy. We thus monitored the phenotype and function of the HCV tetramer-specific cells in these subjects prior to, during and up to 1 year following discontinuation of antiviral therapy. Patient T1 who exhibited a low plasma viral load at the start of therapy (1,170 IU/ml) responded to two HCV tetramers A1/NS3-1436 and A2/NS3-1073. This patient received IFN therapy between 15 and 27 weeks PDV (12 weeks total). Prior to commencement of therapy, A1/NS3-1436 tetramer positive cells were primarily $CD127^{lo}$, $Bcl\text{-}2^{lo}$ (Figure 5A). Within 2 weeks of starting therapy, a rapid shift was observed in the phenotype of NS3-1436 tetramer positive T cells with up-regulation of CD127 and Bcl-2 upon virus elimination (Figure 5A). This patient achieved sustained viral response (SVR) defined as absence of HCV-RNA up to 24 weeks post discontinuation of therapy. A CD127+, Bcl-2+ tetramer population was selected for during therapy and remained detectable up to 58 weeks post discontinuation of therapy (84 wks PDV) (Figure 5A). Similar results were observed in this patient using the A2/NS3-1073 tetramer (data not shown), in patient T2 using B7/Core-41 tetramer (Figure 5B) and in patient T3 who exhibited very high viral load (11×10^6 IU/ml) prior to therapy, but nevertheless responded to treatment and achieved SVR (Figure 5C). Furthermore, although the patients exhibited different levels of PD-1 expression on HCV-specific T cells, PD-1 expression was down-regulated in all patients upon viral clearance (data not shown). The overall expression of CD127, Bcl-2 and PD-1 was not affected in total CD8+ T cells in any of the patients studied during interferon therapy (data not shown). Furthermore, control staining using CMV or Flu tetramers demonstrated that there was no change in phenotype of these T cell populations before, during and after IFN therapy (Supplementary Figure S3) thus confirming that the observed phenotypic changes are unique to HCV-specific T cells and are related to viral clearance rather than immune modulation by IFN-α.

In addition, we monitored the function of HCV-specific T cells using CFSE proliferation assays and ICS. All patients demonstrated a good proliferative response at the early stages of infection which increased following initiation of therapy (Figure 5, bottom panels), suggesting that proliferation might not be predictive of the outcome of acute HCV. In contrast, very weak or no IFN-γ, IL-2 or CD107a expression was detected against the various epitopes tested prior to therapy and cells were primarily mono-functional (IFN-γ producers) (Figure 6). A remarked increase in IFN-g MFI, restoration in all functions and generation of poly-functional T cells was observed in all patients following the initiation of therapy and coincident with virus elimination (Figures 6). Notably, although HCV-specific T cells declined in frequency over time they remained detectable and poly-functional up to one year following discontinuation of therapy in all patients studied (Figures 5, 6). Poly-functional cells constituted 11-20% of specific T cells at the latest follow-up, suggesting a long-lived memory T cell response.

2.1.4.6. Transient restoration of HCV-specific immune responses during therapy followed by loss upon viral recurrence.

We monitored longitudinally the phenotype and function of patient T4 who experienced recurrence in viraemia following the discontinuation of IFN therapy. This patient responded to the A2/NS3-1073 tetramer and exhibited very high viral load (Table 1) (Figure 7A). During the first 17 weeks PDV, the NS3-1073 specific cells were mostly $CD127^{lo}$, Bcl-2^{lo} (Figure 7A). This patient underwent IFN therapy for 16 weeks between weeks 21 to 36 PDV with recurrence of viraemia at 8 weeks post discontinuation of therapy (week 44 PDV). Given the nature of the cohort studied, it is difficult to discriminate whether this recurrence in viraemia is a true relapse or a new infection. We sequenced a stretch of 50 amino acids spanning the NS3-1073 epitope in patient T4 prior to therapy and upon recurrence of viraemia on week 44 PDV and observed the same viral RNA sequence in (13/13) and (14/14) molecular clones, respectively (data not shown), suggesting that this is a recurrence of viraemia and not a new infection.

Interestingly patient T4 rapidly developed CD127+, Bcl-2+ HCV-tetramer positive cells and demonstrated enhanced proliferative capacity at week 28 PDV (7 weeks post beginning of therapy) (Figure 7A). The cells also became poly-functional as they acquired the capacity to produce IFN-γ, IL-2 and CD107a (Figure 7C). However, the proportion of poly-functional T cell declined by week 36 PDV. NS3-1073 specific T cells remained detectable by tetramer 3 weeks later at week 39 PDV, exhibited 65% proliferation (Figure 7A) but were primarily IFN-γ mono-functional at 88% (Figure 7C) and with lower IFN-γ MFI. Furthermore, as this patient relapsed at week 44 PDV, tetramer positive cells and cytokine production were undetectable in peripheral blood (Figure 7A and 7B). Another follow-up time point at week 93 PDV yielded similar results (data not shown). To confirm that the NS3-1073 specific cells were not present at a low frequency undetectable by tetramers, we attempted to generate a T cell line from the peripheral blood of this patient using repeated peptide stimulation at week 93 PDV. These attempts were unsuccessful suggesting that the NS3-1073 specific cells were either completely eliminated from peripheral blood, localized to the liver or were extremely defective in their proliferative capacity.

2.1.4.7. Phenotypic and functional changes observed are not due to changes in targeted epitopes.

To confirm that the phenotypic changes observed overtime are not due to the emergence of escape mutations in this region, we sequenced the HCV-epitope region targeted in the main tetramers used in this study. At least two time points were sequenced for each patient, early upon recruitment and late prior to starting IFN therapy. The following epitopes were sequenced: A2/NS3-1073, A2/NS3-1406 and A1/NS3-1436. As illustrated in Table 3 the dominant viral sequence in all epitopes did not change in any of the patients tested. A mismatch between the autologous virus sequence and the peptide sequence used in the tetramer and ICS assay in patients T1 and T3. In patient T1, a Y → F change was detected in the A1 restricted NS3-1436 epitope, this change was previously

shown not to influence T cell function (36). Similarly, an I → V mutation was observed in the NS3-1073 autolgous virus sequence of patient T3 which is a natural variant of this epitope, previously described (36). It is unlikely that such a mismatch is responsible for the changes in phenotype and function of HCV-specific T cells during therapy.

2.1.5. Discussion

We have performed a comprehensive multi-parametric phenotypic and functional characterization of the HCV-specific immune response during clearly defined acute infection in a unique cohort of IDUs with spontaneous resolution, chronic evolution and during early IFN therapy. We demonstrate that spontaneous resolution is associated with early emergence of poly-functional CD8+ T cells. These poly-functional T cells were defined by the expression of CD127 and Bcl-2. Despite a measurable immune response detected at the early time points, HCV-specific T cells failed to upregulate CD127 and Bcl-2 expression in individuals who developed persistent viraemia and eventually disappeared from peripheral blood. Notably, early therapeutic intervention reconstituted a long-lived poly-functional memory T cell response.

In agreement with previous reports we demonstrate that PD-1 expression does not correlate with the outcome of acute HCV (8, 35), except at the highest viral loads. We also demonstrate that spontaneous resolution correlates with up-regulation of CD127 on HCV-specific cells and intracellular content of the anti-apoptotic molecule Bcl-2, a common characteristic of long-lived memory T cells (30). We further demonstrate that CD127 expression distinguishes a unique subset of HCV-specific memory T cells bearing the phenotypic signature of T_{EM} cells (CCR7-CD45RA-) and yet bearing the functional signatures of both T_{CM} (rapid proliferation, high IL-2 production) and T_{EM} (high IFN-γ production and cytotoxic potential). These cells are poly-functional in nature, defined as the capacity to produce more than one cytokine, in addition to cytotoxic and proliferative capacity (26, 48). Such poly-functional T cells were recently correlated with control of HIV infection (1, 6), response to HIV vaccines (24, 41) and anti-retroviral therapy (44). We establish that these poly-functional memory T cells in the context of HCV are: CD27+, CD28+, CCR7-, CD45RA-, CD127+, Bcl-2+. A similar memory T cell subset at a transitional or early maturation stage (CD27+, CD28+, CD127+) with a short replicative

history and strong telomerase activity was recently described (45). We propose that this poly-functional T cell subset represents transitional effector memory T cells (T_{TEM}) that are long-lived but also constitute the first responders upon antigen encounter in the periphery. Although this T cell subset is distinguished primarily by CD127 expression, and such phenotype was reported in other viral infections like hepatitis B virus (7), it is unlikely that CD127 alone can be used as a marker of such polyfunctional memory T cells or to predict the outcome of HCV infection or therapy. Such simplistic models of defining maturation of virus-specific T cells and their functional capacity are no longer sufficient but a comprehensive systematic phenotypic, functional and even genomic profiling will be essential. Studies using a larger cohort of patients are needed to accurately define the phenotypic and functional signatures of these poly-functional T cells and should be included in monitoring the efficacy of any protective or therapeutic vaccines for HCV as recently suggested for HIV vaccine trials (48).

Several factors may contribute to the induction of poly-functional T cells and establishment of T cell memory (30). The most important factor is unarguably CD4+ T cell help. Indeed, CD4+ T cells play an essential role in establishment and maintenance of CD8+ memory T cells in various models of murine and human infections (49, 52, 53). In particular, acute HCV infections that progress to chronicity have been linked to the inability to sustain a CD4+ helper T cell response (16, 56, 57). Depletion studies in the chimpanzee model have demonstrated that loss of CD4+ T cells results in loss of function and a decline in HCV-specific CD8+ T cells and enhanced emergence of escape mutations in targeted CTL epitopes (20). The role of CD4+ T cell help and the mechanisms of cross talk between CD4+ and CD8+ T cells during the memory phase that probably occur via interaction with dendritic cells still remain to be elucidated (10, 11).

Longevity is one of the unique characteristics of memory T cells. HCV-specific memory T cells were detected in spontaneously resolved infections for up to 20 years in absence of any detectable viraemia (54). Similarly, in the present study, we demonstrate

that although HCV-specific memory T cells declined over time, they remained detectable at a reasonable frequency for up to one year following spontaneous resolution or sustained viral response to interferon therapy. All patients were tested at multiple time points by the most sensitive PCR assays (sensitivity 50 IU/ml plasma) and were consistently negative, suggesting that HCV-specific T-cell memory is antigen independent and that establishment of a long-lived memory response is due to successful viral elimination and prevention of persistent T cell activation and exhaustion. Nevertheless, it is still possible that very low level residual virus or intermittent viral replication (40) contribute to long-term maintenance of virus-specific memory T cell populations.

PEG-IFN-α therapy for HCV is most effective when initiated early during the acute phase (32, 34, 62) but the potential contribution of adaptive cellular immunity in this enhanced response is controversial with several reports demonstrating a decline in the immune response during therapy (31, 33, 37, 43). In the present study, we do not observe any significant drop in the frequency of HCV-specific T cells during therapy. Although, we do observe a gradual decline over time, HCV-specific T cells remained detectable for up to 1.5 years of follow-up. Such differences between the various studies might be due to the therapeutic regimen or the nature of the dominant immune response generated in each patient and how accurately it matches the autolgous virus sequence.

We demonstrate a major change in the immune response following therapy while the circulating viral population remains unchanged, thereby excluding a potential influence for viral escape on the immune response to the epitopes studied. However, we did not perform a detailed analysis of viral escape in these patients and it remains an important mechanism for HCV immune evasion. It is still possible that viral escape did occur in other regions and may have contributed to viral persistence. Furthermore, the increased poly-functionality of virus-specific T cells may induce a new selection pressure and trigger mutational escape. A more comprehensive analysis of poly-functionality in relation to full length viral sequencing is essential to elucidate this point.

We demonstrate that early therapeutic intervention can rescue a poly-functional immune response with the same phenotypic and functional characteristics as memory T cells induced following spontaneous resolution. However, it remains unclear if such reconstituted response is the cause or the effect of enhanced virus clearance during early therapy. We favour the hypothesis that re-constitution of a poly-functional immune response is a consequence of virus elimination and prevention of continued T cell exhaustion similar to acute resolving HCV and other viral infections (30). It is likely that there is a limited window of time when IFN therapy can rescue such a poly-functional response before HCV-specific T cells become severely exhausted and eliminated from the circulation. In agreement with this hypothesis, our results demonstrate that HCV-specific cells eventually become undetectable in patients C1 and C2, who declined HCV therapy. Similarly, several reports in the literature have demonstrated that HCV-specific T cells are mostly undetectable in the peripheral blood of chronically infected individuals and mostly localized in the liver or targeting epitopes that underwent escape mutations, thus preventing exhaustion of the specific T cells.

It is tempting to speculate that the development of a poly-functional T cell response, when treatment is initiated early, will prevent relapse upon discontinuation of therapy. The inability to rescue such a poly-functional response when treatment is initiated later during the chronic phase may increase the possibility of virus recurrence and relapse upon discontinuation of therapy. Indeed, our results demonstrating that patient T4, who exhibited viral recurrence, developed primarily a mono-functional immune response favour this hypothesis. Furthermore, large scale studies demonstrated that the end of treatment response to PEG-IFN-α without ribavirin is approximately 56% when treatment is initiated late in the chronic phase (15) and 83% when treatment is initiated during the acute phase (62). However, the SVR rate is much lower in individuals treated during the chronic phase reaching only 29% (15) versus 71% when treatment is started during the acute phase (62).

The reconstitution of poly-functional HCV-specific T cells after early IFN therapy argues in favour of early administration of interferon therapy. Our results demonstrating that the recovered HCV-specific T cells bear the same phenotype and functional signatures as memory T cells generated upon spontaneous resolution suggest that this immune response may be protective upon re-exposure and further underscores the importance of early therapy administration. Studies in the chimpanzee have demonstrated that prior resolution of one HCV infection can induce protection from chronic infection upon re-exposure (51). Although such protective response is difficult to evaluate in humans, two reports have demonstrated that high-risk IDUs who have already resolved one HCV infection are less likely to be re-infected than individuals who are HCV-naïve, despite repeated high-risk exposures (21, 38). IDUs currently represent the main reservoir of HCV (2, 23). Although this group represents a major public health hazard they have limited access to treatment. We demonstrate that treating this population at an early stage will eliminate the virus and minimize the risk of new transmissions, and we predict that the reconstitution of a poly-functional memory response may protect this population against new infections.

In conclusion, our comprehensive phenotypic and functional characterization argues in favour of a more exhaustive multi-parametric signature analysis for HCV-specific cells at the earliest stages of infection. Our results suggest that poly-functionality rather than one single phenotypic or functional marker is more likely predictive of the outcome of acute HCV. Most importantly, we demonstrate that early administration of IFN therapy can rescue such functional signature and may potentially protect against re-infection upon re-exposure in high-risk populations like IDUs.

2.1.6. Acknowledgements

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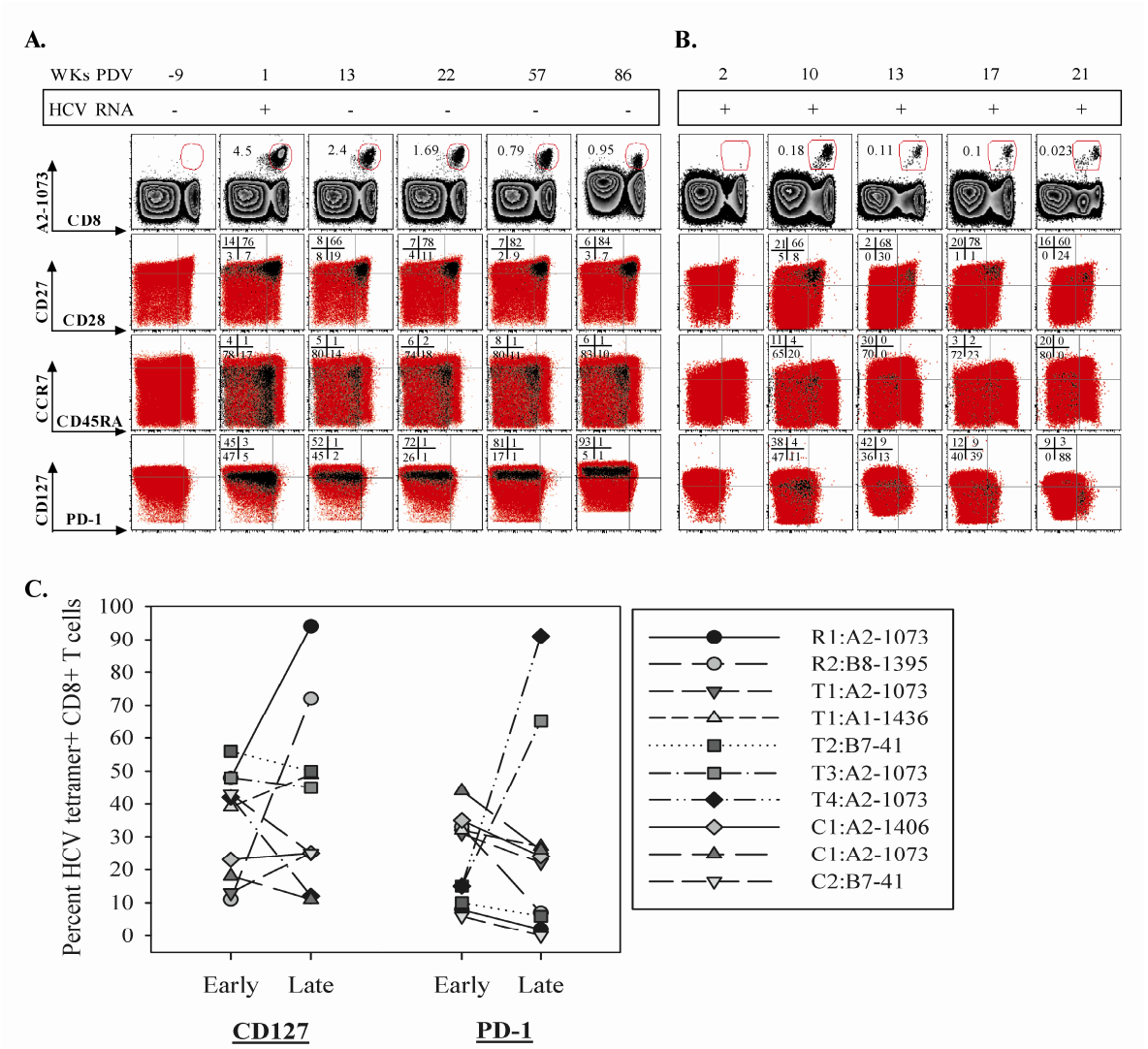
2.1.7. Figures



Badr G *et al.*, 2008

Figure 1

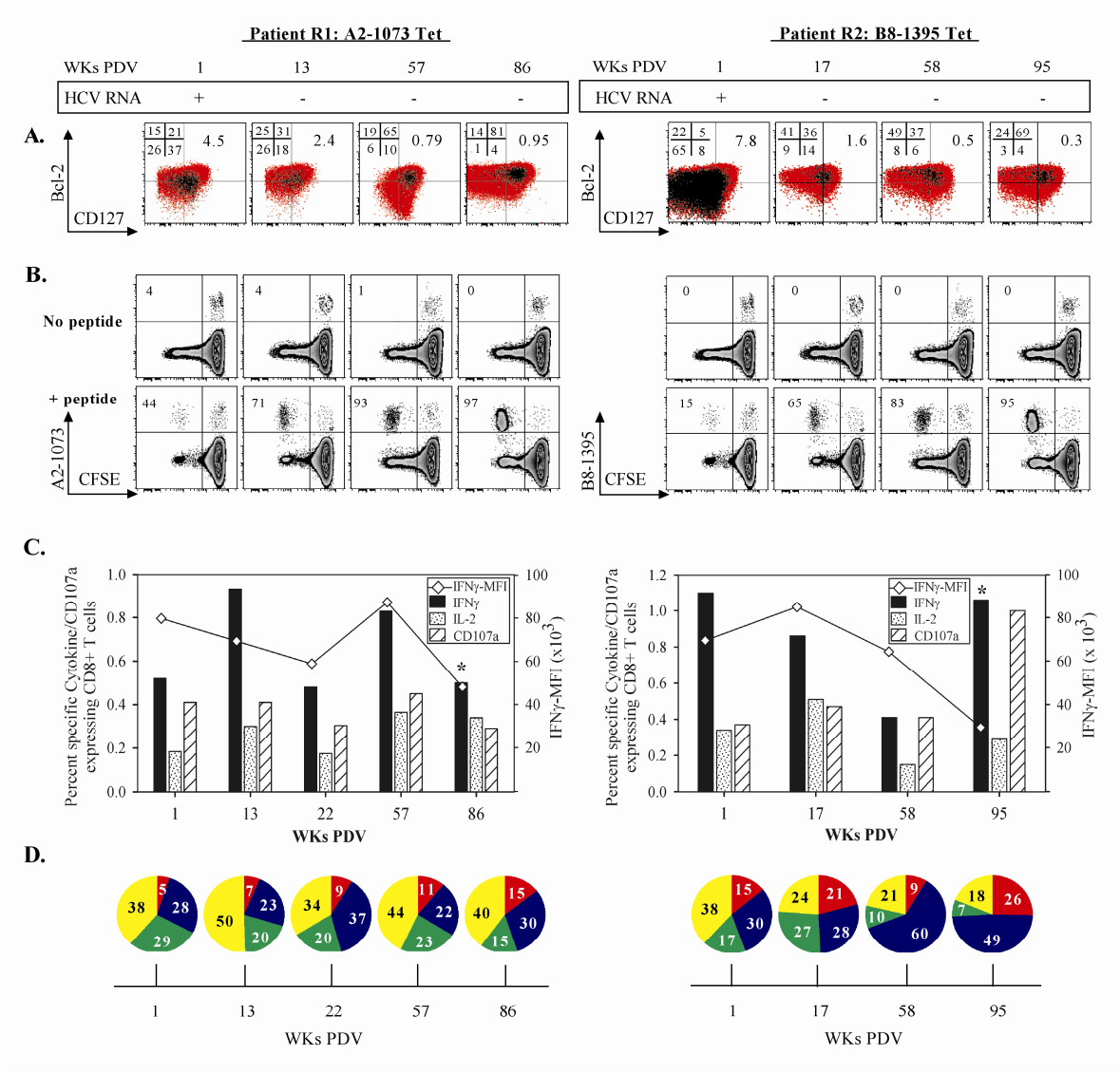


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Figure 2

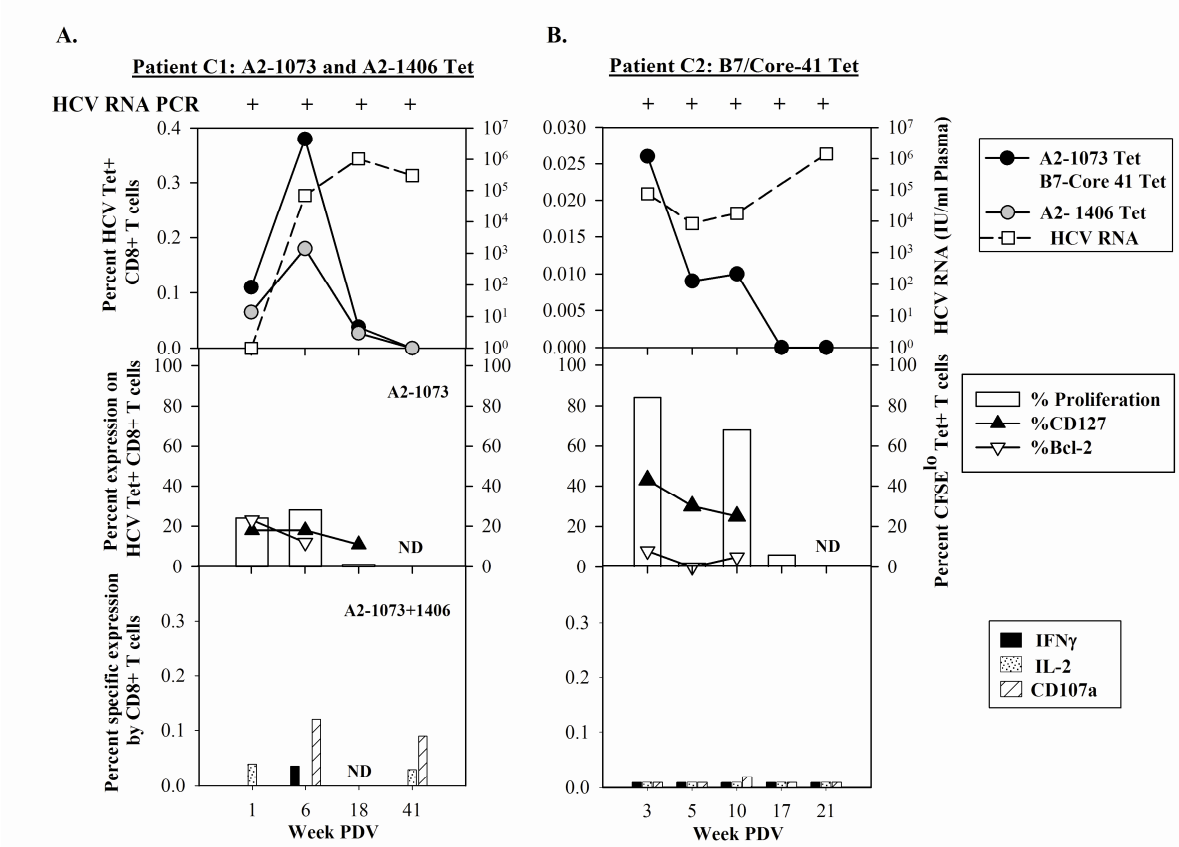

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Figure 3

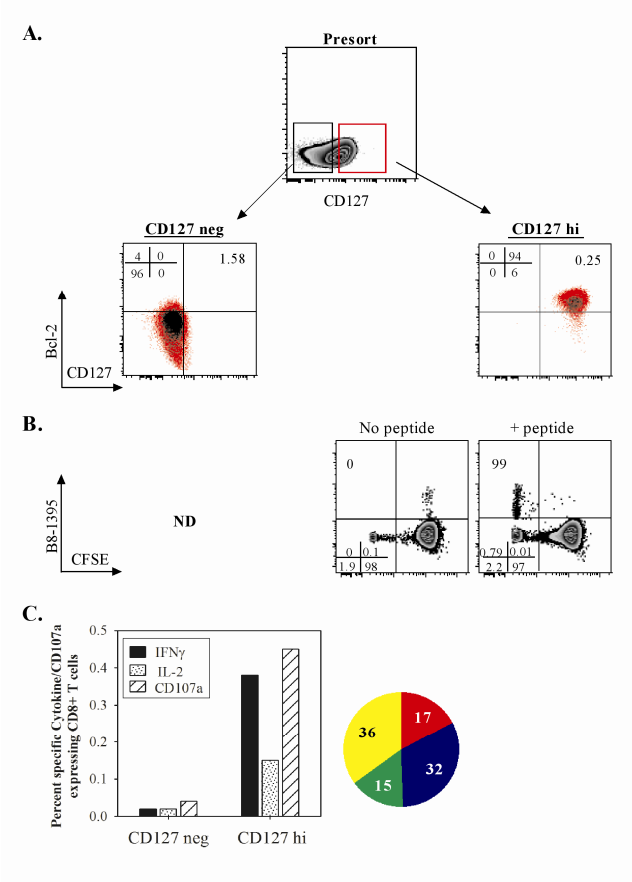

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Figure 4

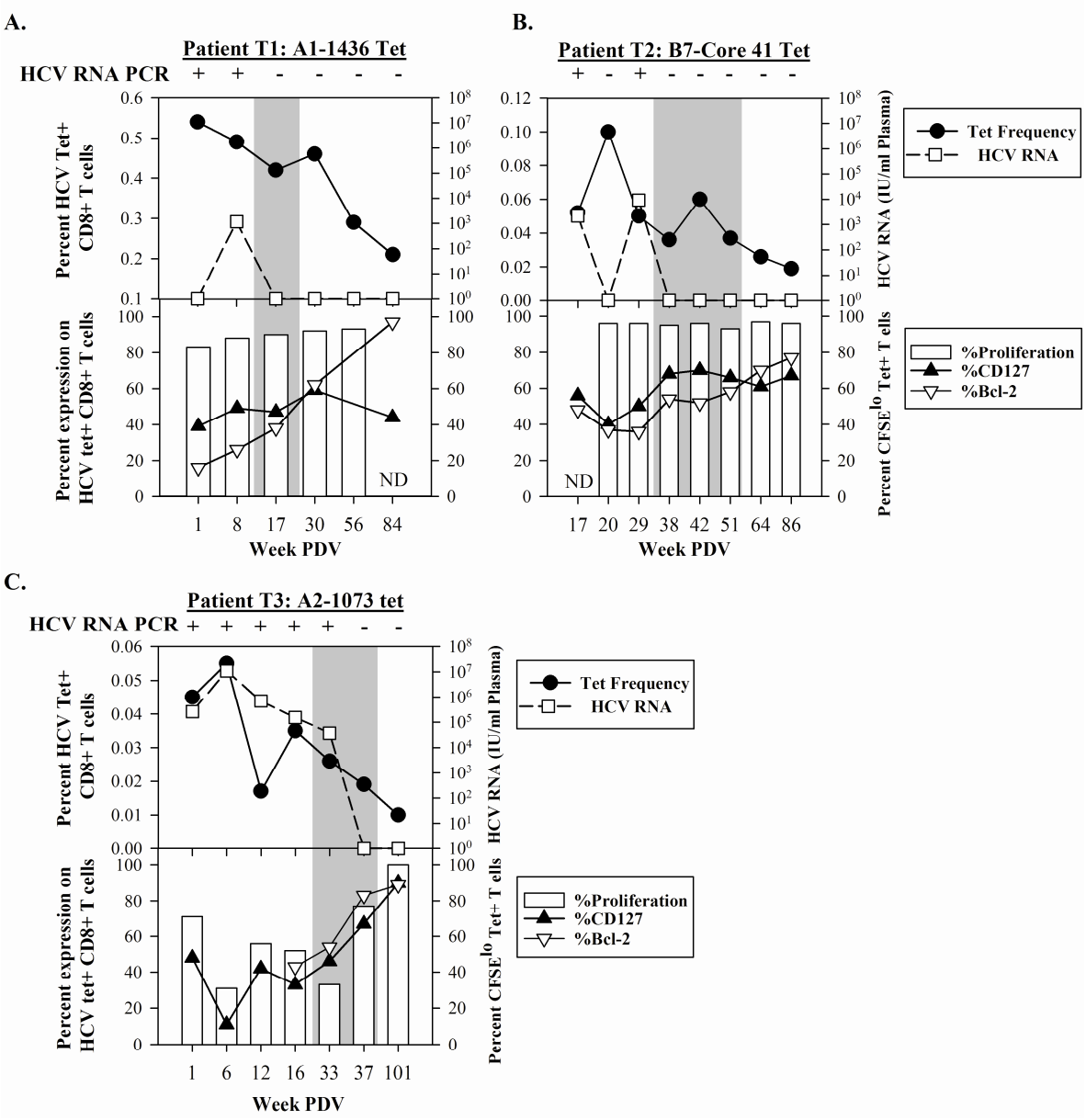

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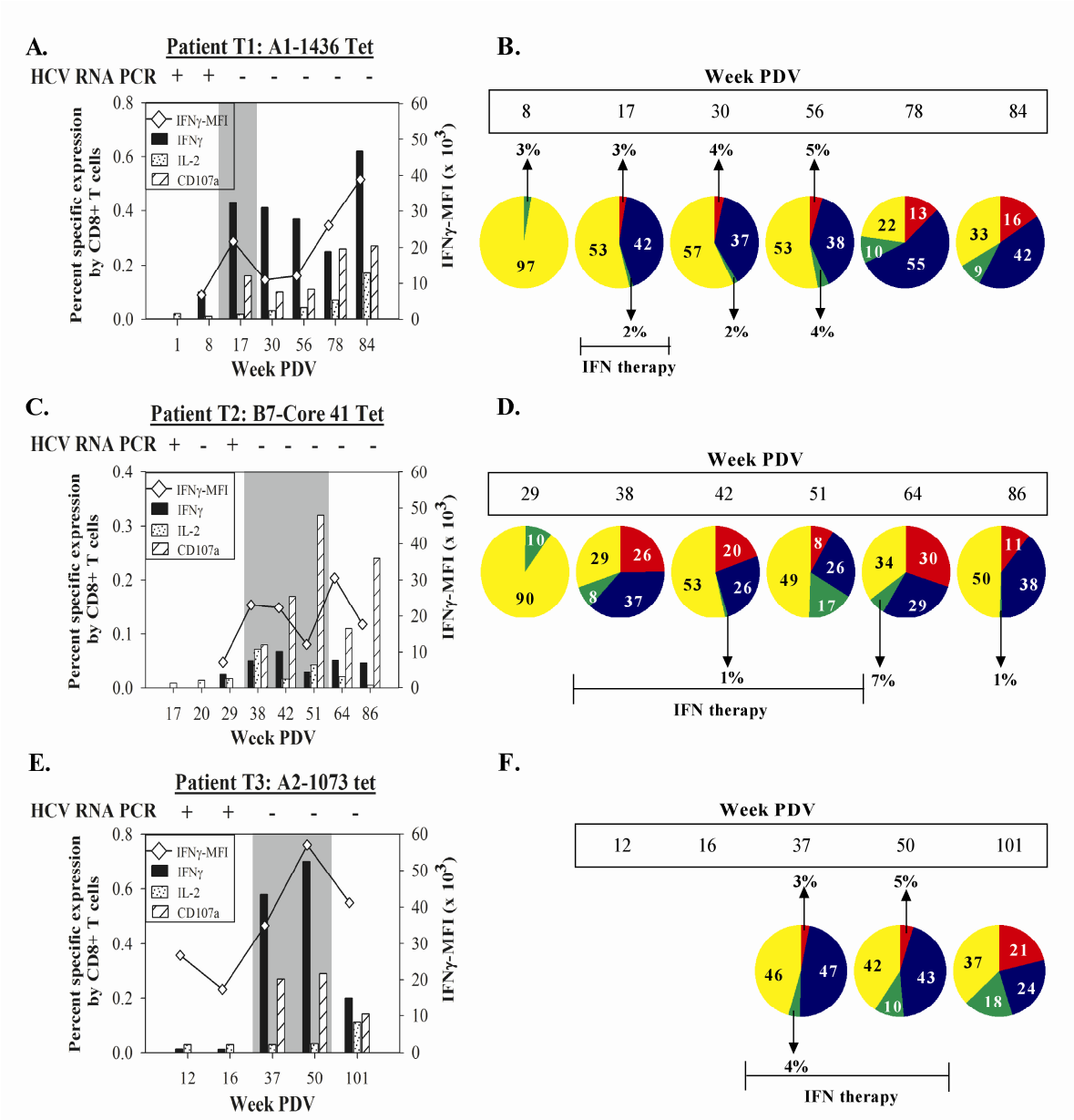
Figure 5



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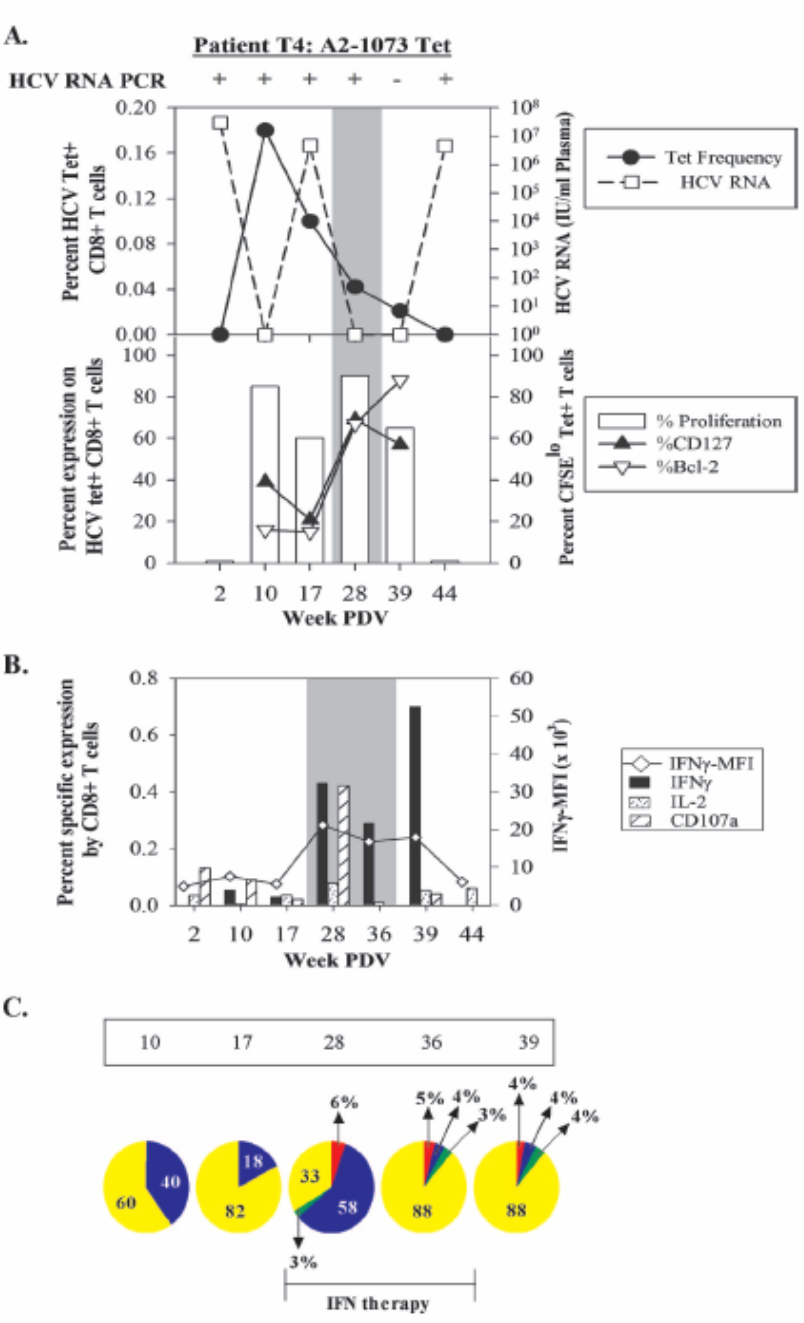

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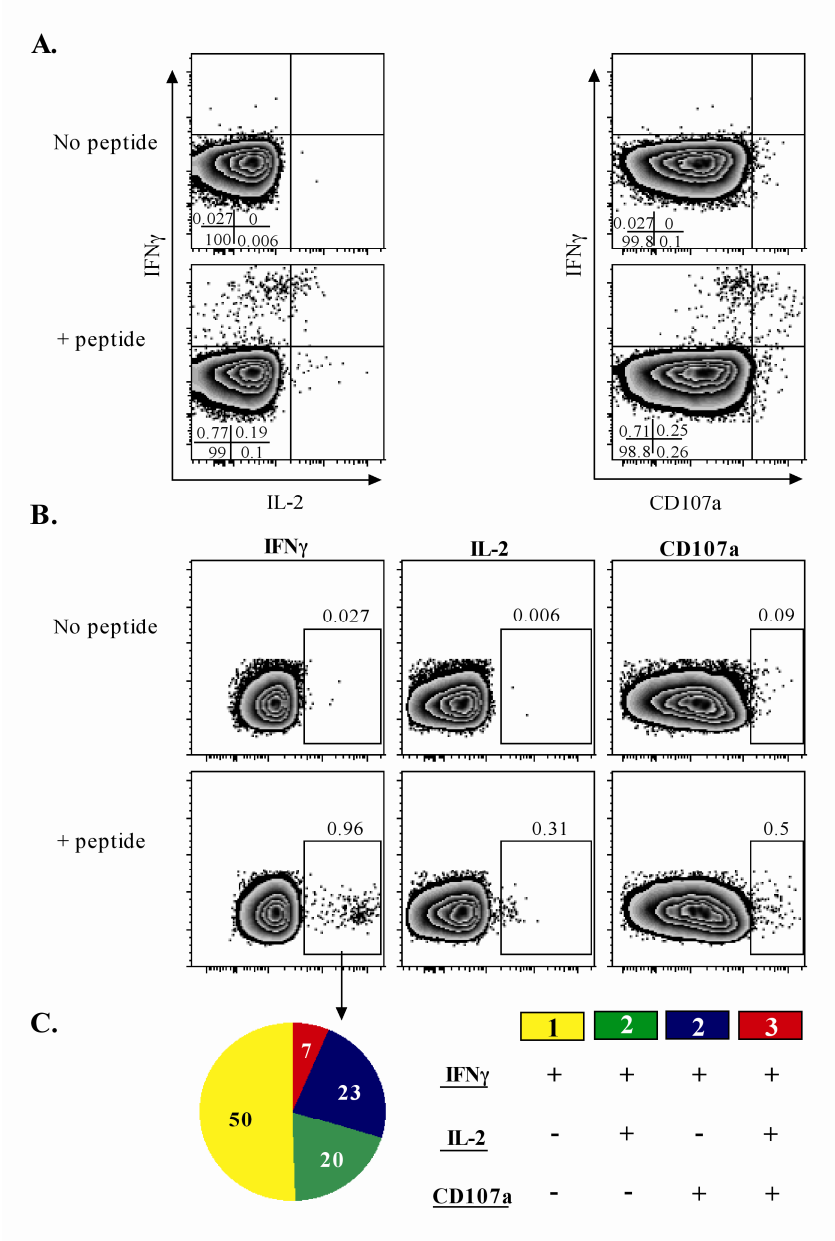

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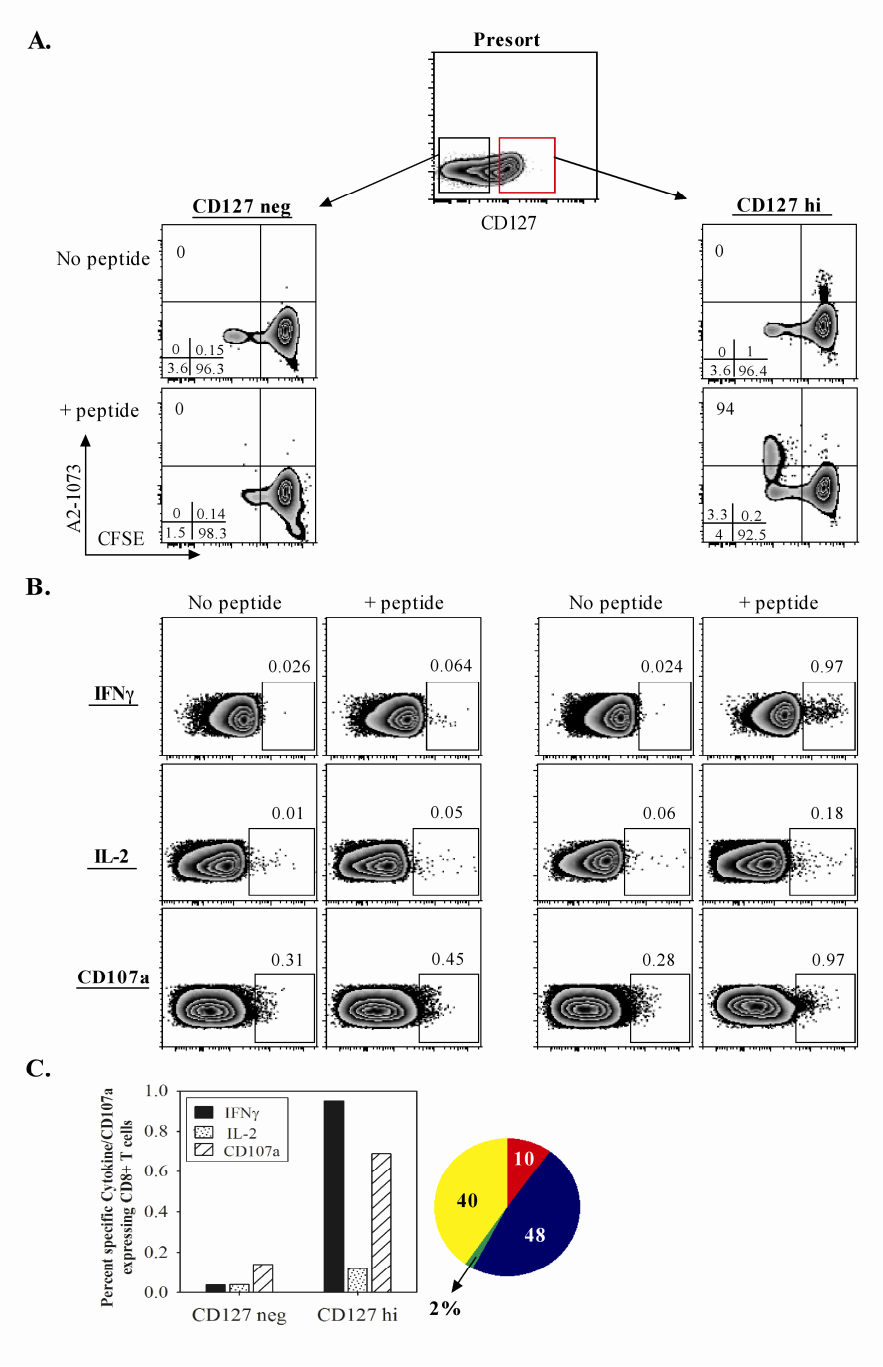
Supplementary Figure S1



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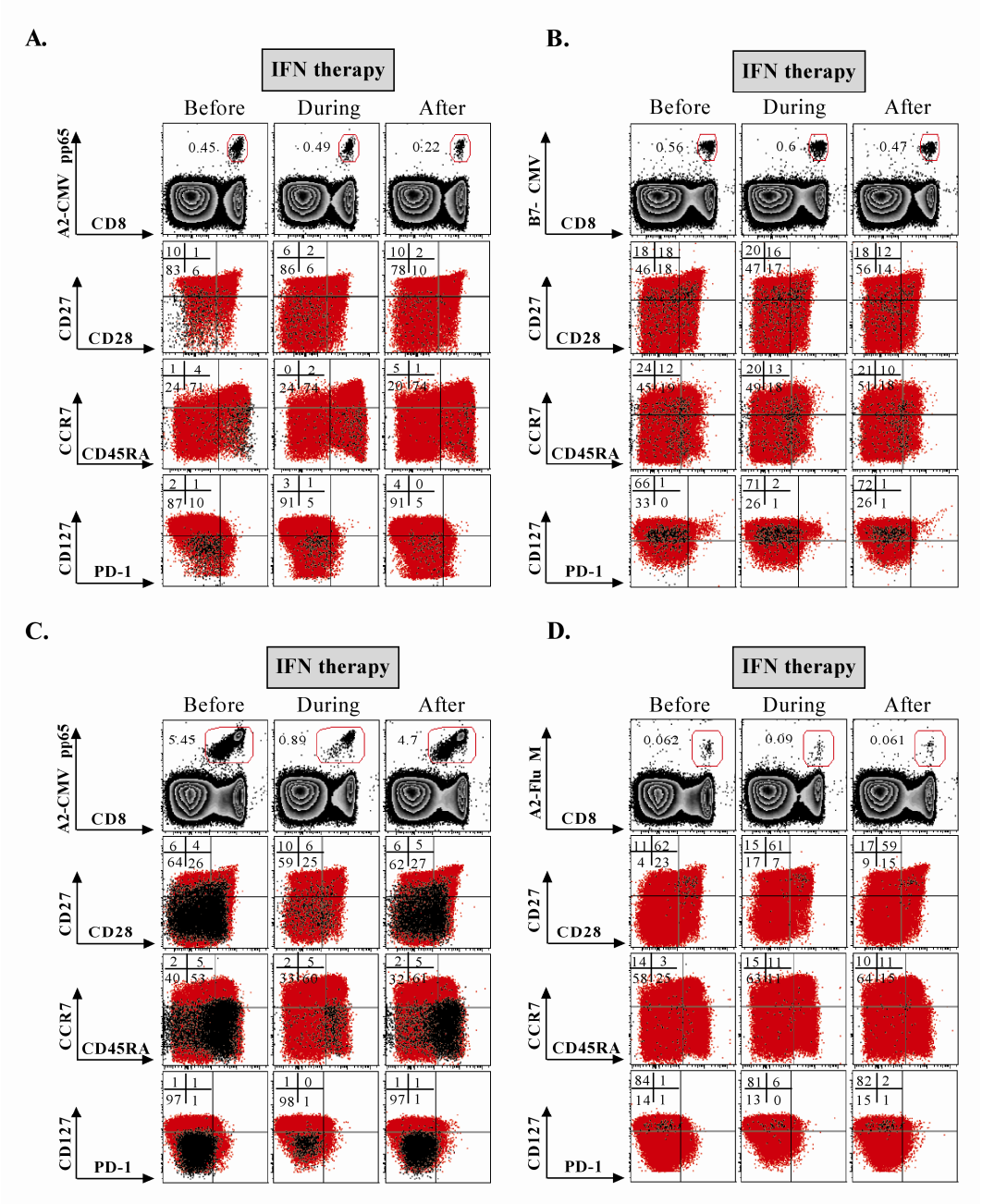
Supplementary Figure S2



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Supplementary Figure S3

2.1.8. Figure Legends

Figure 1. Longitudinal phenotyping of virus-specific T cells during acute HCV. Representative longitudinal tetramer staining and phenotyping data from patient R1 with acute resolving HCV prior to and post detection of viraemia (A) and patient T4 with chronic evolution (B). Tetramer frequency is represented as percent of CD3+CD8+ T cells. Black represents A2/HCV-1073 tetramer positive cells and red represents total CD3+CD8+ T cells in the same donor. Percentages of tetramer positive cells in each quadrant are shown. (C) Summary of longitudinal phenotypic changes in CD127 and PD-1 expression on HCV-tetramer positive cells during acute HCV at the earliest and latest follow-up time point, prior to commencement of therapy. Each symbol represents one HCV-tetramer as indicated in the legend.

Figure 2. Spontaneous resolution of acute HCV infection is associated with up-regulation of CD127 and Bcl-2 and early development of poly-functional HCV-specific T cells. Representative data from patients R1 and R2 using A2/NS3-1073 and B8/NS3-1395 tetramers, respectively. (A) Longitudinal expression of CD127 and intracellular Bcl-2 in tetramer positive cells in patients R1 and R2, tetramer frequency is represented as percent of CD3+CD8+ T cells in the upper right quadrant. Black represents tetramer positive cells and red represents total CD3+CD8+ T cells in the same donor, percentages of tetramer positive cells in each quadrant are shown. (B) CFSE proliferation of HCV specific T cells in absence or presence of the specific peptide, plots are gated on CD3+, CD8+ T cells, numbers in the upper left quadrant represent the percentage of proliferating ($CFSE^{lo}$) tet+ cells. (C) Longitudinal analysis of cytokine production and CD107a degranulation following specific peptide stimulation represented as percent specific expression of each individual cytokine/marker by viable, CD3+ CD8+ T cells. IFN-γ MFI is represented as a line graph. (*) indicates long-term follow-up experiments performed on a different date.

(D) Poly-functionality of HCV-specific T cells, gated on viable CD3+, CD8+, IFN-γ+ cells. Red represents 3 functions (IFN-γ+, IL-2+ and CD107a+), blue represents 2 functions (IFN-γ+ and CD107a+), green represents 2 functions (IFN-γ+ and IL-2+) and yellow represents one function (IFN-γ), numbers on the pie chart represent the percentage of each population.

Figure 3. Chronically evolving acute HCV infection is associated with low expression of CD127 and Bcl-2 and effector functions. Representative data from patients C1 using A2/NS3-1073 and A2/NS3-1406 tetramers (A) and patient C2 using B7/Core-41 tetramer (B). Top panel depicts tetramer frequency relative to HCV-RNA viral load. Middle panel represents longitudinal expression of CD127 and intracellular Bcl-2 in A2/NS3-1073 tetramer positive cells in patient C1 (A) and B7/Core-41 tetramer positive cells in patient C2 (B); proliferation of HCV specific T cells in response to NS3-1073 peptide in patient C1 or Core-41 peptide in patient C2 is depicted as bar graphs representing the percent of proliferating ($CFSE^{lo}$) tet+ cells. Bottom panel represents longitudinal cytokine production and CD107a degranulation following stimulation with a mixture of NS3-1073 and NS3-1406 peptides for patient C1 (A) or Core 41 peptide for patient C2 (B) represented as percent specific expression in viable, CD3+, CD8+ T cells.

Figure 4. CD127 expression delineates a unique poly-functional T cell subset. CD3+ T cells from PBMCs of patient R2 at week 17 PDV were sorted into $CD127^{neg}$ and $CD127^{hi}$ cells and tested for their proliferative capacity and cytokine production. (A) Pre and post sort purity of cells stained with B8/NS3-1395 tetramer, CD127 and Bcl-2. Black represents B8/NS3-1395 tetramer positive cells and red represents total CD3+CD8+ T cells, overall tetramer frequency is represented in the upper right quadrant, percentages of tetramer positive cells in each quadrant are shown. (B) CFSE proliferation of $CD127^{hi}$ cells gated on CD3+CD8+ T cells, percentage of proliferating ($CFSE^{lo}$) tet+ cells is represented in the upper left quadrant, the percentage of total cells in each quadrant is represented in the

bottom left quadrant. Post sort CD127-neg cells were too few to perform this assay. (C) Cytokine production and CD107a degranulation of $CD127^{neg}$ and $CD127^{hi}$ cells stimulated with NS3-1395 peptide. Pie chart represents poly-functional analysis for $CD127^{hi}$ cells, gated on viable CD3+, CD8+, IFN-γ+ cells. Red represents 3 functions (IFN-γ+, IL-2+ and CD107a+), blue represents 2 functions (IFN-γ+ and CD107a+), green represents 2 functions (IFN-γ+ and IL-2+) and yellow represents one function (IFN-γ), numbers on the pie chart represent the percentage of each population. The frequency of IFN-γ producing cells in the $CD127^{neg}$ population was too low to perform the poly-functional analysis.

Figure 5. Early treatment with PEG-IFN-α rescues CD127+ Bcl-2+ HCV-specific T cells. Longitudinal phenotypic characterization and proliferative response of HCV tetramer positive T cells in Patient T1 using A1/NS3-1436 tetramer and treated between weeks 15 to 27 (A), Patient T2 using B7/Core-41 tetramer and treated between weeks 32 and 54 (B) and Patient T3 using A2/NS3-1073 tetramer and treated between weeks 32 and 48 (C). The grey shaded area represent the period of PEG-IFN-α therapy. Top panel depicts the respective tetramer(s) frequency relative to HCV RNA viral load. Bottom panel represents the expression of CD127 and Bcl2 on tetramer positive cells and proliferation of HCV specific T cells in presence of their specific peptides depicted as bar graphs representing the percentage of proliferating ($CFSE^{lo}$) tet+ cells.

Figure 6. Early treatment with PEG-IFN-α rescues poly-functional HCV-specific T cells. Longitudinal analysis of cytokine production, IFN-γ MFI and CD107a degranulation following specific peptide stimulation and poly-functionality in Patient T1 (A, B), Patient T2 (C, D) and Patient T3 (E, F). The grey shaded areas represents the PEG-IFN-α administration period between weeks (15-27) in patient T1; (32-54) in patient T2 and (32-48) in patient T3. For poly-functionality, red represents 3 functions (IFN-γ+, IL-2+ and CD107a+), blue represents 2 functions (IFN-γ+ and CD107a+), green represents 2

functions (IFN-γ+ and IL-2+) and yellow represents on function (IFN-γ), numbers on the pie chart represent the percentage of each population. Polyfucntional analysis was not performed before therapy when the frequency of IFN-γ producing cells was mostly lower than the cutoff threshold (0.02%) to accurately perform the poly-functionality analysis.

Figure 7. Transient restoration of CD127 and Bcl-2 expression and limited poly-functionality in HCV-specific T cells during therapy, followed by loss upon viral recurrence in patient T4. Longitudinal phenotypic characterization and proliferative response of A2/1073 tetramer positive T cells in patient T4. (A) Top panel represents the A2/1073 tetramer frequency relative to HCV RNA viral load. Bottom panel represents the expression of CD127 and Bcl2 on tetramer positive cells, CFSE proliferation of HCV specific T cells in presence of NS3-1073 peptide is depicted as bar graphs representing the percentage of proliferating ($CFSE^{lo}$) tet+ cells. (B) Longitudinal analysis of cytokine production, IFN-γ MFI and CD107a degranulation following NS3-1073 peptide stimulation. The grey shaded areas represent the period of administration of PEG-IFN-α therapy between weeks 21-36. (C) Poly-functionality of HCV peptide-specific T cells in each patient. Red represents 3 functions (IFN-γ+, IL-2+ and CD107a+), blue represents 2 functions (IFN-γ+ and CD107a+), green represents 2 functions (IFN-γ+ and IL-2+) and yellow represents one function (IFN-γ), numbers on the pie chart represent the percentage of each population. The frequency of IFN-γ producing cells at some points prior to therapy was lower than the cutoff threshold (0.02%) to accurately perform the poly-functionality analysis.

Figure S1. Representative intracellular cytokine staining and CD107a degranulation from patient R1. PBMCs were stimulated as described in Methods with either cognate peptide or DMSO control and gated on viable CD8+, CD3+ cells to analyze the expression of each cytokine/marker. (A) Representative expression of IFN-γ, IL-2, CD107a gated on viable CD8+, CD3+ T cells. (B, C) IFN-γ+ gates were then analyzed using PESTLE and SPICE softwares to determine the poly-functionality of the cells.

Figure S2. CD127 expression delineates a unique poly-functional T cell subset in patient R1 with spontaneously resolved HCV infection. CD3+ T cells from PBMCs of patient R1 at week 57 PDV were sorted into $CD127^{neg}$ and $CD127^{hi}$ cells and tested for their proliferative capacity and cytokine production. (A) CFSE proliferation, sorted $CD127^{neg}$ and $CD127^{hi}$ cells, numbers in the upper left quadrant represent the percentage of proliferating ($CFSE^{lo}$) tet+ cells, numbers in the bottom left quadrant represent the percentage of cells in each quadrant. (B) Summary of ICS and degranulation on $CD127^{neg}$ and $CD127^{hi}$ cells. (C) Poly-functional analysis for $CD127^{neg}$ and $CD127^{hi}$ cells stimulated with NS3-1073 peptide, gated on viable CD3+, CD8+, IFN-γ+ cells. Red represents 3 functions (IFN-γ+, IL-2+ and CD107a+), blue represents 2 functions (IFN-γ+ and CD107a+), green represents 2 functions (IFN-γ+ and IL-2+) and yellow represents one function (IFN-γ), numbers on the pie chart represent the percentage of each population. The frequency of IFN-γ producing cells in $CD127^{neg}$ cells was too low to perform poly-functionality analysis.

Figure S3. IFN therapy does not alter the phenotype of non-HCV-specific CD8+ T cells. Control tetramer staining and phenotyping before, during and after IFN therapy is shown for: (A) Patient T1 with A2-CMVpp65 tetramer, (B) Patient T3 with B7/CMV-pp65 tetramer, (C) Patient T4 with A2/CMV-pp65 tetramer and (D) Patient T4 with A2/Flu-M tetramer. Black represents expression tetramer positive cells and red represents expression on total CD3+ CD8+ T cells, in the same patient. Percentages of tetramer positive cells in each quadrant are shown.

2.1.9. Tables

Table 1. Characteristics and demographics of acute HCV infected patients.

Patient ID	Gender (M/F)	Age	Route of infection	HIV Status	Genotype	Highest viral load (IU/ml Plasma)[a,b]	Acute Infection Outcome
R1	M	45	IDU	Negative	1	< 600[a]	Spontanously Resolved
R2	M	39	IDU	Negative	ND	< 600[a]	Spontanously Resolved
C1	M	23	IDU	Negative	1a	1,030,000[b]	Chronic evolution
C2	M	24	IDU	Negative	3a	1,400,000[b]	Chronic evolution
T1	F	29	IDU	Negative	1	1,170[a]	Resolved after therapy
T2	M	32	IDU	Negative	1b	9,300[b]	Resolved after therapy
T3	M	30	IDU	Negative	1a	11,000,000[a]	Resolved after therapy
T4	M	35	IDU	Negative	1a	30,200,000[a]	Therapy Relapser

[a]COBAS Amplicor HCV Monitor test, Version 2.0 (sensitivity 600 IU/ml)

[b]Real time quantitative PCR assay (sensitivity 1000 IU/ml)

Table 2. Patient's HLA and tetramers used.

Patient ID	HLA			HCV Tet	Control Tet
	A	B	C		
R1	A0201, A3101	B4403, B5101	Cw1502, Cw1601	A2/NS3-1073	None
R2	A0101, A2402	B0801, B3906	Cw0701	B8/NS3-1395	None
C1	A0201, A6802	B4402	Cw0501, Cw0704	A2/NS3-1073 A2/NS3-1406	A2/CMVpp65
C2	A0101, A0201	B0702, B4402	Cw0501, Cw0702	B7/Core-41	B7/CMV
T1	A0101, A0201	B2703, B4402	Cw0202, Cw0202	A1/NS3-1436 A2/NS3-1073	A2/CMVpp65
T2	A0301, A2601	B0702, B4002	Cw0202, Cw0702	B7/Core-41	B7/CMV
T3	A0201, A0205	B4901, B5101	Cw0701, Cw1502	A2/NS3-1073	A2/Flu
T4	A0201, A6802	B1801, B4002	Cw0202, Cw0501	A2/NS3-1073	A2/CMVpp65

Table 3. Sequencing of HCV epitopes studied.

Patient ID	Tetramer(s) used	Peptide sequence /autologous sequence (No. of clones)	
		Early	**Late**
C2	A2/NS3-1073	CINGVCWTV --------- (13/13)	CINGVCWTV --------- (8/9) --V------ (1/9)
	A2/NS3-1406	KLVALGINAV ---S------ (7/9) ---S-T---- (2/9)	KLVALGINAV ---S------ (10/10)
T1	A1/NS3-1436	ATDALMTGY --------F (8/8)	ATDALMTGY --------F (5/6) -----V--F (1/6)
	A2/NS3-1073	CINGVCWTV --------- (8/8)	CINGVCWTV --------- (8/8)
T3	A2/NS3-1073	CINGVCWTV -V------- (9/10) -V-----A- (1/9)	CINGVCWTV -V------- (10/10)
T4	A2/NS3-1073	CINGVCWTV --------- (13/13)	CINGVCWTV --------- (10/10)

Supplementary table 1. Primer sequences used for amplification of HCV RNA for epitope sequencing.

Epitope	PCR	Primer sequences (5'-3')	Reference
NS3-1073	First	Forward: GGCYTGCCCGTCTCYGCCCG Reverse: CGGCGCACSGGAATGACATCG	Radziewicz H. *et al.*, 2007, J.Virol. 81:2545-2553
	Nested	Forward: CGGCSTACKCCCARCAGACGMGAGGCC Reverse: CCTCGTGACCARGTAAAGGTCC	
NS3-1406 And NS3-1436	First	Forward: CCATCACGTACTCCACCTACGGCAA Reverse: GCGTGRTTGTCTCAATGGTGAAG	This manuscript
	Nested	Forward: GCCCCATCCYAACATCGAGGAGGT Reverse: TTGCAGTCTATCACMGAGTCGAAG	

2.1.10. References

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Article 2.2.

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Comparison of Immune Restoration in Early versus Late Interferon Alpha Therapy against Hepatitis C Virus

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2.2.1. Abstract

Early interferon-α (IFN-α) therapy against hepatitis C virus (HCV) rescues poly-functional virus-specific memory CD8+ T cells but whether immune restoration is possible during late therapy remains controversial. We compared immune restoration of HCV-specific memory T cells in patients who cleared HCV infection spontaneously and following early or late IFN therapy. Multi-functional, CD4+ and CD8+ memory T cells were detected in spontaneous resolvers and in individuals treated early following an acute infection. In contrast, limited responses were detected in patients treated for chronic infection and phenotype of HCV-specific cells was influenced by autologous viral sequences. Our data suggest that irreversible damage to the host immune system is associated with chronic HCV infection.

2.2.2. Introduction

The majority of acute hepatitis C virus (HCV) infections become chronic with persistent viraemia and a chronic hepatitis (10). Interferon alpha (IFN-α) based therapy is the only approved treatment for chronic HCV, its success rate ranges from 40-90% depending on the infecting genotype (8, 16). Success of therapy is characterized by a sustained viral response (SVR) defined as undetectable HCV RNA in plasma at 6 months post termination of therapy. SVR rates are greatly enhanced if therapy is started between 3 and 6 months following acute HCV but the underlying mechanisms are not well understood (20, 21). We have demonstrated that early interferon therapy for HCV can rescue long-lived poly-functional CD8+ memory T cells (1). Treatment induced memory T cells were identical in phenotype and function to natural memory T cells generated following spontaneously resolved infection. They were $CD127^{hi}$, $Bcl\text{-}2^{hi}$, $PD1^{lo}$ and poly-functional in nature (1). However, whether immune restoration of HCV-specific CD4+ T cells also occurs has not been examined. Furthermore, whether immune restoration is possible following late initiation of therapy during the chronic phase remains controversial. Kamal *et al.* demonstrated that SVR is associated with a recovery in HCV specific CD4+ T-cell responses (11). In contrast, Barnes *et al.* demonstrated that induction of HCV-specific immunity during therapy does not correlate with outcome (2).

2.2.3. Methods, Results and Discussion

The aim of this study was firstly to compare immune restoration of HCV-specific memory CD4+ and CD8+ T cell responses in patients who received early or late treatment. Secondly, we wanted to compare treatment induced/restored memory T cells to natural memory generated following spontaneously resolved acute HCV infection. To address these issues, we performed cross-sectional comprehensive characterization of HCV-specific T cells using CFSE proliferation assays, intracellular staining for cytokines, cytotoxicity and poly-functionality analysis in a cohort of 23 patients who had successfully eliminated HCV-infection under different conditions. Three subgroups were studied: a) patients who spontaneously resolved acute HCV infection without therapeutic intervention (SpR; n=5); b) patients who achieved SVR following IFN therapy initiated during acute HCV (A-SVR; n=5); and c) patients who achieved SVR following IFN therapy initiated during chronic HCV infection > 2 years post first detection of HCV-RNA (C-SVR; n=13). SpR and A-SVR patients were recruited from an acute HCV cohort of intravenous drug users (IDUs) at St-Luc hospital of the CHUM as previously described (1, 5). A-SVR patients received 12-24 weeks of PEG-IFNα–2a (Pegasys) (Roche diagnostics, Welwyn Garden City, Hertfordshire, UK) (180 μg/week) and no ribavirin. C-SVR patients were recruited from patients with chronic HCV who had undergone successful treatment with the standard-of-care therapy protocol (9) at the hepatology clinics of Toronto Western Hospital, Toronto, ON or St-Luc hospital of the CHUM. The infecting genotype had been mainly genotype 1, known to be the most resistant to IFN-α based therapy (15). The distribution was: genotype 1a (n=12); genotype 1b (n=6); genotype 3a (n=3) and undetermined genotype (n=2). Patients' demographics and characteristics are listed in Supplementary Table1.

The time point studied in this cross-sectional analysis was > 6 months post spontaneous resolution or end of antiviral therapy. Immune responses were monitored using four peptide pools representing the NS3 and NS5B regions, the most

immunodominant regions of HCV (7, 12-14) Each pool consisted of 42-49 overlapping peptides, 18 amino acids (AA) long, overlapping by 11 AA as follows: Pool 1 (P1) NS3 (1027-1339), Pool 2 (P2) NS3 (1340-1658), Pool 3 (P3) NS5B (2421-2716) and Pool 4 (P4) NS5B (2717-3012). Peptides were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI resources), Manassas, VA and matched with the infecting viral genotype in each patient (1a, 1b or 3a). All statistical analysis was performed by unpaired Student-t test using GraphPad Prism software version 5.02.

Cross-sectional proliferative responses were first monitored using a CFSE dilution assay as previously described (1). CD4+ T cell proliferation was significantly higher in response to the P1 peptide pool in SpR and A-SVR patients as compared to C-SVR ($p < 0.05$, student t test) and in the A-SVR in response to the P4 peptide pool (Figure 1A). Furthermore, CD8+ T cell proliferative responses were consistently higher in A-SVR as compared to C-SVR in response to all peptide pools and in SpR in response to the P1 pool (Figure 1B). There was no statistically significant difference between A-SVR and SpR patients.

Next, we tested the functionality of HCV-specific CD4+ and CD8+ T cells by simultaneously examining the production of IFN-γ and IL-2, as well as cytotoxicity of CD8+ T cells using the surrogate CD107a degranulation assay (3) in response to the 4 HCV peptide pools as previously described (1). Both IFN-γ and IL-2 responses by CD4+ T cells were consistently higher in response to several peptide pools in the A-SVRs as compared to the other two groups (Figure 2A, 2B). Furthermore, in CD8+ T cells IFN-γ and CD107a responses were consistently higher in the SpR and A-SVR as compared to the C-SVR in response to most peptide pools (Figure 2C-E). In contrast, very limited cytokine responses and/or CD107a responses were detected in the C-SVRs. The CD8+ T cells from the SpR group expressed the highest levels of CD107a, suggesting the cytotoxicity might be more crucial in spontaneous viral clearance as compared to treatment induced clearance.

We analyzed the poly-functionality of HCV-specific T cells. Functionality data was exported from FlowJo and poly-functionality analyzed using a mathematical algorithm for the calculation of the contribution of all possible combinations of mono-, dual- and tri-functional cells. Poly-functionality analysis was not performed for C-SVR patients as frequencies of cytokine producing cells were too low. The CD4+ T cell responses in A-SVR and SpR were primarily single IL-2 producers (Figure 3A), consistent with earlier reports documenting that loss of IL-2 production is one of the earliest functions lost upon dysfunction of virus-specific CD4+ T cells (6, 19, 22). CD8+ T cells in both A-SVR and SpR were primarily CD107a single positive, indicating that cytotoxicity was crucial in both responses. However, in dual function cells, A-SVR were more biased toward IFNγ+, IL-2+ while SpR were more IFNγ+, CD107a+ (Figure 3B). These results suggest that despite an overall similar memory T cell profile, minor functional difference may exist between natural T cell memory and treatment induced memory. It is also possible that IFN therapy, by virtue of its immune-modulatory role, can influence the functional profile of virus-specific memory cells.

To confirm that indeed there was no short-lived recovery in HCV-specific memory T cells in the C-SVR group, we examined HCV-specific immune responses longitudinally in 8 of the C-SVR patients for whom longitudinal samples were available in an IFN-γ ELISpot assay using a panel of 12 peptide pools spanning the entire HCV polyprotein. Three time points were examined: baseline, 5-6 months post start of therapy and at > 6 months post termination of therapy. As previously reported (2, 17), we did not observe any *de novo* HCV-specific T cell responses and no statistically significant enhancement in any of the responses that existed at baseline (Supplementary Figure S1).

Finally, to confirm that HCV-specific responses were limited or severely defective from the start in chronically infected patients, we screened C-SVRs prior to starting interferon therapy with a panel of 10 MHC class I tetramers corresponding to the most dominant HCV-class I epitopes (Supplementary Table S2). No responses were detected

except in 1/13 patients (patient C-SVR-5), who reacted to an A1/NS3-1406 tetramer at a frequency of 0.018% of CD8+ T cells (Figure 4A). Despite the fact that this patient was chronically infected for over 2 years with a viral load of 12 x 10^6 IU/ml, HCV tetramer-specific cells were $CD127^{hi}$, $PD1^{lo}$ (Figure 4A) consistent with a memory phenotype only previously observed in spontaneously resolved individuals. Furthermore, these tetramer positive cells proliferated efficiently at 89% of tetramer+ cells (Figure 4B) and produced cytokines in response to the specific peptide epitope (Figure 4C). In order to understand this confusing result, we sequenced the autologous virus circulating in this patient, 8/8 molecular clones sequences contained a (Y $\Rightarrow$ F) change in residue 9. When the recognition of the tetramer peptide sequence and the autologous viral sequence were compared over several fold dilution in an ELISpot assay, we observed decreased recognition of the autologous viral sequence (Figure 4C). These results suggest that this epitope probably underwent mutation at an earlier time point to the current autologous sequence that is recognized less efficiently. This diminished recognition most likely prevented exhaustion of T cells specific for the original peptide and facilitated their transformation or preservation into long-lived memory T cells. This is consistent with recent results demonstrating that expression of the exhaustion marker PD-1 is likely influenced by the degree of recognition of the autologous viral sequence (18) and might be lost following viral escape mutations (4).

Our results suggest that both HCV-specific CD4+ and CD8+ T cells become persistently defective with prolonged infection. Nevertheless, there is a narrow window of time early during the acute phase where some functional aspects of the HCV-specific T cell response might be rescued. Early therapeutic intervention during this period and subsequent viral clearance seems to prevent T cell exhaustion as is seen with chronic hepatitis C thereby allowing their development into long-lived memory T cells. Similarly, variations in viral sequences leading to loss or diminished recognition of the autologous viral sequence by T cells, decrease T cell exhaustion and permit such T cells to develop into a functional memory T cell pool.

Our data suggest that adaptive immunity may play an active role in clearance of HCV and can be recovered when IFN therapy is initiated early but irreversible damage to the host immune system occurs with chronic HCV infection. The question of whether immune restoration during antiviral treatment of acute hepatitis C is a cause or effect of enhanced response to therapy remains unresolved. We favour the hypothesis that enhanced immune responses may play a role in instigating viral clearance, but that the reconstitution of memory T cells is more likely an effect of that viral clearance. Our data also suggest that there could be minor differences between natural memory and therapy-reconstituted memory T cells and whether they would have the same protective capacity upon re-exposure to the virus requires further investigation.

2.2.4. Acknowledgements

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2.2.5. Figures

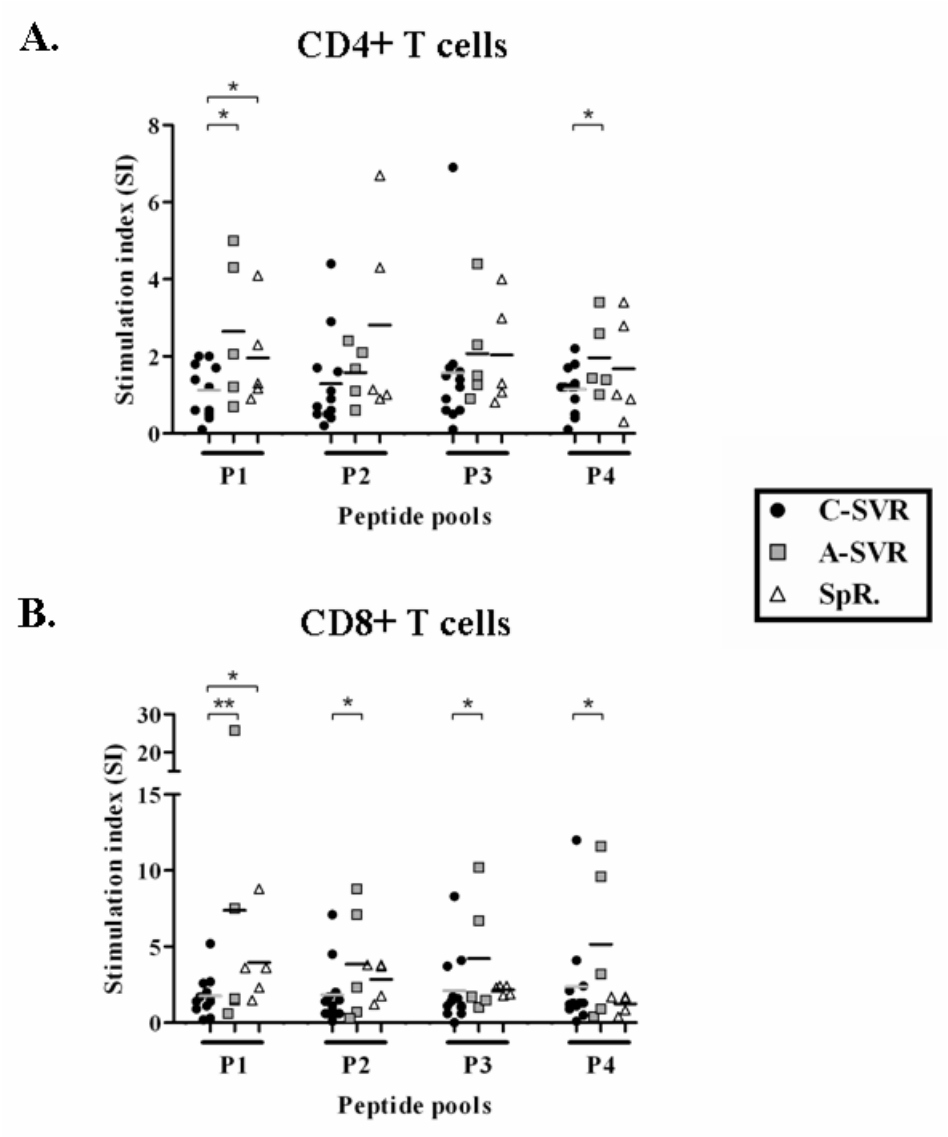

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Figure 1

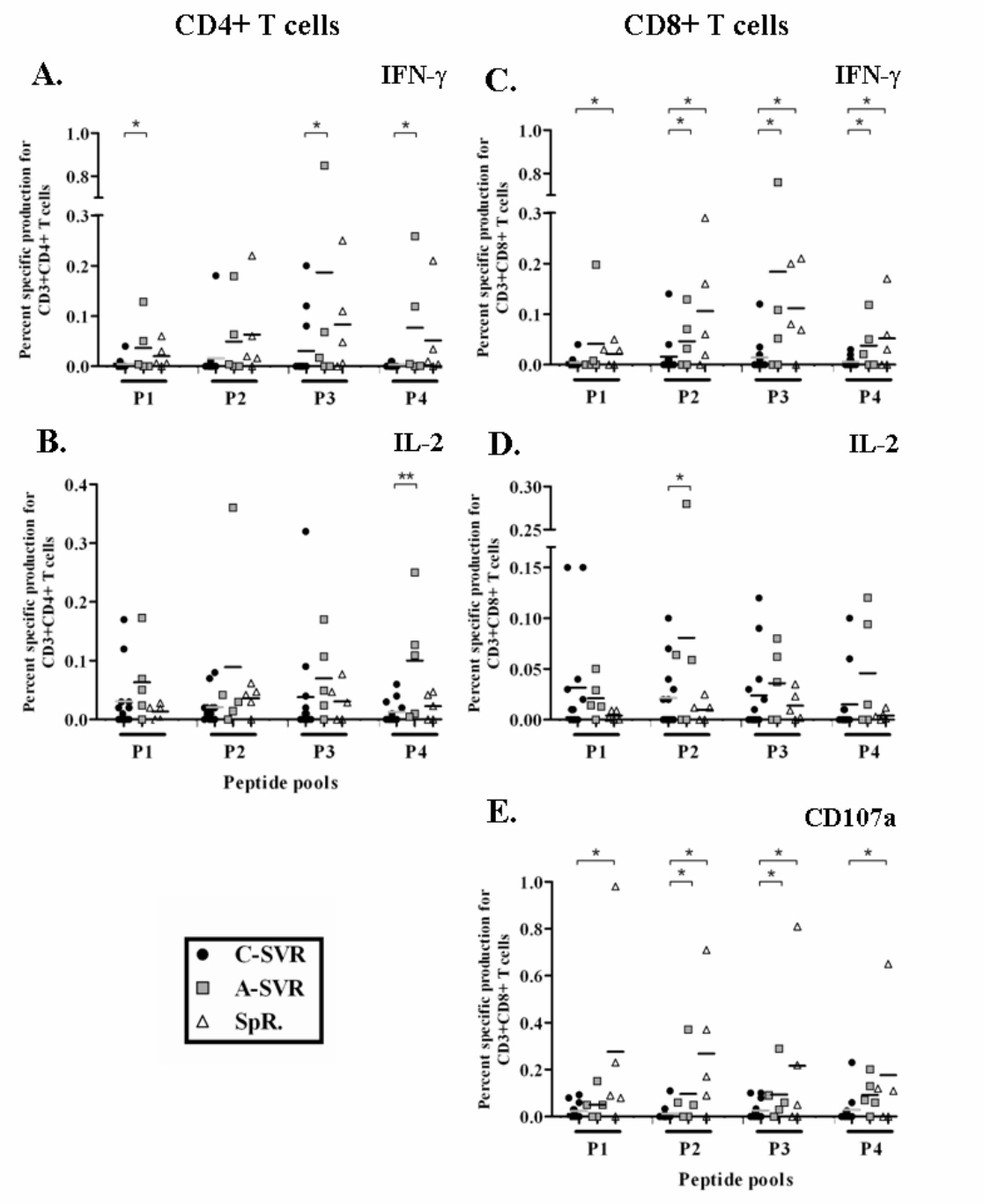

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Figure 2

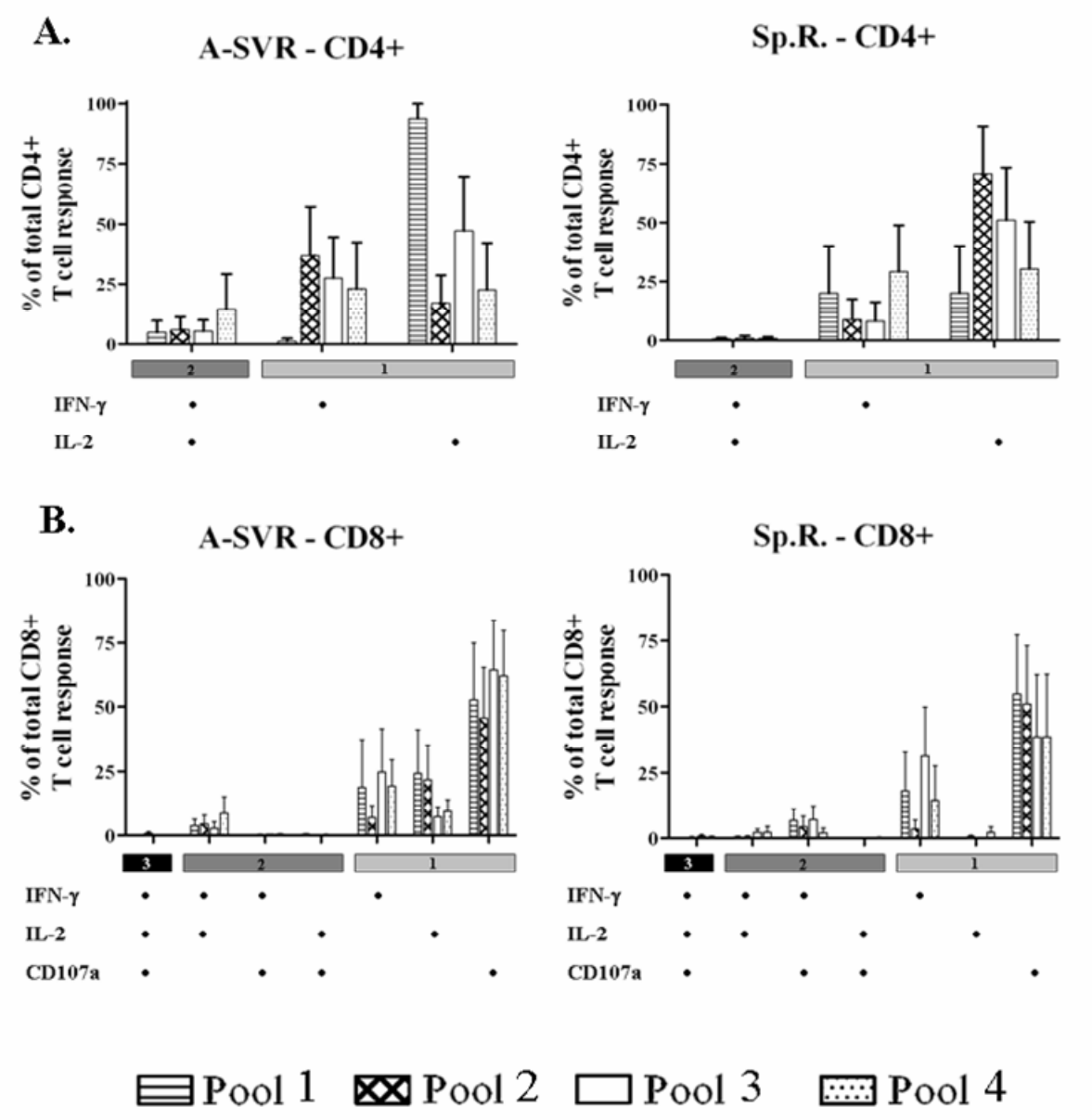

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Figure 3

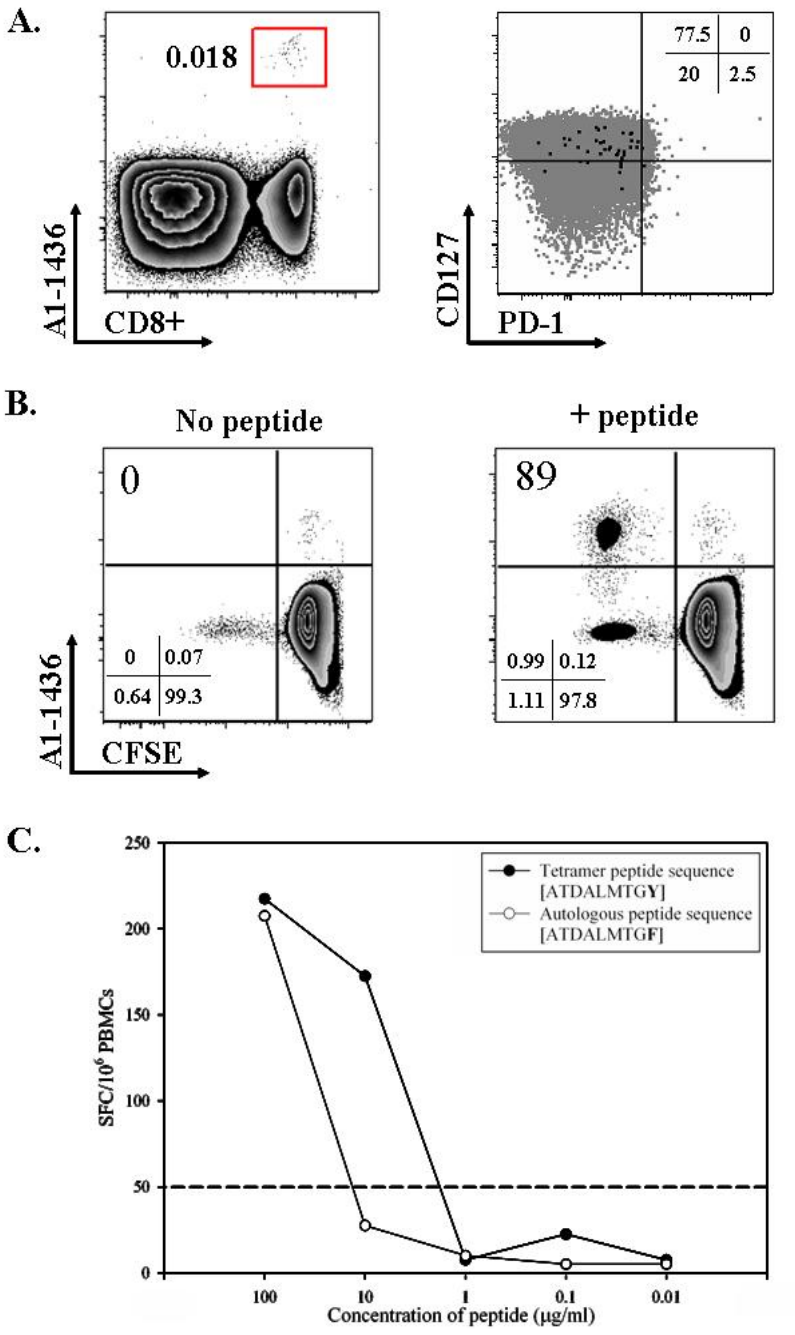

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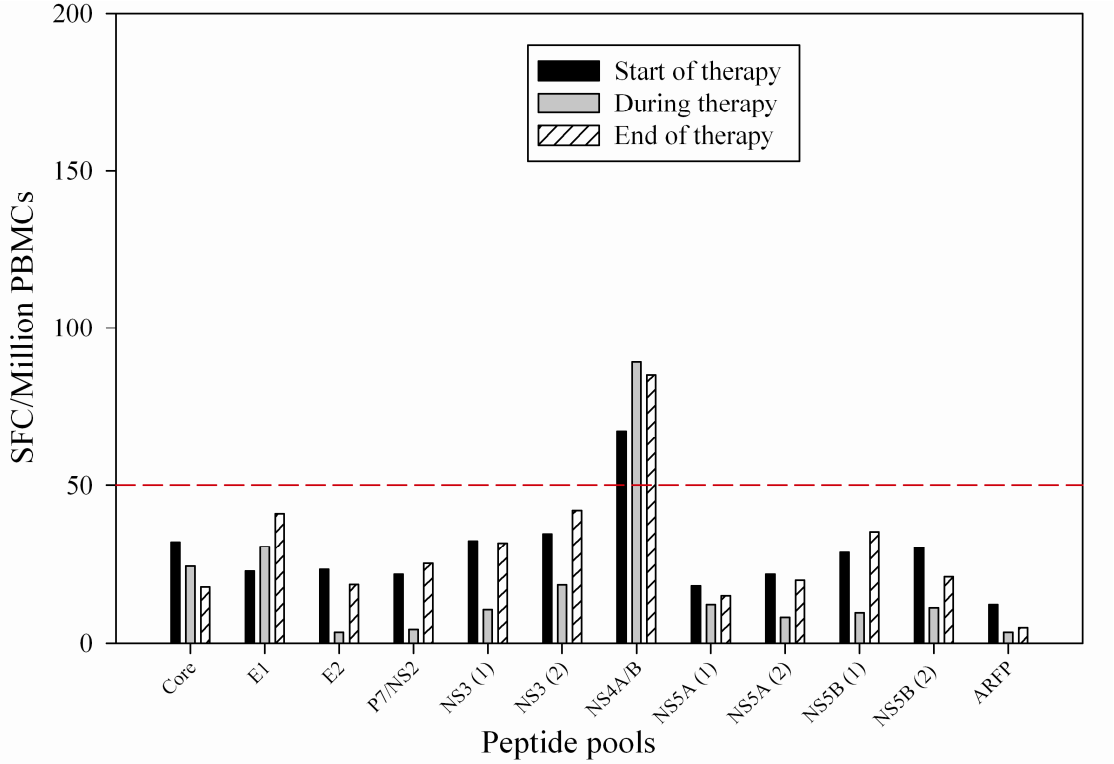
Figure 4



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Supplementary Figure S1

2.2.6. Figure Legends

Figure 1. Limited proliferation of HCV-specific CD4+ and CD8+ T cells in HCV-patients achieving SVR following late therapy (C-SVR) as compared to patients who resolved following early IFN therapy (A-SVR) or who resolved spontaneously (SpR). Cumulative data from CFSE proliferation assays performed on the different patients groups represented as stimulation indices (SI) in response to different HCV peptide pools in (A) CD4+ and (B) CD8+ T cells. (* $p<0.05$, Student-t test)

Figure 2. Higher IFN-γ and CD107a responses in CD8+ T cells and higher IL-2 responses in CD4+ T cells in SpR and A-SVRs as compared to C-SVR. Cumulative data from the different patients groups for IFN-γ, IL-2 and CD107a expression in a standard intracellular staining for cytokines (ICS) assay in response to stimulation with different HCV peptide pools. (* $p<0.05$, ** $p<0.01$, Student-t test)

Figure 3. Minor differences in poly-functionality profiles between SpR and A-SVRs. Poly-functionality data gated on viable, CD3+, CD4+T cells (A) or CD8+ T cells (B). There was no significant difference in the percentages of poly-functional cells between SpR and A-SVRs. For C-SVRs the individual production of cytokines was too low to analyze poly-functionality.

Figure 4. Virus-specific memory CD8+ T cells with long-lived memory phenotype in chronic HCV have limited functionality against the autologous viral sequence. 13 chronic HCV patients were screened using a panel of 10 MHC class I tetramers, patient C-SVR-5 was the only patient where HCV-specific tetramer+ CD8+ T cells were detected using A1/NS3-1436 tetramer. (A) Detection and phenotyping (PD-1, CD127 expression) of A1/NS3-1436 tetramer reactive T cells. Cells are gated on viable, CD3+, CD8+ T cells. Black represents tetramer positive cells and grey represents total CD3+ CD8+ T cells in the same donor, percentages of tetramer positive cells in each quadrant are shown. (B) Proliferation of A1/NS3-1436 tetramer+ cells in response to the reference peptide in a CFSE dilution assay. (C) Dose response for the reference tetramer peptide sequence versus the autolgous virus sequence for the NS3-1436 epitope in patient C-5 using IFN-γ ELISpot assay. Results are represented as specific spot forming cells (SFC)/ 10^6 PBMCs.

Figure S1. No restoration of immune responses against HCV in patients who resolved after receiving IFN-α therapy in the chronic phase (C-SVRs). Results are represented as the mean of C-SVR patients' responses (n=8) in an IFN-γ ELISpot assay against the indicated HCV peptide pools. Each pool consisted of 39-50 overlapping peptides representing the entire HCV polyprotein. Peptides are 18 AA long, overlapping by 11 AA (Biodefense and Emerging Infections Research Resources Repository (BEI resources)). No significant difference was observed in the responses between the different time points tested. The dashed line represents the positivity threshold (50 SFC/ 10^6 PBMCs).

2.2.7. Tables

Supplementary Table S1. Demographic characteristics of patients with HCV

A. Patients receiving IFN therapy during the chronic phase (C-SVR)

Gender M/F	Age Median (Range)	HCV genotype 1a	1b	3a	ND
9/4	47 (34-61)	8	5		

B. Patients receiving IFN therapy during the acute phase (A-SVR)

Gender M/F	Age Median (Range)	HCV genotype 1a	1b	3a	ND
5/0	34 (30-47)	3	1	1	

C. Patients who spontaneously resolved from hepatitis C (SpR)

Gender M/F	Age Median (Range)	HCV genotype 1a	1b	3a	ND
4/1	32 (23-46)	1		2	2

Supplementary Table S2. MHC class I tetramers used in screening HCV-specific CD8+ T-cell responses in chronic patients prior to start of therapy

Restricting HLA Allelle	HCV Protein	AA	Peptide sequence
A1	NS3	1436-1444	ATDALMTGY
A2	Core	131-140	ADLMGYIPLV
	E2	614-622	RLWHYPCTV
	NS5B	2594-2603	ALYDVVTKL
	NS3	1073-1081	CINGVCWTV
	NS3	1406-1415	KLVALGINAV
	NS4b	1807-1816	LLFNILGGWV
A3	E2	630-639	RMYVGGVEHR
B7	Core	41-49	GPRLGVRAT
B8	NS3	1395-1403	HSKKKCDEL

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Chapter 3: Discussion

Results from the longitudinal study that we performed using MHC class I tetramers (Article 2.1) enabled us to define the phenotypic and functional markers of HCV-specific CD8+ T cells associated with patients clearing HCV spontaneously, thus considered as determinants of a successful adaptive immune response against HCV. We demonstrate that spontaneous resolution is associated with early emergence of poly-functional memory T cells that up-regulate CD127 and Bcl-2. This suggests that they represent transitional effector memory T cells (T_{TEM}). Moreover, we were able to demonstrate that early therapeutic intervention during acute HCV-infection can rescue poly-functional memory T cells with similar phenotypic and functional characteristics as observed following spontaneous resolution. Results from our cross-sectional study using peptide pools (Article 2.2) confirmed and extended our conclusions for CD8+ T cells; CD8+ T cells in spontaneous resolvers and SVRs after early therapy (A-SVR) consistently displayed higher functions than chronic SVRs (C-SVR). Similar results were observed for CD4+ T cells.

The possibility that the longitudinal changes observed in the CD8+ T-cell responses in our first study were due to viral escape was ruled out. However, one chronically infected individual in the cross-sectional study who had HCV-tetramer-specific CD8+ T cells that were $CD127^{hi}$ $PD1^{lo}$ did show one amino acid difference between the autologous and the tetramer peptides that led to a diminished recognition of the autologous viral sequence. This could be a possible reason preventing exhaustion and preservation of memory T cells.

Collectively, our results demonstrate restoration of efficient HCV-specific immune responses when interferon therapy is initiated early during the acute phase. This is demonstrated by the rescue of functional HCV-specific T cells similar in phenotype and functionality to those observed in spontaneously resolved individuals, and suggests that they may play an active role in response to therapy. However, such responses were not restored following late therapy suggesting that irreversible damage to the host's defence system precipitates with HCV infection as it proceeds towards chronicity preventing it from playing an active role with late initiation of therapy.

3.1. Successful HCV-specific CD8+ T cells in spontaneously resolved infections are poly-functional memory T cells

We performed comprehensive longitudinal multi-parametric phenotypic and functional characterization of the HCV-specific immune response during clearly defined acute infection in a unique cohort of IDUs with differential outcomes of acute-HCV infection, including spontaneous resolution and proceeding to chronicity. We used MHC class I tetramers to detect the responses of HCV-specific CD8+ T cells targeting defined immunodominant epitopes (Article 2.1). We demonstrate that spontaneous resolution is specifically associated with early emergence of memory CD8+ T cells, defined by the up-regulation of CD127 on cells and intracellular content of the anti-apoptotic molecule Bcl-2, a common characteristic of long-lived memory T cells [293]. Despite a measurable immune response detected at the early time points, HCV-specific T cells failed to upregulate CD127 and Bcl-2 expression in individuals who developed persistent viraemia and eventually disappeared from peripheral blood. In agreement with previous reports we demonstrate that PD-1 expression does not correlate with the outcome of acute HCV [197, 198], except at the highest viral loads.

These cells are poly-functional; defined as the capacity to simultaneously produce more than one cytokine, in addition to cytotoxic and proliferative capacity [294, 295]. Such poly-functional T cells were recently correlated with control of HIV infection [296, 297], response to HIV vaccines [290, 298] and anti-retroviral therapy [291]. We established that these poly-functional memory T cells in the context of HCV are: CD27+, CD28+, CCR7-, CD45RA-, CD127+, Bcl-2+. A similar memory T cell subset at a transitional or early maturation stage (CD27+, CD28+, CD127+) with a short replicative history and strong telomerase activity was recently described [299].

In this context, we demonstrate that CD127 expression distinguishes a unique subset of HCV-specific memory T cells bearing the phenotypic signature of effector memory T

(T_{EM}) cells (CCR7-CD45RA-) and yet bearing the functional signatures of both central memory T cells (T_{CM}) (rapid proliferation, high IL-2 production) and T_{EM} (high IFN-γ production and cytotoxic potential). We propose that this poly-functional T-cell subset represents transitional effector memory T cells (T_{TEM}) that are long-lived but also constitute the first responders upon antigen encounter in the periphery. Although this T-cell subset is distinguished primarily by CD127 expression, it is unlikely that CD127 alone can be used as a marker of such polyfunctional memory T cells or to predict the outcome of HCV infection or therapy. Such simplistic models of defining maturation of virus-specific T cells and their functional capacity are no longer sufficient but a comprehensive systematic phenotypic, functional and even genomic profiling will be essential.

3.2. Similar profile of early-treatment induced memory T cells and normal memory T cells

PEG-IFN-α therapy for HCV is most effective when initiated early during the acute phase [243, 300, 301] but the potential contribution of adaptive cellular immunity in this enhanced response is controversial with several reports demonstrating a decline in the immune response during therapy [58-60, 288]. In the present study, we do not observe any significant change in the frequency of HCV-specific T cells detected by tetramers during therapy in acute HCV patients nor by ELISpot during therapy initiated in chronic patients. Although we did observe a gradual decline over time, HCV-specific T cells remained detectable for up to 1.5 years of follow-up. Such differences between the various studies might be due to the therapeutic regimen or the nature of the dominant immune response generated in each patient and how accurately it matches the autolgous virus sequence.

Our results from the longitudinal study (Article 2.1) demonstrate that early therapeutic intervention can rescue a poly-functional immune response with the same phenotypic and functional characteristics as memory T cells induced following spontaneous resolution. However, it remains unclear if such reconstituted response is the cause or the

effect of enhanced virus clearance during early therapy. We favour the hypothesis that re-constitution of a poly-functional immune response is a consequence of virus elimination and prevention of continued T cell exhaustion similar to acute resolving HCV and other viral infections [293]. It is likely that there is a limited window of time where IFN therapy can rescue such a poly-functional response before HCV-specific T cells become severely exhausted and eliminated from the circulation. In agreement with this hypothesis, we demonstrate that HCV-specific cells eventually become undetectable in patients who declined HCV therapy. Similarly, several reports in the literature have demonstrated that HCV-specific T cells are mostly undetectable in the peripheral blood of chronically infected individuals, mostly localized in the liver or targeting epitopes that underwent escape mutations, thus preventing exhaustion of the specific T cells.

Longevity is one of the unique characteristics of memory T cells. HCV-specific memory T cells were detected in spontaneously resolved infections for up to 20 years in absence of any detectable viraemia [302]. Similarly, in the longitudinal study (Article 2.1), we demonstrate that although HCV-specific memory T cells declined over time, they remained detectable at a reasonable frequency for up to one year following spontaneous resolution or sustained viral response to interferon therapy. All patients were tested at multiple time points by the most sensitive PCR assays (sensitivity 50 IU/ml plasma) and were consistently negative, suggesting that HCV-specific T-cell memory is antigen independent and that establishment of a long-lived memory response is due to successful viral elimination and prevention of persistent T-cell activation and exhaustion. Nevertheless, it is still possible that very low level residual virus or intermittent viral replication [303] contribute to long-term maintenance of virus-specific memory T cell populations.

In the second part of our study (Article 2.2), we performed cross-sectional comprehensive phenotypic and functional characterization of both HCV-specific CD4+ and CD8+ T cells in a cohort of patients who have successfully eliminated HCV-infection

under different conditions. Three subgroups were identified: patients who spontaneously resolved acute HCV infection without therapeutic intervention (SpR), patients who achieved SVR following IFN therapy initiated during acute HCV (A-SVR) and patients who achieved SVR following IFN therapy initiated during chronic HCV infection more than 2 years post first detection of HCV-RNA and diagnosis of chronic infection (C-SVR). The proliferation and functionality were assessed in response to peptide pools corresponding to immunodominant regions representing a third of the entire HCV genome.

Our results show that CD8+ T-cell proliferative responses were consistently higher in A-SVR as compared to C-SVR in response to all peptide pools. CD8+ T cells in the SpR and A-SVR displayed consistently higher production and expression of functional markers (IFN-γ and CD107a) as compared to the C-SVR. Polyfunctionality analysis showed that CD8+ T cells in both A-SVR and SpR were primarily CD107a single positive, indicating that cytotoxicity was crucial in both responses. However, in dual function cells, A-SVR were more biased toward IFNγ+, IL-2+ while SpR were more IFNγ+, CD107a+. These results suggest that despite an overall similar memory T-cell profile, minor functional difference may exist between natural T cell memory and treatment induced memory. It is also possible that IFN therapy, by virtue of its immune-modulatory role, can influence the functional profile of virus-specific memory cells.

We also characterized CD4+ T-cell help, being the most important factor contributing to the induction of poly-functional T cells and establishment of T-cell memory [293]. CD4+ T cells play an essential role in establishment and maintenance of CD8+ memory T cells in various models of murine and human infections [304-306]. In particular, acute HCV infections that progress to chronicity have been linked to the inability to sustain a CD4+ helper T-cell response [158, 114, 135]. Depletion studies in the chimpanzee model have demonstrated that loss of CD4+ T cells results in loss of function and a decline in HCV-specific CD8+ T cells and enhanced emergence of escape mutations in targeted CTL

epitopes [157]. The role of CD4+ T-cell help and the mechanisms of cross talk between CD4+ and CD8+ T cells during the memory phase, still remain to be elucidated [307, 308].

Indeed, we observed significantly higher proliferation in the SpR and A-SVR groups as compared to C-SVR. Both IFN-γ and IL-2 responses by CD4+ T cells were consistently higher in the A-SVRs as compared to the other two groups. In order to assess the contribution of each cytokine to the total memory immune response and to assess poly-functionality of HCV-specific memory T cells generated in the three groups of patients studied, we analyzed the poly-functionality of HCV-specific T cells. Interestingly, CD4+ T-cell responses in A-SVR and SpR were primarily single IL-2 producers, consistent with earlier reports documenting that loss of IL-2 production is one of the earliest functions lost upon dysfunction of virus-specific CD4+ T cells [192, 309, 310].

3.3. Role of viral-sequence variation in memory T-cell preservation

We demonstrate a major change in the immune response following therapy during the acute phase while the circulating viral population remains unchanged (Article 2.1), thereby excluding a potential influence for viral escape on the immune response to the epitopes studied. However, we did not perform a detailed analysis of viral escape in these patients and it remains an important mechanism for HCV immune evasion. It is still possible that viral escape did occur in other regions and may have contributed to viral persistence. Furthermore, the increased poly-functionality of virus-specific T cells may induce new selection pressure and trigger mutational escape. A more comprehensive analysis of poly-functionality in relation to full length viral sequencing is essential to elucidate this point.

Interestingly, one persistently infected patient did show a population of HCV tetramer-specific cells that were $CD127^{hi}$ $PD1^{lo}$ consistent with a memory phenotype observed in spontaneously resolved individuals, despite the fact that this patient was chronically infected for over 2 years and with a high viral load (Article 2.2). Furthermore,

these tetramer positive cells proliferated efficiently and produced cytokines in response to the specific peptide epitope. By sequencing the autologous virus circulating in this patient, a change in one of the anchor residues was observed. Interestingly, we observed decreased recognition of the autologous viral sequence. These results suggest that this epitope probably underwent mutation at an earlier time point to the current autologous sequence that is recognized less efficiently. This diminished recognition most likely prevented exhaustion of T cells specific for the original peptide and their transformation into long-lived memory T cells. This is consistent with recent results demonstrating that expression of the exhaustion marker PD-1 is likely influenced by the degree of recognition of the autologous viral sequence [311].

Our results demonstrate the reconstitution of poly-functional HCV-specific T cells after early IFN therapy, which argues in favour of early administration of interferon therapy. These recovered HCV-specific T cells bearing the same phenotype and functional signatures as memory T cells generated upon spontaneous resolution suggest that this immune response may be protective upon re-exposure, and further underscore the importance of early therapy administration. Studies in the chimpanzee have demonstrated that prior resolution of one HCV infection can induce protection from chronic infection upon re-exposure [312]. Although such protective response is difficult to evaluate in humans, two reports have demonstrated that high risk IDUs who have already resolved one HCV infection are less likely to be re-infected than individuals who are HCV-naïve, despite repeated high risk exposures [173, 313]. We demonstrate that treating at an early stage will eliminate the virus in IDUs and minimize the risk of new transmissions from this population which is the main reservoir of HCV [314, 315]. Further, we predict that the reconstitution of a poly-functional memory response may protect this population against new infections.

Our results suggest, as well, that HCV-specific CD4+ and CD8+ T cells become persistently defective with prolonged infection. Nevertheless, there is a narrow window of

time early in the acute phase where some functional aspects of the HCV-specific T-cell response might be rescued. Early therapeutic intervention during this period and subsequent viral clearance prevents T-cell exhaustion and allows their development into long-lived memory T cells. Similarly, variations in viral sequences leading to loss or diminished recognition of the autologous viral sequence by T cells decrease T-cell exhaustion. Although such T cells may maintain certain antiviral functions and provide partial control of viral replication, they can develop into a ‘non-exhausted’ memory T cell pool.

Conclusion

In conclusion, our comprehensive phenotypic and functional characterization argues in favour of a more exhaustive multi-parametric signature analysis for HCV-specific T cells at the earliest stages of infection. Our results suggest that poly-functionality rather than one single phenotypic or functional marker is more likely predictive of the outcome of acute HCV infection.

Most importantly, we demonstrate that early administration of IFN therapy during the acute phase can rescue such functional signature of HCV-specific adaptive immune responses which we consider an effective adaptive immune response. These responses were similar to those responses observed in individuals who spontaneously cleared HCV. This restoration suggests that the HCV-specific adaptive immune responses might play an active role in the success of therapy and the achievement of a sustained virological response (SVR) when initiated early during the acute phase. However, such responses were not restored following late therapy during the chronic phase, which might be due to irreversible damage to the host immune system that precipitates with chronic HCV infection.

The question of whether immune restoration during treatment in the acute phase is a cause or effect of enhanced response to therapy remains unresolved. We favour the hypothesis that enhanced immune responses may play a role in enhanced viral clearance, but the reconstitution of memory T cells is likely an effect of viral clearance that abrogates viral pressure which might hinder the establishment of such a long-lived memory pool. Nevertheless, such reconstituted memory T cells may play a more central role in preventing viral recurrence upon discontinuation of therapy. Our data also suggest that there could be minor differences between natural memory and therapy-reconstituted memory T cells and whether they would have the same protective capacity against re-infection upon re-exposure to the virus, especially in high risk populations like IDUs, remains elusive.

Taken together, our data suggest that the timing of initiation of IFN-α therapy against HCV is a critical factor in the restoration of an effective HCV-specific immune response that might play an effective role in viral clearance.

Perspectives

We demonstrated the inability of late initiation of IFN therapy during the chronic phase to restore effective HCV-specific immune responses, and thus its failure to play an active role in the success of therapy. It is thus essential to study other factors that might play an important role in the different outcomes of acute infection and the achievement of SVR to therapy during the acute phase, and more important during the chronic phase. In particular, to define what might be directly influencing the outcome of acute HCV infection and response to therapy. An attractive approach is testing the impact on different determinants of the immune system of different regions sequenced from clinical isolates obtained from patients of differential outcomes of acute infection and responses to therapy. Defining these viral determinants is essential in understanding the mechanisms underlying resistance to the available current treatment and would help in the development of novel therapeutic regimens.

To pursuit our characterization of a successful HCV-specific immune response, it is essential to define molecular and/or functional signature of what constitutes a successful/protective immune response against HCV. This information is critical to assess the efficiency of new vaccines or therapeutic strategies aimed at reconstituting an efficient immune response. Such signatures could also be used to predict the outcome of HCV infection and the need for early therapeutic intervention. It is essential to use an integrated functional genomics and protein profiling approach coupled with phenotypic analysis to define signatures of a successful immune response against HCV. Furthermore, it is essential to examine and compare at the genetic and molecular level the characteristics of HCV-specific effector and memory T cells early during the acute phase and following HCV-spontaneous or treatment induced clearance. This will help in determining whether immunological memory reconstituted following therapy is equivalent to spontaneous clearance induced memory. It will also help in verifying whether both types of memory cells would maintain the same functional profile over long periods of time and convey the same level of protection upon re-infection in populations at high risk such as IDUs.

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Appendix I: The candidate's contribution to the articles

Contribution to article 2.1

The candidate, Mohamed S. Abdel-Hakeem, performed the sequencing experimental work and analysed the resulting data. He also participated in the preparation of the manuscript and the included figures.

Contribution to article 2.2

The candidate, Mohamed S. Abdel-Hakeem, performed approximately 70% of the flowcytometry experimental work and analysis, analysed the polyfunctionality data and performed the ELISpot experimental work. He also participated in writing the manuscript and prepared the included figures.