Université de Montréal

Correlates of Protective Immunity against Hepatitis C Virus

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Faculté des études supérieures

Cette thèse intitulée :

Correlates of Protective Immunity against Hepatitis C Virus

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Résumé

Le virus de l'hépatite C (VHC) infecte ~185 millions d'individus dans le monde. Malgré le développement des nouvelles thérapies dirigées contre le VHC, on compte deux millions de nouvelles infections chaque année, en particulier dans les pays sous-développés et les populations marginalisées. Comme pour la plupart des virus à infection chronique, le développement d'un vaccin prophylactique efficace est limité par le manque de caractérisation des déterminants de la mémoire immunitaire protectrice lors des épisodes de réinfection naturelle chez les êtres humains. Le VHC représente un modèle unique au sein des virus à infection chronique pour étudier l'immunité protectrice. En effet ~30% des patients infectés par le VHC peuvent être guéris suite à un premier épisode d'infection spontanément.

Dans cette thèse, nous avons étudié l'immunité protectrice contre le VHC dans une cohorte d'utilisateurs de drogues par injection qui sont à risque d'être infectés ou réinfectés. Notre hypothèse est que la majorité des patients qui ont résolu une première infection par le VHC sont protégés contre le développement d'une infection chronique s'ils sont réexposés. Cette immunité protectrice est associée à la présence des cellules T CD4 et CD8 polyfonctionnelles qui possèdent des fréquences, magnitudes et avidités élevées. La capacité protectrice des cellules T mémoire contre les séquences variables du VHC est dépendante de la diversité et flexibilité du répertoire de leurs récepteurs de cellules T (TCR), qui reconnaissent les séquences variables des épitopes ciblés.

Notre premier objectif était de définir et détailler les déterminants de l'immunité protectrice conférée par les cellules T spécifiques du VHC. Nos résultats ont montré que la protection pendant l'épisode de réinfection était associée à une augmentation de la magnitude et du spectre des réponses spécifiques par les cellules T CD4 et CD8 polyfonctionnelles, ainsi que par l'apparition d'une population de cellules T tétramère+ CD8+ effectrices qui expriment faiblement le marqueur CD127 ($CD127^{lo}$) lors du pic de la réponse. Chez les patients qui ont développé une infection chronique pendant l'épisode de réinfection, nous avons observé une expansion très limitée des cellules T CD4 et CD8. Le séquençage des épitopes ciblés par les cellules T CD8 chez ces patients qui sont non-protégés a montré que les

séquences de ces épitopes sont différentes des séquences de référence qui étaient utilisées pour tous les essais immunologiques de cette étude.

Le deuxième objectif était d'analyser la dynamique du répertoire des TCRs des cellules T CD8 spécifiques chez les patients protégés versus les patients non-protégés. Nos résultats ont montré que le répertoire des cellules T CD8 spécifiques est plus focalisé que chez les patients protégés. En plus, nous avons observé que les clonotypes qui forment le répertoire chez les patients protégés sont distincts de ceux chez les patients non-protégés. Ces clonotypes chez les patients protégés ont montré de plus grandes avidité et polyfonctionnalité que leurs homologues chez les patients non-protégés.

En conclusion, nos résultats suggèrent que la protection contre le développement d'une infection chronique pendant l'épisode de réinfection par le VHC est associée à une augmentation de la magnitude, du spectre et de la fonctionnalité des réponses des cellules T spécifiques, ainsi qu'à un répertoire des TCRs plus focalisé composé des clonotypes distincts qui possèdent de plus grandes avidité et polyfonctionnalité que chez les patients non-protégés. L'homologie des séquences des souches virales entre les différents épisodes de l'infection est un déterminant majeur de l'établissement d'une protection efficace. Ces résultats ont donc des implications très importantes pour le développement d'un vaccin prophylactique contre le VHC et d'autres virus à infection chronique.

Mots-clés : Virus de l'hépatite C (VHC) – Mémoire immunitaire – Réinfection par le VHC – Immunité protectrice – Répertoire des cellules T

Abstract

Hepatitis C virus (HCV) currently infects ~185 million individuals worldwide. Despite the development of new effective antivirals against HCV, it is estimated that two million new infections occur annually, especially among marginalized populations and in developing countries. As with many chronic viral infections, the development of an effective prophylactic vaccine is hampered by the limited knowledge of determinants of a protective immune response in humans upon natural exposure to the virus. HCV represents a unique model to study protective immunity in chronic viruses, since ~30% of the patients are able to clear the primary infection spontaneously.

In this thesis, we proceeded to study protective immunity against HCV in cohorts of injection drug users (IDUs) who are continuously at risk of infection and reinfection by HCV. We hypothesized that the majority of spontaneous resolvers of a primary HCV infection are protected against chronicity of infection upon re-exposure. Protective immunity would likely be associated with the highest breadth, frequency and functional avidity of HCV-specific polyfunctional CD4 and CD8 memory T cells. The protective capacity of memory T cells upon infection with novel HCV variants will depend on the diversity and flexibility of the T cell repertoire that can recognize these viral variants and/or the capacity to generate *de novo* T cell responses specific for these variants.

The first aim of the project was to define and dissect correlates of protective immunity conferred by HCV-specific T cells in reinfected individuals. Our results showed that protection from chronicity upon reinfection was associated with an increased magnitude and breadth of the HCV-specific polyfunctional CD4 and CD8 T-cell responses, as well as the appearance of a $CD127^{lo}$ tetramer+ CD8+ effector T-cell population at the peak of the response. Individuals who developed persistent viremia upon HCV reinfection demonstrated very limited or no expansion of HCV-specific T cells. Sequencing of the cytotoxic T-lymphocyte (CTL) epitopes targeted by the memory immune response in unprotected individuals revealed that the sequence of the autologous reinfecting virus was different from the reference sequence used in our immunological assays.

The second aim was to analyse the dynamics of the T-cell receptor (TCR) repertoire of HCV-specific CD8 T-cells in protected versus unprotected patients and in relation to infection

with variant viral sequences. Our results demonstrate that for epitope-specific CD8 T cells a more focused TCR-repertoire of distinct clonotypes was associated with protection. These T-cell populations showed higher functional avidity and polyfunctionality compared to their counterparts in non-protected patients. The clonotypes forming the effector T-cell population during reinfection were recruited from the memory pool, rather being *de novo* responses generated from the naïve pool.

In conclusion, our results suggest that protection from persistence upon HCV reinfection is associated with an enhanced magnitude, breadth and quality of the HCV-specific T-cell response, as well as a more focused TCR-repertoire of distinct clonotypes with high functional avidity. The degree of homology between viral strains causing the consecutive episodes of infection is a critical determinant for protection. These findings provide a first insight into the correlates of protective immunity against HCV and have important implications for the rational design of effective prophylactic vaccines against HCV and other chronic viruses.

Keywords:
Hepatitis C Virus (HCV) – Memory immune response – HCV reinfection – Protective immunity – T-cell receptor (TCR) repertoire

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List of abbreviations

Ab	Antibody
Ad	Adenovirus
ADCC	Ab-dependent cellular cytotoxicity
Ag	Antigens
AIDS	Acquire immunity deficiency syndrome
AP-1	Activator protein 1
APC	Antigen presenting cells
Bcl-2	B-cell lymphoma-2 regulator protein
BrdU	Bromodeoxyuridine
CCL	C-C motif chemokine ligand
CCR7	C-C motif chemokine receptor type 7
CDR	Complementarity-determining regions
CLDN	Claudin
CMI	Cell-mediated immunity
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
CypA	Cyclophylin A
DAA	Direct-acting antiviral agents
DC	Dendritic cells
dsRNA	Double-stranded RNA
EBV	Epstein–Barr virus
EGFR	Epidermal growth factor receptor
ELISPOT	Enzyme-linked immunospot
EOMES	Eomesodermin
EphA2	Ephrin receptor type A2

Fab	Ag-binding fragment of the antibody
Fc	Constant fragment of the antibody
FDA	Food and Drug Administration
fDC	Follicular DCs
FoxP3	Forkhead transcription factor 3
GATA3	GATA-binding protein 3
GC	Germinal centre
GSEA	Gene set enrichment analysis
GWAS	Genome-wide association studies
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVpp	HCV pseudo-particles
HCVcc	Cell-cultured HCV
HEV	High endothelial venules
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HSV	Herpes simplex virus
HVR	Hypervariable region-1
IDU	Injection drug users
IFN	Interferon
Ig	Immunoglobulin
IKKε	IkB kinase-ε
IL	Interleukin
IL7Rα	IL-7 receptor alpha chain
IRES	Internal ribosome binding site
IRF	Interferon regulatory factor
ISG	Interferon-stimulated genes

JAK	Janus kinase
JFH1	Japanese fulminant hepatitis-1
kb	Kilo base-pairs
KIR	Killer cell immunoglobulin-like receptors
LCMV	Lymphocytic choriomeningitis virus
LDL	Low density lipoproteins
LN	Lymph nodes
LTNP	Long-term non-progressors
LVP	Lipoviroparticles
MAVS	Mitochondrial antiviral signaling protein
mDC	Myeloid DCs
MHC	Major histocompatibility complex
MVA	Modified vaccinia Ankara
nAb	Neutralizing Abs
NANB	Non-A, non-B
NCR	Non-coding regions
NK	Natural killer cells
NKG2D	Natural killer group 2, member D
NR	Non-response
NS	Non–structural
OCLN	Occluding
ORF	Open reading frame
PAMP	Pathogen Associated Molecular Patterns
PBMCs	Peripheral blood mononuclear cells
PD-1	Programmed death-1

pDC	Plasmacytoid DCs
PEG	Polyethylene glycol
pMHC	Peptide-MHC
p.ri.	Post reinfection
PRR	Pattern recognition receptor
RdRp	RNA-dependant RNA-polymerase
RIG-I	Retinoic-acid-inducible gene I
RORγt	Retinoic acid receptor-related orphan receptor-γt
SNP	Single-nucleotide polymorphisms
SR-BI	Scavenger receptor class B type I
ssRNA	Positive single-stranded RNA
STAT	Signal transducer and activator of transcription
SVR	Sustained virological response
TANK	TRAF family member-associated NFκB
TBK	TANK-binding kinase 1
T_{CM}	Central-memory T cells
TCR	T-cell receptor
T_{eff}	Effector T cells
T_{EM}	Effector-memory T cells
T_{EMRA}	Terminally-differentiated T cells
Tfh	Follicular helper T cells
TGF	Transforming growth factors
Th	T helper cells
Tim-3	T-cell immunoglobulin and mucin domain 3
TJ	Tight junction
TLR	Toll-like receptors
T_{mem}	Memory T cells

TNF Tumor necrosis factor

TRAF TNF receptor–associated factor 3

TRAIL TNF-related apoptosis-inducing ligand

Tregs Regulatory T cells

TRIF TIR domain-containing adapter inducing IFN-β

Tyk Tyrosine kinase

UTR Untranslated region

VLDL Very-low-density lipoproteins

VLP Virus-like particles

VV Vaccinia virus

WHO World Health Organization

WT Wild-type

YF Yellow fever

To freedom fighters all over the world,
especially heroes of The Egyptian Revolution,
January 25th, 2011.
To Mom, family & friends in Egypt.
To my second family & friends in Canada

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﴾ … and you (mankind) have been given but a little knowledge ﴿ Quraan (17 :85)

« Seek knowledge even at the farthest place » Prophet Muhammad (PBUH)

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All praise to ALLAH with Whose Benediction the good deeds are accomplished.

CHAPTER 1:

INTRODUCTION

(Parts of this introduction were used in the review article

"Protective Immunity against Hepatitis C: Many Shades of Gray",

***Frontiers in Immunology*, 2014**)

The rational design of prophylactic vaccines against chronic viral infections necessitates dissection of the correlates of protective immunity that remain undefined to date. Hepatitis C virus (HCV) represents a unique model among chronic viruses where two dichotomous outcomes of infection are observed: spontaneous resolution or chronic infection. Thus it represents an ideal model for understanding determinants of the memory immune response that can confer protection upon re-exposure to the virus in a human cohort.

This introduction discusses what is known about primary and memory immune responses against chronic viral infections, followed by a more specific review of the literature about the primary and memory immune responses against HCV infection. It also gives a brief account of different aspects of HCV; characteristics of the virus, the infection caused by HCV, treatment strategies and HCV-vaccine trials.

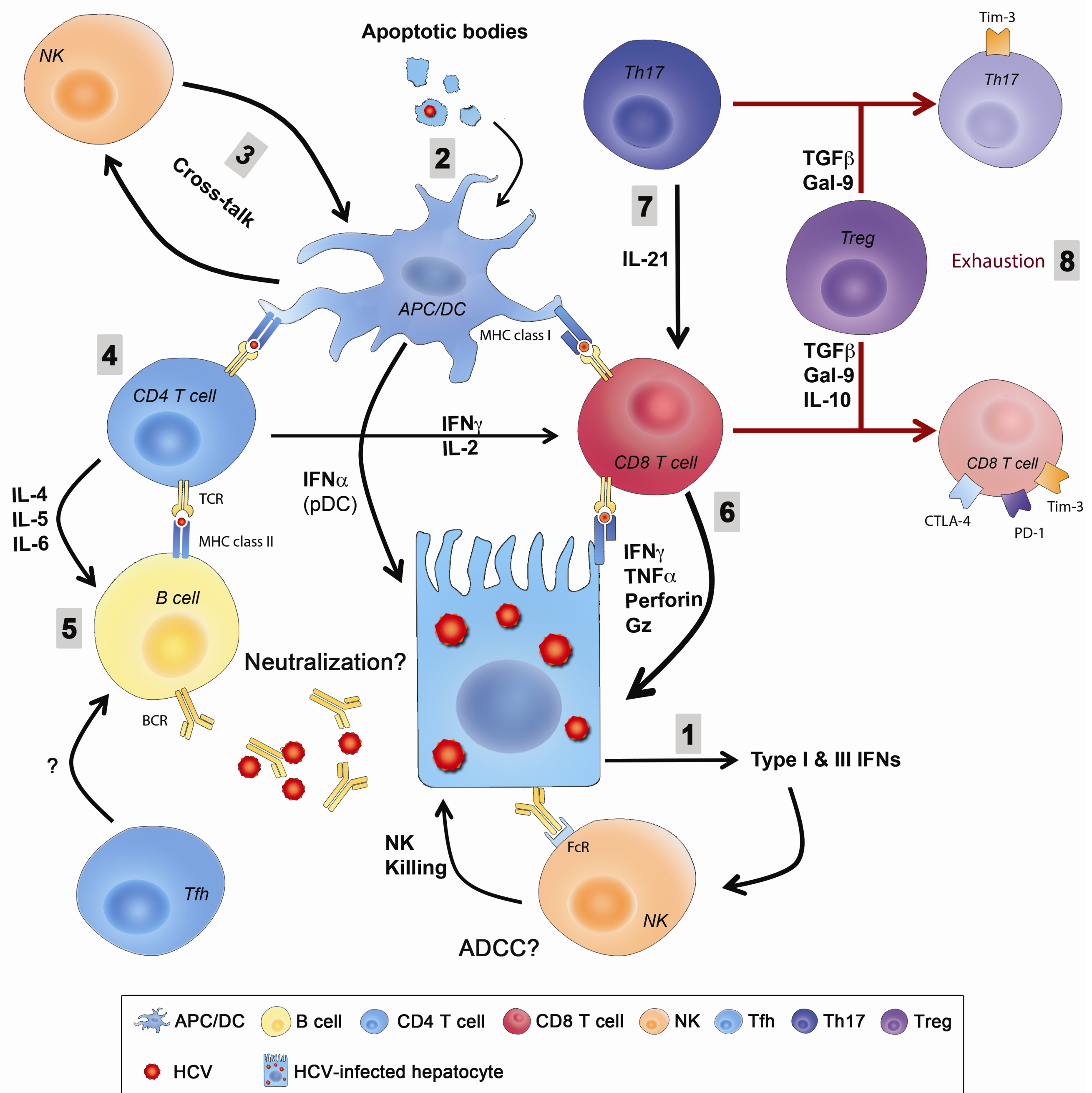
1.1. Immunity against primary viral infection

The human immune system is composed of two interconnected lines of defence; innate immunity and adaptive immunity. Innate immunity is non-specific, as it recognizes common motifs shared by interrelated pathogens known as “Pathogen Associated Molecular Patterns” (PAMPs), while adaptive immunity is highly specific and recognizes specific pathogen derived peptides known as antigens (Ags) or epitopes. Furthermore, adaptive immunity is privileged by its ability to generate immunological memory, which has the capacity to respond more rapidly to subsequent exposures to an Ag that has been previously encountered (also known as, the recall response).

Innate immunity is the first line of defence triggered by foreign pathogens including viruses. Cells of innate immunity include monocytes, natural killer cells (NKs) and dendritic cells (DCs) [1]. NKs have direct cytotoxic action and can kill infected cells. In addition, they possess antiviral action through secretion of inflammatory cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) [2], as well as having the ability to stimulate maturation of DCs that represent the link between innate and adaptive immunity (Figure 1) [3]. NK cell activity is triggered by reduced expression of the major histocompatibility complex (MHC) class I molecules on the surface of infected cells, known as the “missing self” hypothesis. The extent of NK cell activation is determined by the balance of interaction between the activating and inhibitory receptors on NK cells and their corresponding ligands expressed on target cells. These receptors include members of the killer cell immunoglobulin-like receptors (KIRs) family and the NKG2 family [4, 5]. DCs are the main professional antigen presenting cells (APCs) involved in priming naïve adaptive immune cells known as T cells [6]. There are two main populations of DCs: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). The mDCs are the major APCs involved in antigen uptake and presentation, while pDCs mainly produce IFN-α and act as overall orchestrators of the immune response. DCs become activated upon recognition of PAMPs by cell surface and/or intracellular receptors [7].

Adaptive immunity has two arms that evolved to provide distinct functions; humoral immunity and cell-mediated immunity (CMI). The humoral immune response is comprised of

B cells that produce antibodies (Abs) that mainly target extracellular microorganisms (e.g. bacteria), but also neutralize intracellular pathogens (e.g. viruses) by targeting surface epitopes to prevent viral binding and entry into target cells. Abs also help in the destruction of infected cells by Ab-dependent cellular cytotoxicity (ADCC), where specific Abs bind to cell surfaces using their variable Ag-binding fragment (Fab) recognizing a specific Ag and their constant portion (Fc) binds to the Fc-receptors on NK cells and/or monocytes and activates their cytotoxic functions [6]. Cell-mediated immunity mainly targets intracellular pathogens (e.g. viruses) and abnormal cells like cancer cells, and it is comprised of CD8 T cells, known as cytotoxic T lymphocytes (CTLs) and CD4 T cells that act as helper T cells. CD8 T cells recognize and kill cancer cells and virus infected cells expressing foreign Ags/epitopes on their cell surface in the form of peptides bound to MHC class I molecules. CD8 T lymphocytes kill infected/cancerous cells by secreting pore-forming molecules inducing cell lysis (granzymes and perforin) or through ligand-mediated initiation of programmed cell death (e.g. Fas-FasL interactions). CD8 T cells also secrete cytokines (e.g. IFN-γ and TNF-α) that have direct antiviral effects and can activate other effector cells [8]. CD4 T cells, were previously divided into two major helper subsets; T helper 1 cells (T_h1), that help priming and sustaining CTL responses, and T_h2 that secrete specific cytokines that help activate B cells and promote them to differentiate into plasma cells and produce Abs [9]. More recently other CD4 T-cell subsets were identified that regulate different aspects of the immune response and include T_h17 cells that mediate inflammation and response to microbial pathogens, regulatory T cells (T_{regs}) that regulate different immune responses and follicular helper T cells (T_{fh}) cells that regulate formation of germinal centers for the initiation of antibody responses.



NK
Apoptotic bodies
Th17
Tim-3
Th17
3
Cross-talk
2
7
TGFβ
Gal-9
IL-21
Treg
Exhaustion 8
APC/DC
MHC class I
4
CD4 T cell
TGFβ
Gal-9
IL-10
IFNγ
IL-2
CD8 T cell
CD8 T cell
IFNα
(pDC)
TCR
6
CTLA-4
Tim-3
PD-1
IL-4
IL-5
IL-6
MHC class II
IFNγ
TNFα
Perforin
Gz
B cell
5
Neutralization?
BCR
1
Type I & III IFNs
?
FcR
NK
Killing
Tfh
NK
ADCC?
APC/DC
B cell
CD4 T cell
CD8 T cell
NK
Tfh
Th17
Treg
HCV
HCV-infected hepatocyte

Figure 1: Innate and adaptive immune responses in humans using hepatitis C virus (HCV) as a model.

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Legend: (1) Pathogen entry and replication induces the production type I IFNs that are also secreted by pDCs and create a defensive state and stimulate NK cells that kill infected hepatocytes. (2) APCs (especially DCs) uptake apoptotic bodies from destroyed infected cells and present epitopes to both CD4 and CD8 T cells in the context of MHC class II and MHC class I, respectively. (3) The cross-talk between DCs and NKs regulates the function of both cells enhancing antigen presentation and priming, as well as NK-mediated killing. (4) CD4 helper T cells support responses of CD8 T cells and B cells through production of Th1 and Th2 cytokine, respectively. (5) Abs produced from B cells could have an essential role in pathogen clearance by directly neutralizing the pathogen or by mediating ADCC. Tfh cells play an essential role in mediating the selection and survival of B cells. (6) CD8 T cells eliminate pathogen-infected cells through direct cytotoxic mechanisms (cytolytic granules, as perforin and granzyme) or non-cytolytic mechanisms through secretion of the antiviral cytokines IFNγ and TNFα or through ligand-mediated initiation of programmed cell death (e.g. Fas-FasL interactions). (7) CD8 T cell functions are sustained and enhanced by IL-21 mainly produced by Th17 cells. IL-21 is also essential to rescue virus-specific T cells from exhaustion caused by persistent exposure to HCV antigens. Reduced IL-21 production or Th17 cell numbers results in an increased exhaustion status and expression of exhaustion markers like PD-1, Tim-3, CTLA-4, and others. (8) Regulatory T cells might be a cause in the failure of the primary immune response by secreting the regulatory cytokines TGFβ and IL-10, or secretion of Gal-9 that enhances apoptosis of Tim-3+ CD4 and CD8Tcells.

ADCC, antibody-dependentcellular cytotoxicity; APC, antigen-presenting cell; CTLA-4, cytotoxic T lymphocyte antigen-4; DC, dendritic cell; FcR, receptor for the constant fragment of the antibody; Gal-9, galactin-9; Gz, granzyme; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; NK, natural killer; PD-1, Programed death-1; pDC, plasmacytoid DC; Tfh, T follicular helper cells; TGF, transforming growth factor; Th17, T-helper 17 cell; Tim-3, T cell immunoglobulin and mucin domain 3;TNF, tumor necrosis factor;Treg, regulatoryT cell.

1.2. Immunological memory and vaccines against viruses

Immunological memory is the ability to mount a specific accelerated recall response by antigen experienced T- and B-lymphocytes against a previously encountered pathogen [11-13]. The memory immune response is also characterized by the increased frequency of antigen-specific lymphocytes with improved recognition and function [14, 15], as well as enhanced trafficking, migration and localisation at the site of infection [16, 17].

Most of our current knowledge about immunological memory against viruses is based on studies of the lymphocytic choriomeningitis virus (LCMV) infection in mouse models. LCMV is an excellent infection model, since its different strains have distinct courses of infection; where the Armstrong strain causes an acute infection cleared within a few days, as opposed to LCMV-clone 13 that leads to chronic infection and serves as a model that would correspond to human chronic viral infections (e.g. HIV and HCV) [18]. It is only recently that these findings were validated in human viral infections [19].

1.2.1. Generation of memory immune responses

Naïve B cells are activated and proliferate when they encounter their specific antigen for the first time. Activated B cells either remain in the marginal zone of T cells within lymph nodes and differentiate into short-lived plasma cells or migrate to B cell follicles initiating a germinal centre (GC) reaction. Within the GC, B cells undergo somatic hypermutation, affinity maturation and selection leading to the generation of high affinity memory B cells. The GC formation requires help from T_{fh} CD4 T lymphocytes [20, 21].

Development of memory T cells (T_{mem}) includes three phases (Figure 3);

Phase I (Expansion): activation and expansion of Ag-specific T-cell clones that have recognized their specific Ag within the context of MHC molecules, and with adequate costimulation these T cells differentiate into effector T cells (T_{eff}).

Phase II (Contraction): after resolution of infection the majority (~90%) T_{eff} die by apoptosis.

Phase III (Maintenance): establishment of the T_{mem} niche (from the remaining ~10% of Ag-specific T cells) [22].

Two models for the development of T_{mem} were proposed, the linear/progressive model and the asymmetric/bifurcative/stochastic model. Evidence supporting both models is equivocal, thus it remains unclear whether the pathway is linear or asymmetric, and whether it is one-way or reversible. The linear model proposes that activated naïve T cells develop into T_{eff} then a proportion of those T_{eff} differentiate into T_{mem} that are either effector-memory T cells (T_{EM}) or central-memory T cells (T_{CM}) (Figure 2). This was supported by microarray analysis of the gene-expression program of T cells in the LCMV model showing that acquisition of T_{mem} cell traits was gradual and was still occurring several weeks after viral clearance. This suggests that the 5-10% of the surviving T_{eff} are promoted to T_{mem} by completing a gradual program of differentiation and acquisition of all memory properties [23]. An interesting study of SIV-infected monkeys treated with bromodeoxyuridine (BrdU) demonstrated the progression of labelled cells, from T_{CM} to T_{EM} and finally to terminally-differentiated T cells (T_{EMRA}) representing the end-stage of T-cell development [24] (different T-cell subsets are explained in detail in section 1.2.4.). These results suggest that memory subsets are not independent, but rather share a common differentiation pathway, supporting the linear model [25]. On the other hand, the asymmetric model suggests that a single naïve T cell once primed gives rise to a heterogeneous population of Ag-specific T cells, and that the different populations of T cells of all phases are programmed for their final fate shortly following antigenic stimulation [26]. This early imprinting of a specific developmental program is reflected in changes in the gene expression that occur shortly after priming of naïve T cells [27]. Asymmetric T-lymphocyte division during early steps of initiation of the adaptive immune response were demonstrated in a mouse model of *Listeria monocytogenes* [28].

Important issues concerning generation of immunological memory remain to be resolved. First, what factors determine which cells would express memory T-cell markers versus those that become short-lived effector cells? Second, are those factors intrinsic, extrinsic or both? Finally, is it predetermined for CD8 T cells to become T_{mem} from the earliest point of priming or is it a stochastic process occurring later during the activation process? [11].

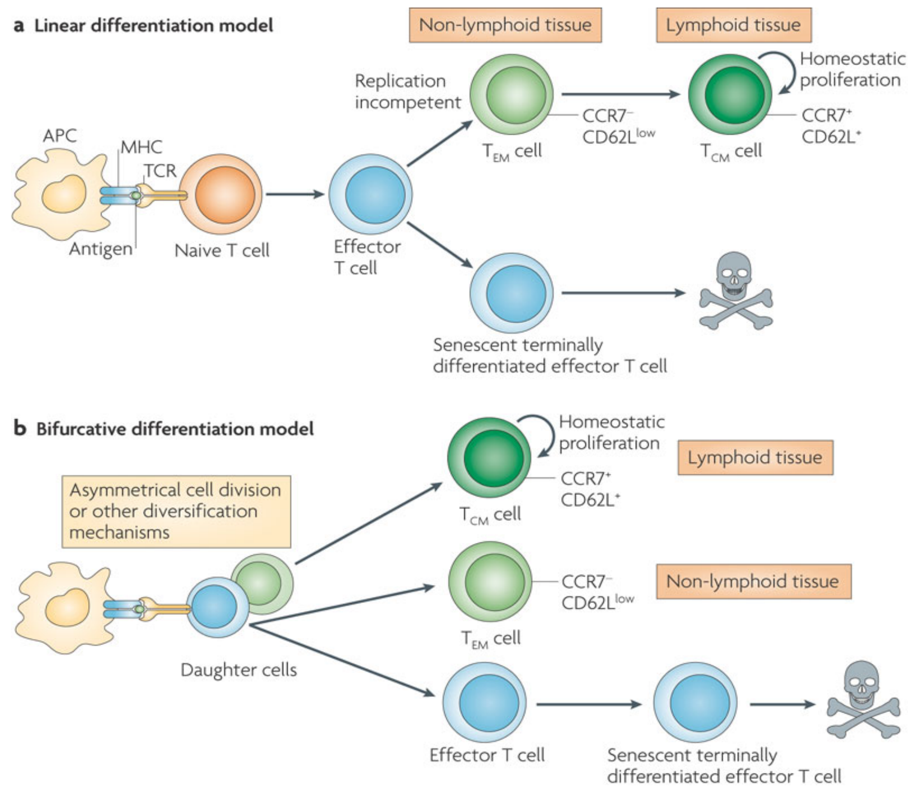

Figure 2: Possible models for memory T-cell differentiation.

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Legend: (A) The linear model versus (B) the asymmetric model for the generation and differentiation of memory T-cell populations.

1.2.2. Maintenance of immunological memory

The T_{mem} population remains stable at a nearly constant number for many years. BrdU labelling of T cells in a mouse model showed that T_{mem} cells maintain their numbers by continuously dividing [30]. Homeostatsis of T_{mem} is achieved through slow turnover balanced by both survival and death [11]. Survival of T_{mem} will depend on intrinsic properties of T cells, but also on the space available in the anatomic niche that is accessible to memory cells [31]. The dogma of the space limitation is challenged by a recent study showing that the memory niche is subject to expansion [32].

Homeostatsis of T_{mem} is regulated by common γ-chain cytokines as interleukin-7 (IL-7), IL-15 and IL-21 [33]. IL-7 is essential for the generation and basic survival of T_{mem}. This was partially attributed to its ability to sustain the expression of B-cell lymphoma-2 regulator protein (Bcl-2), which is an important anti-apoptotic protein [34, 35]. Expression of the IL-7 receptor alpha chain (IL-7Rα/CD127) was not sufficient for the formation of a CD8 T_{mem} population during viral infection [36], but it was necessary for T_{mem} generation and identifies T cells that give rise to a long-lived memory population [37]. IL-15 is another important cytokine for homeostasis, but mainly for the stage of maintenance of T_{mem} [38]. This was demonstrated in mice deficient in IL-15 or IL-15Rα, since they could still generate LCMV-specific CD8 T_{mem}, but they declined in number faster than in normal mice and regained their proliferative capacity when transferred to wild-type (WT) mice with intact IL-15/IL-15Rα [39-41]. Under steady-state conditions, T_{mem} slowly turn over, even in the absence of the Ag, depending on the constitutive production of IL-7 and IL-15 [42]. IL-21 was also shown to be important for maintenance of T_{mem} by preventing their exhaustion [43].

Whether maintenance of B and T cell memory requires continuous Ag stimulation (or even intermittent stimulation) is still controversial. In several mouse models it has been shown that T cells may persist indefinitely in the absence of Ag [44, 45]. This was confirmed by observations in humans, where specific memory was preserved despite lack of exposure to the pathogen for many years. Long-term immunological memory was reported for measles on the Faroe Islands (where the population was not exposed to the pathogen for 65 years) [46], yellow fever in Virginia (75 years) [47], or polio in remote Eskimo villages (40 years) [48].

However, these studies did not specify whether those memory cells would be protective [49, 50]. In contrast, results from other human studies suggest that the presence of Ag plays a role in maintaining memory (reviewed in [13]). Epidemiological studies confirm this concept by demonstrating that the levels of memory responses are higher in people who continue to live in endemic regions than in individuals who leave those areas [11]. To reconcile those seemingly contradicting findings, it was proposed that even in the absence of reinfection and re-exposure to Ag there is maintenance of Ag presence on follicular DCs (fDCs) trapped in immune complexes in the GCs for months or even years after the resolution of infection [51, 52]. Another possible mechanism is that lymphocytes might undergo low affinity interactions with self-Ags [53]. A third explanation could be that there is periodic re-exposure to the antigen(s) due to asymptomatic exposures to the same pathogen (or a closely similar pathogen).

Longevity of memory also depends on inherent properties of the lymphocytes, as their division potential. Telomere shortening is an important mechanism for limiting the number of cell-divisions, where rapidly dividing cells/populations reach senescence before slowly dividing ones [54]. Despite up-regulation of telomerase to limit telomere shortening, it will ultimately reach the Hayflick limit and cell division will be arrested [55]. Different proliferation rates are required for continuous replenishment of memory pools according to their life spans; where T_{EM} have a proliferation rate of 4.7% per day compared to 1.5% for T_{CM} [56].

1.2.3. Characteristics of a memory immune response

The memory immune response is characterized by having more rapid kinetics upon re-exposure to the same Ag. Many studies in animal models reported this finding; for example, rechallenging chimpanzees with HCV showed that memory immune cells responded in a much shorter time than that required for naïve cells to respond to primary infection [57].

A second important characteristic of the memory immune response is the increased frequency of the specific lymphocytes targeting the Ag. It was shown in the LCMV model that CD8 T cell precursors specific for a certain epitope occur at a frequency of about 1 in 10^5 CD8 T cells (~100-200 cells). At the peak of the immune response these specific cells expanded to

reach ~10^7 cells. After the resolution of infection, a memory pool of ~ $5x10^5$ cells was present [45]. Other reports confirmed that Ag-specific cells are 20- to 1000-fold more prevalent after primary exposure than in naïve mice [14].

Third, memory immune cells show improved recognition and responsiveness to their specific Ags upon re-exposure. The nature and magnitude of stimulatory and co-stimulatory signals required for activation of lymphocytes differ between naïve and memory populations. Memory B cells were shown to respond more effectively than naïve B cells and to lower concentrations of Ag and with less help from helper CD4 T cells [58]. This was suggested to be due to somatic mutations of immunoglobulin (Ig) genes that take place upon primary exposure [59]. Memory T cells also have enhanced proliferation and more rapid differentiation into effectors in response to lower doses of Ag when compared to naïve T cells [15]. Some of these changes have been attributed to alterations in T-cell signalling, thus leading to less stringent requirements for Ag presentation, costimulation and help [60].

Fourth, altered trafficking contributes to the accelerated secondary response. Where T_{mem}, T_{EM} in specific, are more predominant in non-lymphoid organs and preferentially migrate through different peripheral tissues enabling them to perform surveillance where infections usually start and concentrate at the initial site of Ag encounter which is the most probable site of reinfection [61]. In contrast, naïve cells mainly circulate the blood and lymph nodes (LNs) [62]. This is attributed to lower level of expression of the LN-homing receptors such as the C-C motif chemokine receptor type 7 (CCR7) and L-selectin (CD62L) (both detailed in section 1.2.4) [16].

Fifth, memory T cells were shown to have improved effector functions than naïve cells. As naïve cells get primed during the primary immune response they are educated through the signals they receive, and as they differentiate into memory cells their gene expression is reprogrammed to enable the production of more cytokines. CD4 T cells that are able to produce high levels of one cytokine only (IL-2) when they are naïve, gain the capacity to produce other cytokines at high levels as they develop into differentiated T_{mem}, becoming polyfunctional rather than monofunctional like their naïve counterparts [17]. Similarly, genes encoding IFNγ and cytotoxic proteins are not expressed in naïve CD8 T cells, but constitutively expressed in CD8 T_{mem} [23].

Sixth, differences in proliferation kinetics have been observed for T_{mem} in comparison to naïve T cells. This could be attributed to the difference in telomere length, which is shorter in T_{mem} than naïve cells, and is reflected in the more rapid turnover of T_{mem} and their limited number of cell divisions [12]. Nevertheless, heterogeneity of proliferative capacity exists among the different subpopulations of both T_{mem} and naïve T cells [56, 63].

Finally, the relative contributions of humoral versus cell-mediated immunity (antibodies versus CD8 and CD4 T cells) during primary and secondary viral infections seem to be different. Using smallpox as a model, it was shown that mice lacking Ab responses had comparable rates of viral clearance during primary infection as WT mice indicating that CD8 T cells had the predominant role [64]. In contrast, in a reinfection setting pre-existing neutralizing Abs (nAbs) represented the first line of defense against the virus [50].

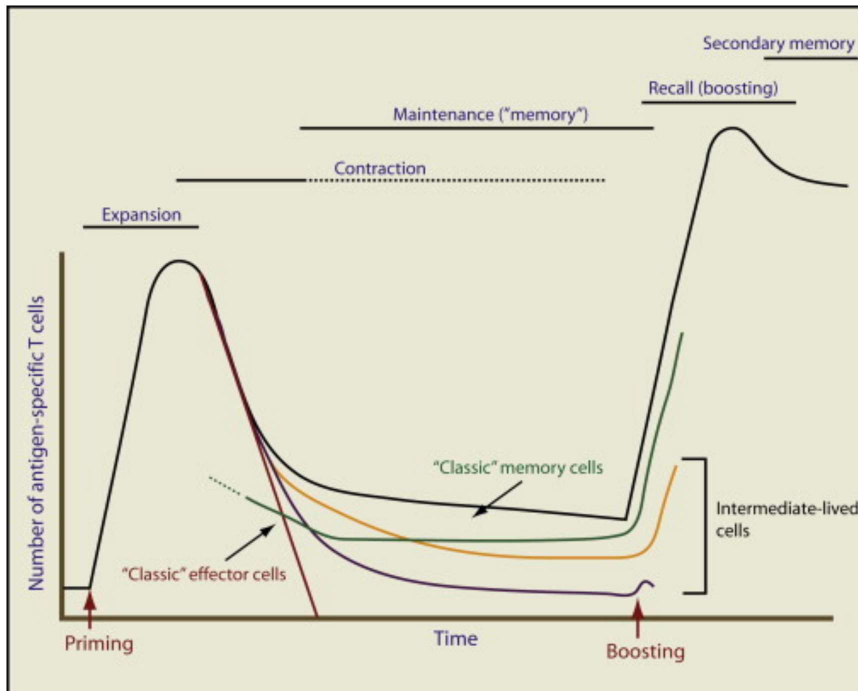

Figure 3: Generation and maintenance of T-cell memory.

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Legend: The diagram depicts the dynamics of Ag-specific T cells at various stages after priming and boosting the immune response. The total number of T cells (black line) is the sum of the effector T cells activated during the primary response (red line) and the memory population generated following the clearance of the Ag (green line). Some proposed intermediate populations are also represented (yellow and violet lines).

1.2.4. Memory T cell subsets

Memory T cells represent a heterogeneous population. The different subsets of T_{mem} are associated with the expression of specific surface receptors and intracellular molecules/transcription factors, and this is linked to their differential requirements for stimulation, survival, homing and some effector functions [66].

These subsets are mainly classified according to the expression of the surface molecules involved in homing to lymph nodes; CCR7 and CD62L. CD62L mediates attachment and rolling to walls of high endothelial venules (HEVs). CCR7 binds to the C-C motif chemokine ligand 19 (CCL19) and CCL21 on endothelial cells causing arrest of movement and extravasation [67]. Combined expression of adhesion molecules and chemokine receptors allow tissue-specific targeting of T cells [68]. $CD62L^{hi}$ $CCR7^{hi}$ T_{mem} cells are located predominantly in the lymph nodes and constitute the central memory T population. Effector memory T cells are $CD62L^{lo}$ $CCR7^{lo}$ and mainly circulate the blood and peripheral tissue [69]. Distinct CD8 T-cell subsets have differential functional profiles, where T_{CM} mainly produce IL-2 and T_{EM} produce effector cytokines as IFNγ and TNFα [70].

Additional markers better distinguish the different subsets. These include markers involved in T-cell activation (e.g. CD45RA or CD45RO), costimulation/maturation markers (e.g. CD27 or CD28), regulation markers (e.g. programmed death-1 (PD-1)) and cytokine receptors (e.g. CD127/IL-7Rα). General phenotypic profiles emerge within the heterogeneity of T-cell population (Figure 4). T_{CM} (CCR7+ CD45RA- CD27+ CD28+) express low levels of PD-1, but high levels of CD127 and CD62L, in contrast, to terminally-differentiated cells (T_{EMRA}) having an opposite phenotype (CCR7– CD45RA+ CD27– CD28–) [66].

Ag-specific CD8 T cells display unique profiles depending on their viral specificity; where HCV-specific CD8 T cells are predominantly CCR7+ CD27+ CD28+, whereas for human immunodeficiency virus (HIV) they are mainly CCR7– CD27+ CD28– and for cytomegalovirus (CMV) they are mainly CCR7– CD27– CD28– [71]. Interestingly, the phenotypic profiles of CD4 T cells specific for the corresponding viruses are also present with similar distributions [64, 72, 73]. This may be related to the nature of the infection (resolved, latent or chronic), as well as the site of infection.

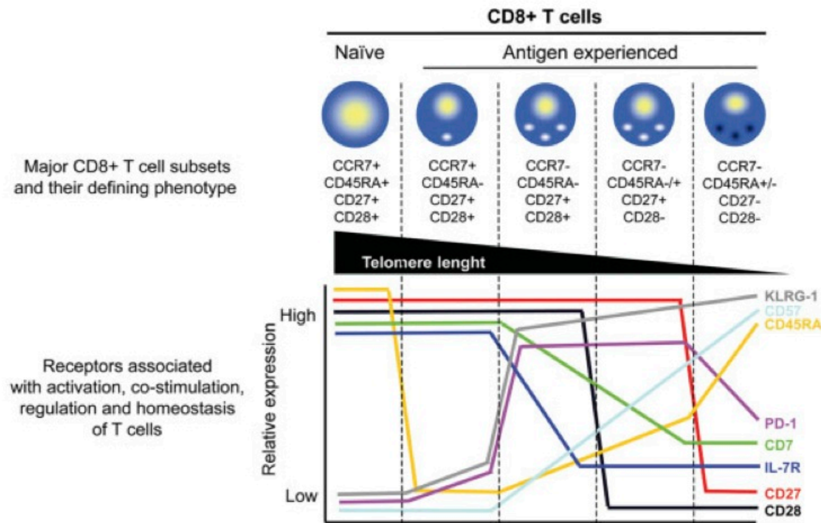

Figure 4: Different subsets of human T cells and their phenotypic attributes.

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Legend: Different homing and activation markers that distinguish the various stages of differentiation of CD8 T cells (upper half), and the level of expression of these markers, as well as markers of activation, co-stimulation and regulation at the different phases of differentiation (lower graph).

1.2.5. Immunological memory at the clonal level

Another level of complexity is that T_{mem} subsets and even T-cell populations specific for a single epitope are formed of heterogeneous clones of T cells bearing different T-cell receptors (TCRs). It is the additive function of these clones and their differential capacity to recognize antigen that would finally determine the efficacy of the memory response [13]. However, some Ag-specific CD8 T-cell clones can dominate the total effector CD8 response, especially during the initial phase of the response [74-76]. The clonal repertoires of T_{mem} subsets could be different (e.g. the repertoire of T_{EM} could be distinct from the T_{CM}-repertoire) [77].

1.2.5.1. Generation of TCR diversity

The adaptive immune system has evolved to generate a large number of T-cell clonotypes, each expressing a unique TCR. The TCR is formed of a heterodimer of the α and β chains (Figure 5). The variable region of both chains is generated by somatic recombination of genes encoded by the variable (V) and junction (J) gene segments, in addition to the diversity (D) gene segment for the β chain. Regions of hypervariability are encoded within the V gene segments, these are known as complementarity-determining regions (CDRs). CDR3 of the β-chain is formed by combinatorial and junctional variation and is characteristic for a specific clonotype. During T-cell development, gene segments recombine and are spliced to form a unique TCR for each T-cell clone [78]. To add to the diversity, the recombination process involves random addition and deletion of nucleotides to/from the ends of V, J and D gene segments. Mathematically, this would lead to the generation of a TCR repertoire of > 10^{15} different TCRs in humans. However, positive and negative selection in the thymus leaves only ~10^{7} T-cell clonotypes with unique TCR amino acid sequences forming the human repertoire [79].

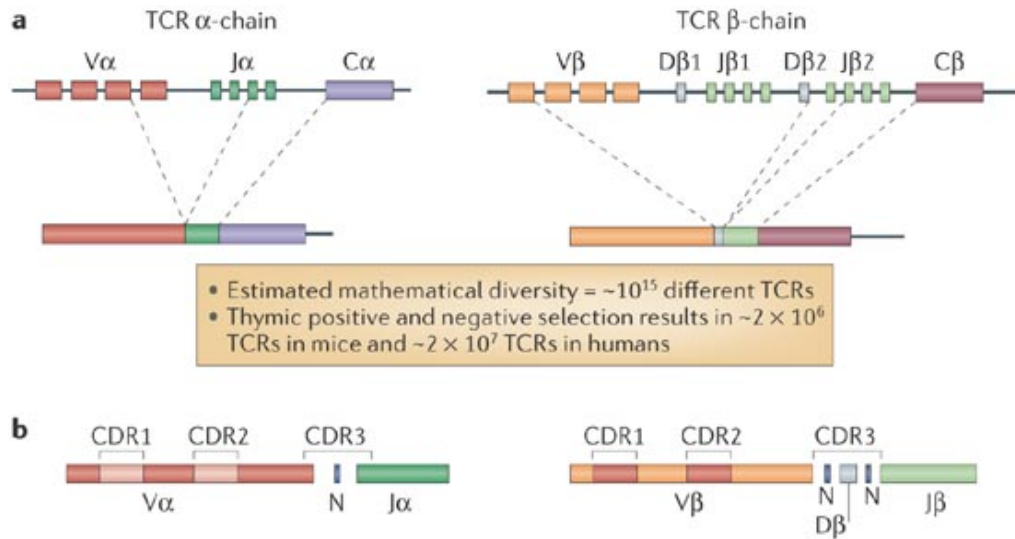

Figure 5: Generation of TCR diversity by recombination of TCR gene segments.

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Legend: (A) TCRs are formed of heterodimers of both α and β chains. They are generated by gene recombination of the V and J gene segment (and for the β-chain, also the D gene segment). During T-cell development, genes segments recombine and are spliced together to form a single functional αβ TCR unique for each T-cell clonotype (B) Regions of hypervariability (CDRs) are encoded in the V gene segments. The diversity of the TCR repertoire is further increased due to lack of precision during gene rearrangements.

1.2.5.2. Impact of TCR diversity on the viral immune response

Given the huge number of different T-cell clonotypes with unique TCRs present in the repertoire, it would not be expected that the same clonotype could be found in different individuals. A rarer event would be that a specific clonotype would be responding to a particular epitope in several individuals. Surprisingly, specific T-cell clonotypes were detected recurrently in different individuals against the same viral epitopes, referred to as a “public” T-cell clonotype [79]. Several models and explanations have been proposed to explain this phenomenon [80, 81]. A decisive factor in the selection of the clonotype(s) that would participate in the initial response against a specific epitope is the TCR fitness measured by its functional avidity. Functional avidity is a determinant of T-cell efficacy that reflects the ability of specific T-cell clonotypes to recognize peptide-MHC (p-MHC) complexes. Higher avidity of a clonotype/TCR would mean the recognition of p-MHC at lower densities on cell surfaces, leading to more rapid proliferation and more rapid induction of effector responses. This is usually associated with prompt elimination of virus-infected cells [82]. Consequently, these T cells with high avidity have a selective advantage over other clonotypes and would proliferate more rapidly shaping the dominance of clonotypes in the responding T-cell pool. Thus, despite the presence of a great variety of T-cell clonotypes with TCRs that could interact with a specific Ag, not all of them would be present in the pool of the responding T_{eff} population [81].

Memory T-cell populations are composed of the clones that expanded during the initial exposure to the pathogen. The longevity of specific memory and the stability of the repertoire are limited by the senescence of the different clones, which might be still replenished by homeostasis. Senescence of the individual clone is governed by a number of factors including: avidity to the epitope and competition from other clones. Other general factors include the nature of the pathogen and the microenvironment of the clone [13]. Longevity of persistence of an individual clone in the memory repertoire was measured in the context of Epstein–Barr virus (EBV) infection. Many clones were detected for at least a year and some persisted for over 3 years [74, 83], and the overall repertoire was stable for 19 months [77]. Nevertheless, some changes could occur due to switch in response towards a different set of Ags [84]. Clonal survival is probably shaped by the avidity of each clone to the epitope, so that the

largest responses are the ones with high avidity. Nevertheless, dominant clones show the highest proportional reduction over time [85], as their accelerated exhaustion was observed in mouse models [86]. Another factor affecting clonotype survival is the persistence of the corresponding epitope. Persistence of epitopes in the chronic phase of viral infections (e.g. HIV, EBV and CMV) can result in deletion of their specific highly reactive clonotypes [18]. Antigen decay was associated with dynamic changes in the TCR repertoire and gain of function within HIV-specific CD8 T populations attributed to functional improvement of the clonotypes originally present and/or recruitment of additional clonotypes with higher functionality [87].

The lack of consistent correlations between the quantitative parameters of the overall specific CD8 T-cell responses and control of viral infection indicated that not all specific T cells have equivalent efficacies. Dissecting the responses according to the fittest clonotypes provided better correlations with the control of the viral replication, indicating that it is more precise [66]. T-cell avidity, diversity and cross-recognition were shown to be crucial for superior control of HIV replication by Ag-specific T cells and their polyfunctionality [88]. Furthermore, it was shown that both avidity and polyfunctionality are interrelated, since Ag-sensitivity is a major determinant of CD8 T-cell polyfunctionality and HIV-suppressive activity [89]. A study rechallenging chimpanzees with HCV after clearance of a primary infection demonstrated that rapid resolution of viremia after rechallenge temporally coincided with the expansion of the dominant T_{mem} clonotype that was associated with the clearance of the primary infection [90].

Another important difference between T-cell clonotypes is their ability to tolerate mutations in the targeted peptides [91]. HIV-positive patients who are long-term non-progressors (LTNPs) to AIDS (*i.e.* had better long-term control on the virus) possessed highly cross-reactive clonotypes that recognize the different variants of targeted viral epitopes efficiently [92]. Studies analysing HCV-specific immune responses at the clonotypic level showed that one of the mutations in a specific epitope (NS3-1406) was aimed at exploiting a virtual gap or a “hole” in the TCR repertoire, thus avoiding recognition [93]. Rechallenging a chimpanzee that spontaneously resolved a primary HCV infection demonstrated that higher diversity within the TCR CDR3 region correlated with viral clearance and better control on the

evolution of escape mutations within the targeted epitope. Despite preferential expansion of the dominant clonotypes at the peak of the immune response to reinfection, the repertoire was diverse in the memory phase following primary infection and following clearance of the secondary viral infection. This indicates that focusing/narrowing the repertoire at the peak of the recall response was only temporary [94]. Altogether, these findings underscore the importance of studying the protective memory immune response at the clonal level.

1.2.6. *Immunological memory and vaccination*

Vaccination is the most efficient and cost-effective intervention strategy for prophylaxis against pathogens. It represents the main pillar for public health measures to contain infectious diseases. The only infectious diseases that were successfully eradicated are those against which an effective vaccine was developed and accompanied with successful vaccination campaigns worldwide (e.g. Smallpox). Ironically, successful vaccines were developed without understanding the molecular basis for protection or immunological memory. We realize now that vaccines are an effective and well-controlled alternative to a primary infection for inducing immunological memory, without suffering from the high rates of morbidity and mortality associated with many pathogens.

The majority of available antiviral vaccines are protective against lytic viruses causing acute infections. The main correlate of protection conferred by these vaccines is the generation of nAbs that inhibit viral binding and entry into target cells [95]. Another factor contributing to the success of these vaccines is the presence of only a few closely related strains in nature, thus the inclusion of few cross-reactive strains in the vaccine provided broad protection against the majority of strains (e.g. polio, human papilloma virus (HPV) and rotavirus) [96-100]. This memory immune response provides sterilizing immunity, where the immune response neutralizes the pathogen before establishing an infection. However, for chronic viruses the realistic goal would be to have protective immunity that could prevent the establishment of persistent infection.

1.2.7. Challenges for developing vaccines against chronic viruses

Early vaccines were the result of an empirical design based on clinical observations of patients infected with the corresponding pathogen. However, several challenges impede the successful design of vaccines against chronic viruses, since major questions remain unanswered. First, how to design a vaccine for viral infections where the CD8 T-cell response is the major determinant of viral clearance and protection upon re-exposure? Second, how to overcome the high variability of viral-sequences for viruses that continually mutate, as HIV and HCV? Third, is the memory immune response always protective in case of chronic viruses? The answers to these critical questions are essential for the rational design of an effective prophylactic vaccine [101]. But, the most challenging issue remains the definition of the characteristics of the protective immune response, specifically, the properties of the T_{mem} that would be protective upon exposure and/or re-exposure to chronic viruses.

Whether the memory immune responses observed for the different chronic viruses would be protective upon re-exposure is debatable, since strong memory responses do not necessarily provide protective immunity, as it was shown for different human pathogens [102]. The discrepancy between the demonstration of immunological memory and protective immunity may reflect the time needed to produce sufficient levels of nAbs or effector T cells following exposure to the pathogen versus the incubation period of the pathogen [103]. Thus, an important factor for memory immune cells to be protective would be their ability to differentiate into effector cells upon re-exposure fast enough to outpace the virus and prevent the establishment of persistent infection [104]. It is also difficult to verify whether the memory immune response would be protective for some viruses upon re-exposure, since the virus is never eliminated from the body of the host after the primary infection due to being chronic or latent (e.g. HIV and herpes simplex virus (HSV), respectively). For other viruses as HCV (where ~30% of patients do clear the virus spontaneously and with no latency), it has been very challenging to study a reinfection setting.

The setback caused by the STEP trial for developing an HIV vaccine by Merck & co. (New Jersey, USA) underscores the importance of defining the correlates of memory immune responses, especially for complex chronic viruses like HIV and HCV [105]. This vaccine was tested in a population at risk and aimed at inducing CMI to reduce viremia, consequently

slowing disease progression and transmission [106]. Despite being immunogenic in 77% of the vaccinees, it did not prevent infection or reduce viremia in the vaccinated group, and there were even more infection cases in the vaccinated group compared to the placebo group [107, 108].

1.2.8. Protective CMI mechanisms for successful vaccines

The Food and Drug Administration (FDA) defines a correlate of protection as a parameter that has been shown to be associated with protection from clinical disease [109]. Deeper understanding of correlates of protection of successful vaccines would ensure better design of next-generation vaccines [110]. With the recent advances in flow cytometry, genomics, proteomics and other immunology and molecular biology techniques, it is inevitable to revise our strategies for vaccine design based on lessons learned from successful vaccines on how to trigger an efficient memory CMI response. More important, what are the correlates of this memory immune response associated with protection against the different pathogens with their varying disease courses and pathologies?

Early protection provided by most available vaccines has been mainly attributed to the presence of nAbs or antibody-dependent mechanisms [95]. The contribution of CMI to the long-term protection conferred by those vaccines has not been thoroughly studied [103]. Studies using yellow fever (YF) 17D vaccine as a model were able to shed light on some of the determinants of protection of CMI. Yellow fever 17D vaccine is an ideal example of a successful vaccine where 540 million doses have already been administered [111]. Despite existing in seven different genotypes differing by 25% at the nucleotide sequence level, the YF 17D vaccine provides effective protection against all of them [111]. The nAb response is a correlate of protection, yet, the CD8 T cell response seems to contribute by mediating viral clearance. A system-biology approach demonstrated that both innate and adaptive immunity collaborate in providing protection. Both humoral and CMI are implicated in the protection orchestrated by transcription factors as signal transducer and activator of transcription-1 (STAT1) and interferon regulatory factor-7 (IRF7) [112]. The vaccine was shown to activate multiple DC subsets via several toll-like receptors (TLRs) (specifically, TLR-2, 7, 8 and 9), thus stimulating polyvalent immunity [113].

1.2.9. Human models for studying protective immunity against chronic viruses

The LCMV model was invaluable in shaping our knowledge about anti-viral immunity against chronic viruses and characteristics of the memory immune response. However, these findings require validation in a human model due to caveats of the LCMV model that hinder applying this knowledge directly for developing human vaccines. First, the mouse is a short-lived species when compared to humans, thus, whether a successful memory generated in the mouse would be paralleled by a similar memory in humans is questionable. Second, many differences exist between the immune system and the cell biology of mice versus humans. Third, mice used for these studies are inbred and they do not reflect the genetic diversity found in human populations. They are also kept in a restricted semi-sterile environment with limited exposure to environmental factors and other pathogens that shape the natural immune response. Finally, LCMV causes systemic infection and affects many organs [114, 115]. Whether similar findings would be observed for chronic viruses causing localized infections needs verification. One example reflecting these discrepancies is the observation of experiments for blocking inhibitory receptors. In human HCV infection cytotoxic T lymphocyte antigen-4 (CTLA-4) blocking synergized with PD-1 blocking in restoring CD8 T cell functions, versus LCMV infection in mice where there was no synergism [116].

A better understanding of the mechanisms and correlates of the protective memory against chronic viral infections in humans would enable the rational design of a vaccine capable of stimulating such protective memory. With more elaborate immunological tools available, we need to define these correlates, not only at the cellular level (phenotype and function) but also at the molecular level (signalling and transcriptome), and not only at the cell-population level but also at the clonotypic level.

We remain in a quest for a model of a human chronic virus that would permit studying the correlates of protective immunity under real-life settings. HCV represents an excellent model for studying protective memory immune responses in a natural human infection. First, the infection with HCV has the advantage that the natural immune response in humans can clear the virus spontaneously [117]. Second, the infection has a clear dichotomous outcome

during the acute phase of infection (first 6 months of infection); either clearance or persistence. Third, epidemiological studies have reported that in populations at risk, such as injection drug users (IDUs), patients who were able to clear the first infection could be infected for a second time (reinfected) as they continued their high-risk behaviour. Among those reinfected patients, many successfully cleared the second infection [118, 119]. These cohorts represent a unique opportunity to study protective memory immune responses in a real-life human infection context, where humans (rather than in-bred laboratory animals) are infected with variant quasispecies (rather than single reference sequences) and are under natural environmental conditions (rather than controlled laboratory conditions). Therefore, HCV represents a unique setting for identifying correlates of protective immunity against chronic viruses in humans.

1.3. Hepatitis C Virus

HCV is a non-cytopathic hepatotropic virus that infects the liver [120]. HCV was first cloned in 1989 in the laboratory of Dr. Michael Houghton at Chiron Corporation (California, USA), from a chimpanzee infected with the plasma of a patient infected with non-A, non-B (NANB) viral hepatitis [121, 122]. This discovery impacted public health strategies, where screening blood for this new virus (given the name hepatitis C) became a regular procedure at blood banks since 1992 [123].

1.3.1. HCV epidemiology and transmission

The World Health Organization (WHO) estimates HCV prevalence at 3.1% worldwide (~185 million individuals) [124]. The prevalence is higher in developing countries, the highest being in Egypt (15-20%) (Figure 6) [125, 126]. In the absence of a prophylactic vaccine, two million new HCV infections are estimated to occur every year worldwide [127]. In Canada, the prevalence of HCV is 0.8% (~275,000 individuals), of whom 30% are IDUs [128, 129]. These numbers might not be representative, since many HCV patients live in secluded areas in developing countries or belong to marginalized groups in developed countries. Thus, despite the universal distribution of HCV, it is uneven [130].

The main mode of transmission of HCV is direct exposure to infected blood [131-133]. Other modes of transmission account for a small percentage (e.g. the second and third most common modes of transmission, perinatal transmission and sexual transmission, account for 4% and 1.5% of the cases, respectively) [134-136]. Currently, there are 2 patterns for HCV transmission; in the developing world unsafe therapeutic injections and transfusions account for ~40% of new cases, while in developed countries, sharing contaminated needles between IDUs accounts for ~68% of new cases. In Canada, most new HCV infections occur among IDUs and aboriginal populations [126].

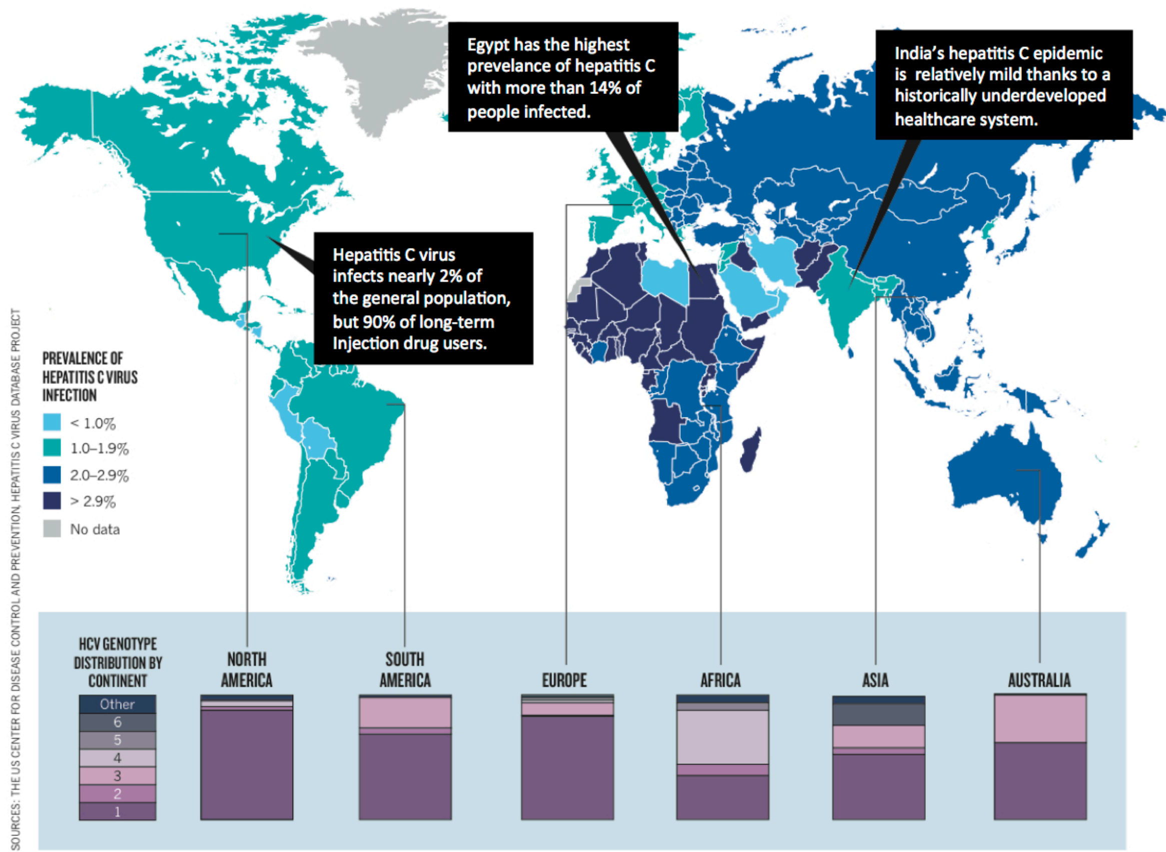

Figure 6: Prevalence of HCV and genotype distribution worldwide.

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Legend: World map showing the prevalence of HCV infection in different countries. The panel at the bottom shows the distribution of genotypes per continent.

1.3.2. HCV genome and proteins

HCV genome is an uncapped positive single-stranded RNA (+ssRNA) of approximately 9.6 kilo base-pairs (kb) [138]. The genome represents an uninterrupted open reading frame (ORF) encoding a polyprotein precursor [121] which is processed upon translation first by cellular then by viral proteases into ten proteins: three structural proteins (Core, E1 and E2), the viroporin (p7) and six non–structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Figure 7). The genome also contains two non-coding regions (NCRs) flanking the ORF, the 5'untranslated region (5'UTR) and the 3'UTR [139].

The core: The core protein amino acid sequence is highly conserved among different HCV genotypes [140]. The core is involved in the formation of the HCV nucleocapsid [141] and was shown to modulate the host's intracellular signalling pathways [142].

The envelope: The two envelope glycoproteins (E1 and E2) form complexes which are the building unit of the envelope [143], and are necessary for viral entry [144, 145].

P7: The P7 protein is a small hydrophobic polypeptide suggested by many reports to function as a viroporin or ion channel [146, 147].

NS2: NS2 is essential for viral replication *in vivo* [148]. NS2/3 possesses an autoprotease activity necessary for the polyprotein processing between NS2 and NS3. This activity resides in the C-terminal half of NS2 and the N-terminal third of NS3 [149, 150].

NS3: NS3 is a complex multi-functional molecule essential for both viral protein processing and RNA replication [150], with a serine protease activity located in the N-terminal third and RNA helicase/NTPase activity residing in the C-terminal two-thirds of the protein [151]. The helicase domain unwinds RNA-RNA substrates in a 3' to 5' direction [152]. The serine protease catalyses the processing of the polyprotein downstream of the NS3 region [140].

NS4A: NS4A is the co-factor for the NS3 serine protease [153].

NS4B and NS5A: NS4B and NS5A are poorly characterized and their functions are not fully elucidated. However, studies show that NS4B induces the formation of a membranous-web compartment where viral replication takes place [154, 155], and cell-culture

adaptive mutations mapped to the NS5A enhance RNA replication suggesting its importance for viral replication [156-158].

NS5B: NS5B is the viral RNA-dependant RNA-polymerase (RdRp) responsible for HCV-RNA replication [141]. As with other RNA viruses the HCV RdRp 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', that differ in nucleotide sequence by >10% [130].

The untranslated regions (UTRs): 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 [159-161]. The 3'UTR is formed of three regions; a short variable region of ~30 nucleotides, a poly–U/UC stretch and a highly conserved 98-base sequence designated the 'x-tail' [162, 163]. The poly–U/UC region is recognized by the retinoic-acid-inducible gene I (RIG-I) pattern recognition receptor (PRR) [164]. Current evidence suggest the importance of 3'UTR in viral replication [165].

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, thus classifying HCV into genotypes 1 to 7 which differ from each other by 31–33 % at the nucleotide level, and more than 60 different subtypes (e.g. 1a, 1b, 1c, etc.) with 20–25 % differences in sequences [166-169]. The different genotypes show marked differences in geographic distribution (Figure 6) and response to therapy [170].

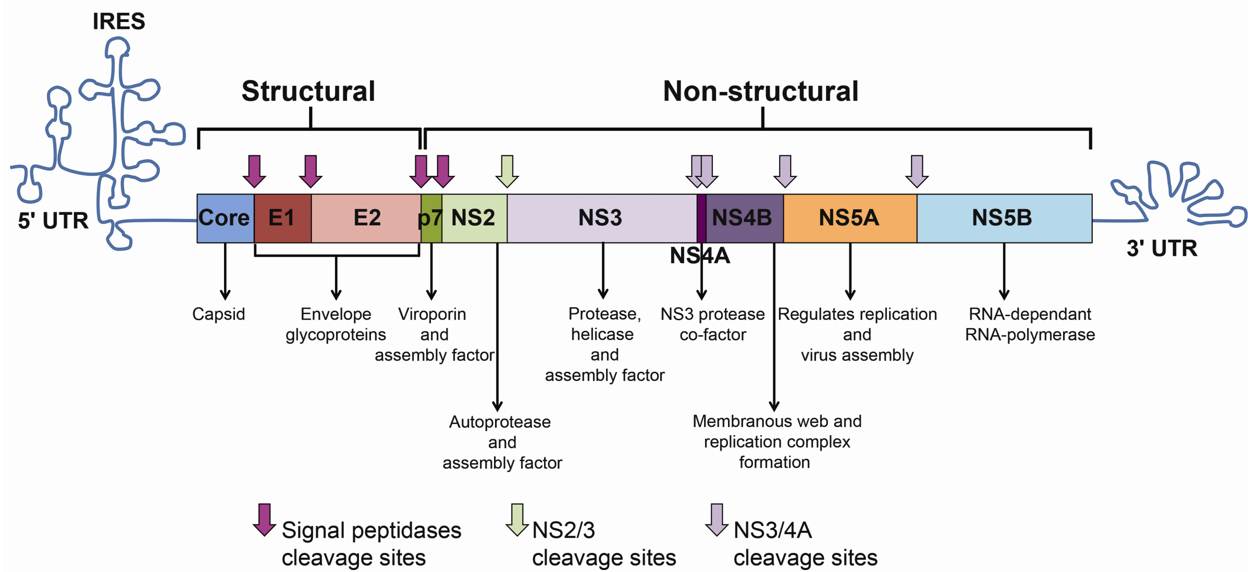

Figure 7: HCV genome and polyprotein.

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Legend: HCV genome RNA is composed of an ORF flanked by 5′ and 3′ UTRs (blue). IRES-mediated translation of the ORF leads to the formation of a polyprotein that is processed into ten viral proteins. Cleavage of the core protein from E1 involves a cellular signal peptidase, which also cleaves E1, E2 and p7 from the polyprotein (pink arrows). In an autocleavage mechanism the NS2-NS3 protease cleaves itself (green arrow). The NS3 protease located in the first one-third of NS3, assisted by its membrane-bound cofactor, NS4A, cleaves the remaining proteins NS3, NS4A, NS4B, NS5A and NS5B (violet arrows).

1.3.3. HCV life cycle

Major advances in our knowledge about the life cycle of HCV were achieved since 2005, with the development of the first *in vitro* culture system able to produce complete infectious virus particle by Dr.Takaji Wakita using the Japanese fulminant hepatitis-1 (JFH1) isolate [171].

Receptors involved in binding and entry of a given virus determine its tissue tropism and host range [140]. All the receptors and co-receptors that have been discovered for HCV are expressed on hepatocytes. The first described receptors for HCV were CD81 [172] and scavenger receptor class B type I (SR-BI) [173]. Tight junction (TJ) proteins Claudin-1 (CLDN1) and occludin (OCLN) have also been identified as receptors/co-receptors for HCV. It has recently been reported that epidermal growth factor receptor (EGFR) and ephrin receptor type A2 are also required for HCV entry and possibly modulate the interaction between CD81 and CLDN1 [174] (Figure 8 – Main panel). Binding is followed by fusion of the viral envelope with the cellular membrane by clatherin mediated endocytosis then fusion with the endosomal membrane and the viral genome is released into the cytosol [144].

Translation of the HCV genome (lacking a 5' cap) depends on the IRES [160], which binds the 40s ribosomal subunit directly without the need for pre-initiation factors [175]. The major product of HCV ORF translation is a large polyprotein that is subsequently processed into mature structural and non-structural proteins. Junctions between structural proteins are processed by host signal peptidases from the endoplasmic reticulum. The viral non-structural proteins are processed by two proteases encoded by HCV; the NS2/3 autoprotease [140, 141] and the NS3/4A serine protease (Figure 7) [176].

Replication starts by the NS5B RdRp synthesis of a complementary full-length negative strand RNA using the genome as a template, then using this negative strand as a template for the production of many copies of the genomic positive strand in a replication complex [177, 178] that is associated with lipid-raft based membranes (Figure 8 – Lower left then right panels) [179, 180].

Data from expression systems show that once an HCV nucleocapsid is formed at the cytoplasm-endoplasmic reticulum interface it acquires an envelope as it buds through an

intracellular membrane [181-183] producing particles of heterogeneous size (40-70 nm) that are released from the cell through the secretary pathway [140]. HCV nucleocapsid is built from units of the core protein with RNA, surrounded by a membrane derived from the human-cell with embedded heterodimers of the envelope glycoproteins (E1 and E2) [138]. The virions associate with low-density and very-low-density lipoproteins (LDL and VLDL) forming lipoviroparticles (LVPs) that are pleomorphic (Figure 8 – Upper right panel) [184].

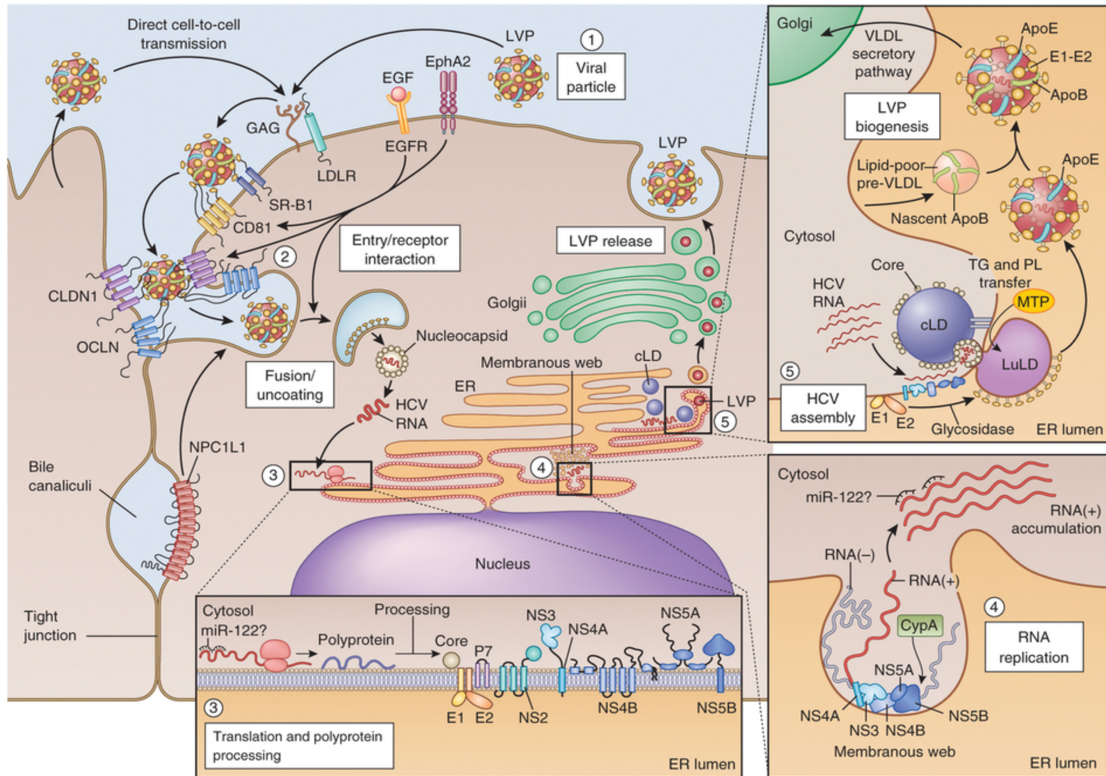

Figure 8: HCV viral life cycle.

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Legend: Interaction of extracellular HCV LVPs (1) with cellular surface receptors initiates the entry process (2), which can also occur by direct cell-to-cell transmission. After fusion and uncoating, the incoming HCV genome is translated and the resulting polyprotein processed (bottom panel, (3)). Replication takes place in ER-derived membrane spherules (membranous web, bottom right panel (4)). In the assembly and release process (top right panel, (5)), core protein is transferred from LDs to form nucleocapsids that, assisted by NS5A, are loaded with RNA. The p7, NS2 and NS3-NS4A proteins are also involved in coordination of assembly. HCV virion morphogenesis is coupled to the VLDL pathway, and particles are produced as LVPs. EphA2, ephrin receptor type A2; GAG, glycosaminoglycans; LD, lipid droplet; PL, phospholipids; TG, triglycerides.

1.3.4. HCV infection and its outcome

Due to the asymptomatic nature of HCV infection, less than 15% of the infected persons worldwide are aware of their carrier status [186], leading to the spread of infection and missing the opportunity of early treatment [187, 188]. The majority of acutely infected HCV patients (~70%) progress to chronicity [117], and up to 20% of patients with chronic viral hepatitis C subsequently develop complications including end-stage cirrhosis, liver failure and hepatocellular carcinoma (HCC) [189]. These patients represent a quarter of the cirrhosis and HCC cases worldwide [190], making hepatitis C the most common indication for liver transplantation (40-50%) [191, 192].

Several factors influence the outcome of HCV infection. Ethnicity and accompanying co-morbidities (e.g. HIV infection, alcohol abuse, renal diseases, obesity or liver fibrosis) are considered the most relevant host factors [193]. African Americans were shown to have a resolution rate of one-third that of Caucasians [194]. This was explained in light of the findings of genome-wide association studies (GWAS) that demonstrated a correlation between specific single-nucleotide polymorphisms (SNPs) near the IL28B gene locus and the spontaneous resolution of infection [195]. The different alleles for these SNPs are distributed unequally among the different ethnicities [195, 196]. Recently, another SNP near the genes for HLA class II, the DQB1*03:01 was independently associated with spontaneous resolution of HCV infection [197]. Other genetic determinants were shown to affect HCV-infection outcome due to having an impact on HCV-specific immune responses; Khakoo *et al*., reported that individuals homozygous for KIR2DL3 and HLA-C1 alleles were more likely to clear HCV infection than individuals with other KIR2DL:HLA-C combinations [198]. KIR2DL3 has a lower affinity for HLA-C1 than other KIR2DL alleles, so HLA-C1-mediated inhibition of NK cells is thought to be weaker in individuals homozygous for KIR2DL3 and HLA-C1 (i.e. easier NK activation) [199]. Also, some HLA class I alleles (e.g. HLA-B*27) [200] and HLA class II alleles (e.g. HLA-DRβ1*0101) demonstrated a strong association with HCV clearance, whereas others (e.g. HLA-DRβ1*0701) were associated with HCV persistence [201-203].

Nevertheless, the outcome of HCV infection is determined by a ménage-à-trois between host genetics, the virus and HCV-specific immunity. The viral interplay with the host and HCV-specific immunity will be discussed in details later in this chapter.

1.3.5. Treatment of HCV infection

1.3.5.1. Available treatments

IFN-α was shown to have a success rate of 6-12% in curing patients with NANB hepatitis in 1986, even before HCV was cloned [204]. Prolonging the treatment duration to 12 months, addition of the antiviral ribavirin and covalently binding IFN-α to polyethylene glycol (PEG) boosted the success rate to ~56% against genotype 1 (which is the most resistant genotype to treatment) [205-207]. However, some associated side effects are severe, as lymphopenia, depression and anxiety that might develop into suicidal tendencies [208].

Since 2011, the two new anti-HCV protease inhibitors; Boceprevir and Telaprevir have been available on the market. They were included in a triple therapy regimen (with PEG- IFN-α and Ribavirin) to increase the cure rates to ~70%. Nevertheless, this added to the existing side effects and the high cost of the standard of care treatment [209, 210].

Responses to IFN-α based therapy fall into three main categories; sustained virological response (SVR), which is equivalent to success in clearing HCV, relapse and non-response (NR). The most significant viral factor correlating with SVR is the genotype. Genotype 1a is the most resistant genotype with an SVR rate of 42-46% [205, 207], versus genotypes 2 and 3 that have the highest SVR rates. Other viral factors associated with a higher SVR rate are lower baseline viral load [193] and greater quasispecies diversity pre-commencement of treatment [211]. Ethnicity (correlated with the IL28B SNPs) and absence of accompanying co-morbidities are considered the most relevant host factors [193, 196]. Timing of initiation of treatment is a crucial factor, as well, where early initiation of therapy during the acute phase markedly increases the SVR rate (e.g. for genotype 1a it increases to ~88%) [212, 213].

1.3.5.2. Novel HCV treatments

The ideal HCV treatment would be an all-oral, IFN-free regimen, with SVR rates approaching 100%, minimum side effects and minimum development of resistance.

1.3.5.2.1. Direct-acting antiviral agents (DAAs)

DAAs target HCV proteins in an attempt to counteract their actions. NS3/4A protease inhibitors act by inhibiting the NS3/4A serine protease, thus inhibiting the processing of the HCV-polyprotein into mature non-structural proteins. After the approval of Telaprevir and Boceprevir by the FDA in 2011, Simeprevir was approved in 2013 and there are still several anti-NS3/4A molecules that are in phase III clinical trials as Faldaprevir and Vaniprevir. Another class, NS5B polymerase inhibitors bind the attractive target that represents the RdRp, thus halting HCV-replication. They are either nucleotide analogues (e.g. Sofosbuvir) or non-nucleoside analogues (e.g. ABT-333 and BI207127) [185]. Sofosbuvir is a very promising drug that was approved in 2013, with pan-genotypic action, high resistance barrier and a good safety profile. It showed SVR rates of ~90% for genotype 1 infected patients in clinical trials, when combined with IFN and Ribavirin for twelve weeks [214]. NS5A inhibitors (e.g. Daclatasvir and ABT267) emerged despite the fact that NS5A lacks enzymatic activity and its function(s) are not fully elucidated. Screening processes have shown these compounds to be potent antivirals causing extremely rapid HCV decline and their inhibition of HCV-replication was shown to be NS5A-dependant [185]. Results from a phase IIb clinical trial for a combination of an all-oral/IFN-free regimen are promising. Two drugs from Abbvie (Chicago, USA) combined with Ribavirin showed 95% SVR rate in a group of patients infected with HCV genotype 1. The combination of the non-nucleoside polymerase inhibitor ABT-333 and the protease inhibitor ABT-450 combined with Ribavirin showed SVR rates of 83-100% in difficult-to-treat patients and naïve patients, respectively. The addition of the NS5A inhibitor ABT-267 improved efficacy in difficult-to-treat patients [215]. Another study showed that regimens combining Daclatasvir and Sofosbuvir could achieve very high SVR rates, even in patients who had previous failure with Telaprevir/Boceprevir and PEG-IFNα combinations. Among gentotype 1 patients, 98% of 126 previously untreated patients and 41 patients who

did not have SVR with Telaprevir/Boceprevir and PEG-IFNα achieved SVR after 12 and 24 weeks of treatment, respectively [216].

1.3.5.2.2. Host targeting agents (HTAs)

A number of host factors were found to influence HCV replication as Cyclophylin A (CypA) and micro-RNA miR-122. This encouraged the search for inhibitors against those molecules. The CypA inhibitor, Alisporivir, showed a pan-genotypic action and a high resistance barrier. Nevertheless, it is currently on clinical hold due to the emergence of several cases of acute pancreatitis. Small molecules that would inhibit HCV uptake and entry into cells by targeting receptors and/or coreceptors (e.g. ITX-5061 and Erlotinib) are also in clinical trials [185]. Numerous side effects would be expected for this class of antivirals, taking into consideration that those human factors might have many other functions and effects on various physiological processes.

1.3.5.2.3. Type III interferon

IFN-lambda (IFNλ) is currently examined in clinical trials for use as a treatment for HCV infection [217]. IFNλ is more specific in its actions compared to IFNα, owing to the limited distribution of its receptor on a specific set of cells, unlike IFNα that has a ubiquitous receptor distribution. Thus, it is expected to have fewer side effects, whereas studies have shown that efficacy would not be compromised.

1.4. Immunity against primary HCV infection

1.4.1. Innate sensing of HCV

RNA viruses are recognized by TLRs and/or nucleic acid-binding proteins [218, 219]. In hepatocytes, pathways downstream of TLR-3 and RIG-I are the two major signalling pathways triggered by foreign double-stranded RNA (dsRNA), including HCV RNA (which has secondary dsRNA structures and a dsRNA replication intermediate) [130, 220-222] culminating in the secretion of type I IFNs [223, 224] (Figure 9). This induces the expression of hundreds of interferon-stimulated genes (ISGs) in the infected cells and sends a danger signal to neighbouring uninfected cells inducing ISGs expression in them, as well. This creates a general antiviral state in the liver that limits HCV RNA replication and cell-to-cell spread [225, 226]. Upregulation of ISGs is detected in liver cells early after HCV infection irrespective of the final outcome of the infection, suggesting that most HCV isolates are resistant to the antiviral effects of this line of defence [227, 228].

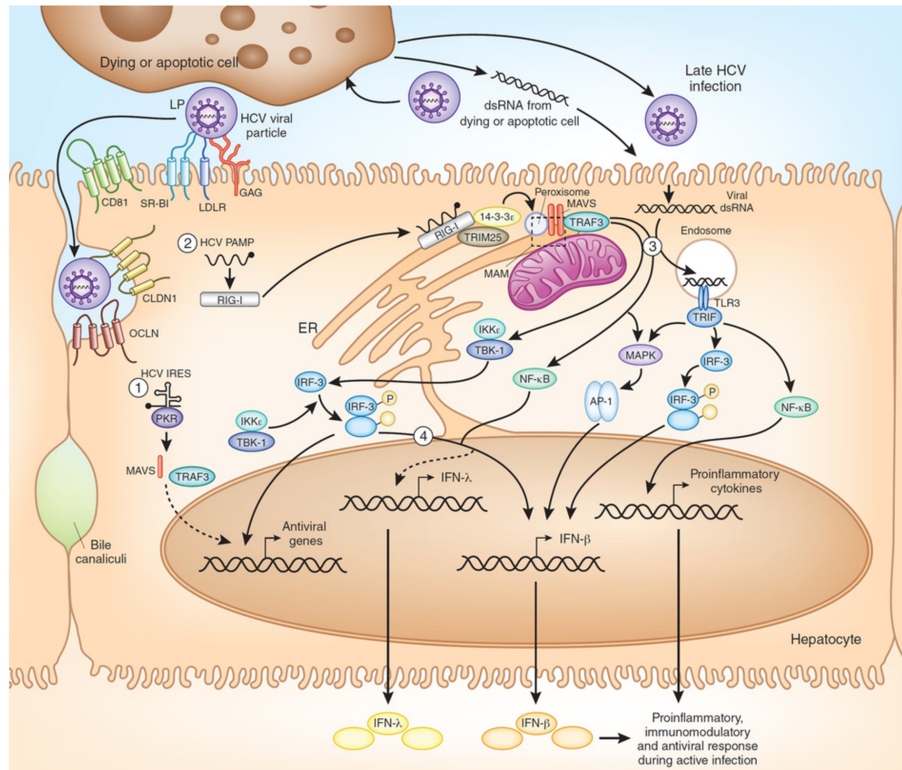

Figure 9: Sensing HCV in hepatocytes.

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Legend: Innate sensing of HCV in the infected hepatocyte occurs through the combined actions of PKR (1), RIG-I (2) and TLR3 (3). These proteins recognize specific features of HCV, including the dsRNA in the IRES, poly U/UC PAMP and dsRNA that accumulates with HCV replication. This recognition leads to downstream signaling that results in the induction of antiviral genes, IFN-β and other proinflammatory cytokines. The mechanisms activating IFN-λ during HCV infection are not fully characterized (4). AP-1, activator protein 1; CLDN1, claudin 1; IKKε, IkB kinase-e; LDLR, low-density lipoprotein receptor; OCLN; occludin; SR-BI, scavenger receptor class B member 1; TBK-1, TANK-binding kinase 1; TRAF3, TNF receptor–associated factor 3; GAG, glycosaminoglycans.

1.4.2. Innate immune response against HCV

Natural killer cells are more abundant in the liver than the blood of healthy individuals, but their frequency is even higher in the liver and lower in the blood in chronic HCV patients [230]. One of the determinants of HCV outcome is the threshold of NK activation. This activation is governed by the combination of the alleles of the KIRs expressed on NKs and the HLA molecules expressed on other host cells, as well as their interactions [198]. NK cells from HCV infected patients show a higher level of activation markers as NKp30, NKp44, NKp46, NKG2C and NKG2D than healthy individuals. Hyperactivation of NKs was observed in HCV-infected patients irrespective of the outcome of the infection [231, 232].

Being the bridge between innate and adaptive immunity, DCs have an impact on the immune response against HCV. The frequencies of mDCs and pDCs were shown to correlate with the outcome of infection, where normal frequencies were associated with spontaneous resolution of HCV infection versus lower frequencies that were associated with chronic infection [233]. Sustained hyper-responsiveness of DCs was associated with resolution of HCV infection [234]. Nevertheless, the cross-talk between NKs and DCs remains intact even in patients who develop persistent HCV infection [235].

1.4.3. Adaptive immunity against HCV

Cell-mediated immunity is of special importance in immunity against non-cytopathic viruses like HCV [236]. Evidence for the importance of T-cell responses, is supported by the association of specific alleles of MHC class I and II with clearance of HCV infection [237]. This importance was confirmed by depletion studies in chimpanzees [238, 239], as well as temporal association of appearance of HCV-specific T-cell responses and HCV clearance, versus the absence of these responses in infections that progress to chronicity [239].

Studies during the acute phase of HCV infection showed that chimpanzees and humans capable of resolving primary HCV infection mounted an early, robust, broad/multispecific and polyfunctional HCV-specific immune response for both CD8 and CD4 T cells (Figure 10) [240-246]. Development of chronic infection was consistently associated with the failure to initiate or sustain such a T-cell response [96, 246, 247]. The role of anti-HCV Abs in the

spontaneous clearance of HCV is less defined, since neutralizing Abs appeared after clearance of the virus in many studies [248, 249].

1.4.3.1. Anti-HCV cell-mediated immunity

1.4.3.1.1. Role of CD8 T cells in the clearance of primary acute HCV

Early onset of CD8 T response against HCV is important for clearance of the virus. Evidently, the only chance for the immune system to overcome HCV is during the early phase of infection before it has the chance to establish infection and produce enough viral proteins that would antagonize and/or exhaust functions of the immune system, as well as generate a high number of viral mutants that would enable selection for resistant variants [244]. The magnitude of HCV-specific T-cell responses strongly correlated with resolution of infection [250]. Breadth of the CD8 T-cell response and the relative dominance of the targeted epitopes were both shown to be determinants of the outcome of HCV infection [240, 243].

The initial decrease in HCV titer in infected patients and chimpanzees coincided with the appearance of IFNγ-secreting HCV-specific T cells in the blood and liver [57]. However, individual cytokines/functions did not necessarily correlate with the outcomes of HCV infection. Flow cytometric analysis of multiple effector functions performed simultaneously identified the presence of a polyfunctional population of virus-specific CD8 T cells that correlated with spontaneous clearance of HCV [251]. The functions included secretion of effector cytokines as IFNγ and TNFα, and IL-2 that helps in amplifying the immune response, as well as the increased surface expression of CD107a. CD107a is a surrogate marker of cytotoxicity since it is a measure of degranulation capacity required for the release of the cytotoxic/cytolytic proteins, as perforin and granzymes [251]. Vigorous and multispecific proliferative responses were observed for spontaneous resolvers and were maintained after HCV clearance [252]. Differences in T-cell responses become increasingly apparent as the infection progresses. In patients who become chronically infected T-cells showed decreasing functions, less polyfunctionality and diminished proliferative capacity [253]. HCV clearance was associated with responses mainly directed against non-structural proteins [244, 254].

Genetic restriction was shown to have an impact on HCV-specific CD8 T-cell responses (e.g. HLA-B*27 demonstrated a strong association with viral clearance) [200]. MHC class I tetramer staining showed a strong correlation between infection outcome and the frequency of CD8 T cells targeting a specific HCV epitope. The detection of high frequencies of HCV-specific tetramer-positive cells in the blood and liver of humans and chimpanzees is temporally and kinetically associated with the control of viraemia [130]. In spontaneously resolved patients up to 8% of the total CD8 T cells in the blood targeted a single immunodominant HCV epitope [243] and even higher frequencies could be detected in the livers of HCV resolving chimpanzees [255]. These tetramer positive cells displayed markers of T-cell activation [246, 256]. Furthermore, multi-specificity of targeted epitopes is more pronounced in humans and chimpanzees able to clear the infection, with up to nine different epitopes recognized simultaneously by CD8 T cells [240, 243]. Unlike the observation that specific HCV regions were more immunodominant and correlated with spontaneous clearance of the infection [250], there was lack of consistency in the targeted epitopes among the different individuals who resolved infection, even those who shared HLA alleles [96, 257].

A distinct phenotype of CD8 T cells was associated with spontaneous clearance of HCV infection. HCV-specific CD8 memory T-cell population generated following clearance of a primary HCV infection were shown to be $CD127^{hi}$ and $Bcl2^{hi}$ indicating they are destined to become long-lived memory T cells [251]. It was demonstrated that early expression of CD127 predicted resolution of acute infection, while loss of this marker was associated with viral persistence [258, 259].

1.4.3.1.2. Role of CD4 T cells in the clearance of primary acute HCV

Chimpanzee studies showed that CD8 T cells alone could not clear highly mutable viruses as HCV, indicating that help from CD4 T cells is essential. The loss of helper CD4 T cell responses during acute infection correlated with HCV persistence [238, 246]. Human studies confirmed the same findings, where an initially functional CD8 T-cell response would wane resulting viral recurrence upon the loss of CD4 T-helper response [243, 246, 247]. HLA class II restricted CD4 T-cell responses were shown to be of direct impact on the outcome of acute HCV infection [247, 260] and some HLA class II alleles were associated with resolution

while others were associated with chronicity [201-203]. In human IDU cohorts, the indispensable role for helper CD4 T cells was confirmed in HIV/HCV co-infected patients, as the reduction of CD4 T-cell count upon HIV infection was associated with a lower level of protection against HCV infection [118].

A successful helper T-cell response is characterized by being early, of high magnitude and multi-specific. In patients that proceed to chronicity either one or more of these were lacking [130]. Early CD4 responses were temporally and kinetically correlated with control of viremia. Patients who cleared HCV spontaneously demonstrated CD4 T cells with a high magnitude of IFN-γ production [243], whereas loss of IL-2-secreting CD4 T cells was associated with developing chronic HCV infection [261]. Broad CD4 T-cell proliferative responses of high magnitude were associated with spontaneous clearance of HCV. Those responses appeared early in patients who resolved the infection and were maintained [243, 247]. On the contrary, proliferating CD4 T cells were mostly absent in chronic patients, and even if they could be detected early in the infection, they disappeared rapidly with viral persistence [262, 263].

The frequency of HCV-specific CD4 T cells targeting a specific epitope is several folds higher in individuals who resolved infection versus those who became chronic. CD4 helper T cells targeting several immunodominant epitopes were associated with spontaneous resolution, in a human study up to 14 epitopes were targeted mapping to the core and non-structural regions [264]. The phenotype of HCV-specific CD4 T cells is also important, since there is an association between the prevalence of CD4 T-cells with a Th1 cytokine-profile and resolving acute HCV infection [244, 265]. The Th1 responses had to be maintained to achieve long-term control of the virus [247].

In conclusion, an early functional CD4 T-cell helper response directed against multiple immunodominant epitopes predicts control of viremia, while in cases that develop chronic disease CD4 helper T-cells are either absent or functionally altered or target fewer epitopes [247, 263, 264, 266]. 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 [130].

Other CD4 T cell populations, specifically regulatory T cells (T_{regs}) and T-helper 17 cells (Th17), were also shown to impact the outcome of primary HCV infection. The percentage of CD4+ CD25+ T_{regs} expressing the forkhead transcription factor 3 (FoxP3) was shown to be elevated and more suppressive in chronic versus spontaneously resolved HCV patients or healthy individuals [267, 268]. Decrease in IFNγ secretion from CD4 T cells and the simultaneous increase in IL-10 and TGFβ production was associated with the development of chronic infection [263]. Our group has recently shown that imbalance between Th17 and T_{regs} could be a cause for failure of CD4 T-cell help during acute HCV. Specifically, inability to expand IL-21 producing Th17 cells was a key determinant of developing a chronic disease course [269].

1.4.3.2. Humoral immunity during primary HCV infection

Although, HCV RNA reaches high serum titres by week two post infection, anti-HCV antibody response (seroconversion) is usually not detected before week eight [224, 270]. Early studies showed that antibodies targeting the hypervariable region-1 (HVR1) of the E2 glycoprotien of HCV are neutralizing *in vitro* and *in vivo* [271, 272], but select for mutations in the envelope region [273]. Chimpanzee studies showed that clearance was not associated with the generation of antibody responses [274, 275]. The presence of antibodies targeting HVR1 during acute infection was even associated with the development of chronic infection in one study [276]. In humans, Ab-responses are delayed, have a low titre and rapidly decline in individuals who clear HCV spontaneously [248, 249, 277]. One study showed that nAbs appeared in patients after HCV has already established a chronic infection and were unable to clear the virus and selected for escape mutants [278]. However, another study showed that early induction of nAbs was associated with spontaneous resolution of HCV primary infection [279]. Law *et al.* reported the identification of monoclonal nAbs that could protect against heterologous viral infection [280]. Broadly nAbs confer protection against most pathogens, including viruses. However, the most evident exception is HIV (a chronic virus with a high rate of sequence variation), where broadly nAbs do not necessarily correlate with the control of infection or protection against superinfection [281].

One of the major limitations for understanding humoral immunity against HCV is the lack of proper tools to measure the levels of nAbs precisely. The current method measures neutralization by the degree of neutralization of HCV pseudo-particles (HCVpp), which are retroviral particles pseudotyped with HCV E1-E2 envelope proteins. The sequences of the included envelope proteins were derived from reference sequences, and thus do not necessarily represent the autologous E1/E2 sequences circulating the patient [282]. A major advancement was the generation of an HCVpp library of 19 genetically-distinct genotype 1 HCVpp. This library comprises the natural variability of genotype 1 E1/E2 sequences [283]. A recent study using this HCVpp library demonstrated that resolution of HCV infection was associated with a broad nAb response generated early during the infection [284].

Some major limitations exist for protective humoral immunity against HCV. First, the envelope proteins are not highly immunogenic, causing the Ab response to be slow and weak during primary infection [283]. Second, Abs mostly target the HVR of the envelope protein, which facilitates the selection of viral sequences that are highly resistant to Ab neutralization [285]. This selection was shown to take place as the CMI collapsed and the infection entered the chronic phase [286]. Third, the epitopes targeted by nAbs are shielded by heavy glycosylation and complexation with host lipoproteins [287].

The role of antibodies in the resolution of HCV infection needs further investigation, since the results of *in vivo* studies performed to date are inconclusive.

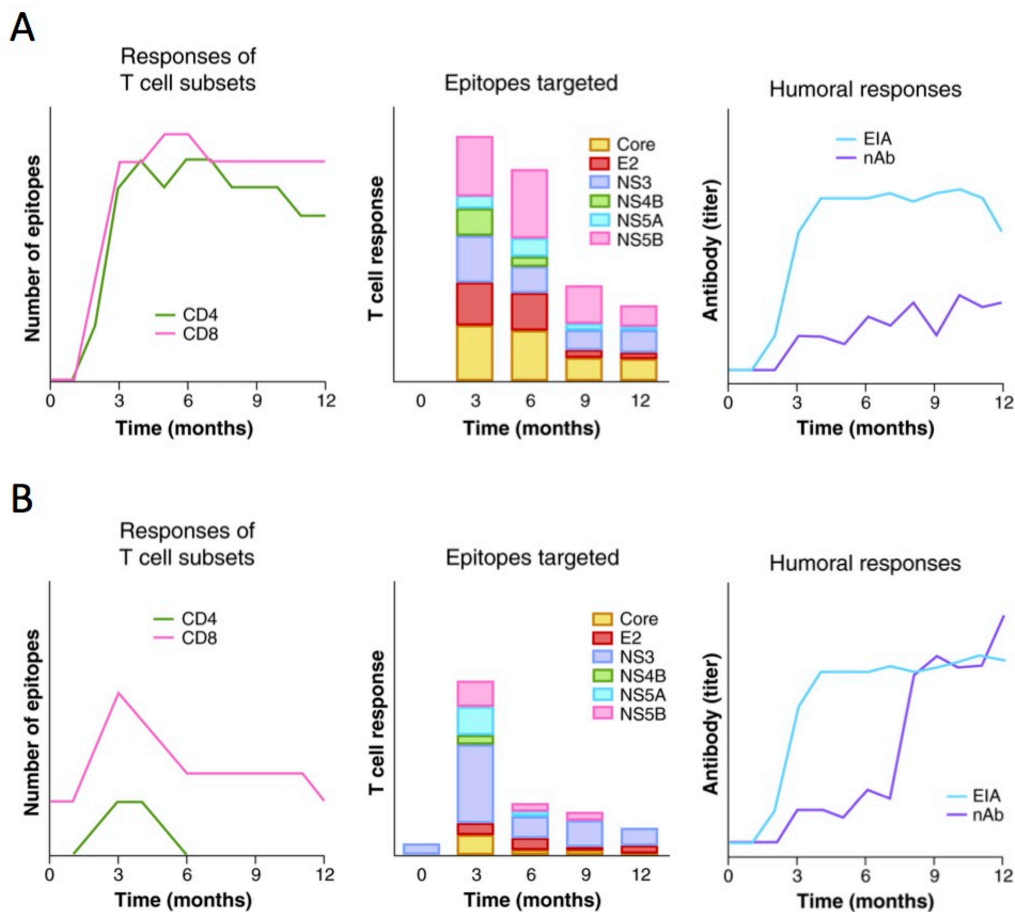

Figure 10: Successful versus unsuccessful immune responses during acute HCV infection.

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Legend: (A) Early, robust, broad and sustained CD4 and CD8 T cell responses are able to control and clear HCV viraemia. (B) Weak CD4 and CD8 T cell responses from the beginningis associated with lack of control of viremia. 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. The role of humoral immunity remains controversial and needs further investigation.

1.4.4. Failure of immune responses against HCV

Failure of the HCV-specific immune response could be due to primary failure of components of the immune system from the beginning of infection or their exhaustion over time. Nevertheless, even a functional immune response could be subverted by different viral evasion strategies adopted by HCV.

1.4.4.1. Primary failure of the HCV-specific immune response

Several defects have been associated with failure to control primary HCV infection. Those defects were either in the cells/functions of innate or adaptive immunity or both.

NK cells' numbers and functions, especially cytotoxic activity, were shown to be lower in chronic hepatitis C patients as compared to healthy donors [288-290]. In chronic HCV patients a reduction in number of both mDCs and pDCs was observed [291-293]. Also, functions of mDC and pDC were impaired in chronic HCV-infection (e.g. Ag-presentation, TLR-sensing and allostimulatory functions) [294-296] [296, 297].

Primary defects in adaptive immune cells' frequency and/or functions were reported in chronically infected patients. CD4 T-cell responses are very weak or absent in individuals with chronic HCV [73], as indicated by decreased cytokine production (especially IL-2) [261]. CD8 T cells in chronic patients were also reported to be anergic or of an arrested developmental phenotype [71, 298]. Other studies showed them to be impaired in cytotoxic function and proliferative capacity [256, 298], thus were described as being in a 'stunned' state [243, 246].

One plausible cause of failure might be the increased exhaustion of adaptive immune cells. Progressive functional exhaustion and subsequent deletion of HCV-specific CD4 and CD8 T cells is a hallmark of HCV infection developing into persistent infection [298, 299]. HCV-specific CD8 T cells from chronic individuals showed high expression of PD-1 [300], and their functions were restored by its blockade [301]. Recently, it was proposed that PD-1 and CTLA-4 contribute synergistically to CTL exhaustion [116]. The downregulation of T-cell responses follow a similar pattern in patients with HCV and LCMV-infected mice, where virus-specific T cells show increased expression of inhibitory molecules as PD-1, CTLA-4, T-cell immunoglobulin and mucin domain 3 (Tim-3) and CD244 [116, 299, 302, 303], and some

of their corresponding ligands were shown to be upregulated in the liver and plasma [269, 304]. Those exhausted T cells lost their functions sequentially; first IL-2 production, followed by cytotoxicity, then IFNγ and TNFα production [305]. This could be attributed to the persistence of the Ag, where it was shown in the LCMV model that prolonged exposure to viral antigens is the main cause for reduced frequency and impaired effector functions of virus-specific CD8 T cells [306, 307]. The defects in HCV-specific CD8 T cells in patients with persistent infection were shown to correlate with HCV titer having the original sequence of the targeted epitope [308]. Confirming this finding, HCV-specific CD8 T cells whose epitope has already mutated, did not show exhaustion in chronic patients and even displayed markers of long-lived memory cells (e.g. CD127), since they were not subjected to such pressure from their corresponding antigens [251, 309].

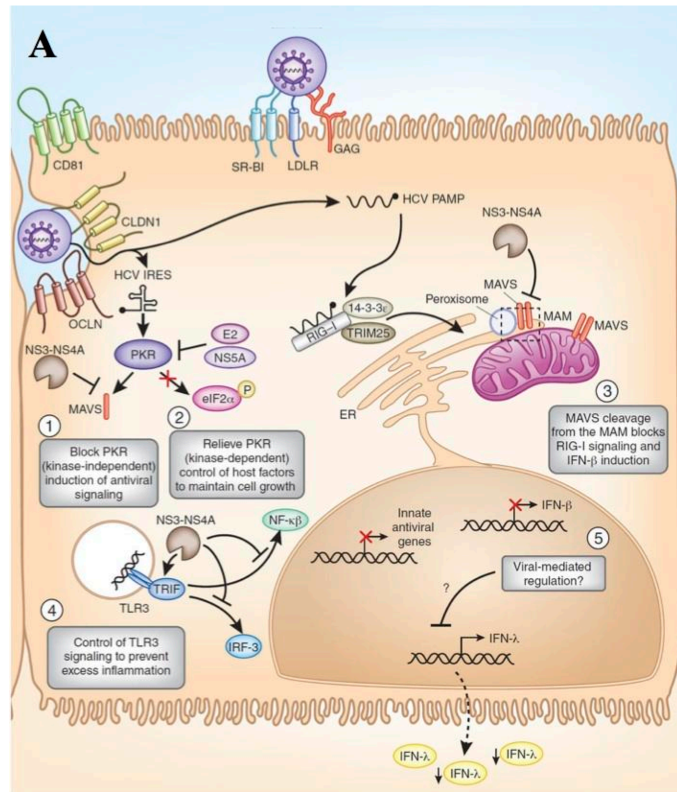
Suppression of HCV-specific immunity can contribute to chronicity, where the percentage of CD4+ CD25+ FoxP3+ T_{regs} producing the anti-inflammatory cytokines IL-4 and IL-10 were higher in chronic patients versus spontaneously resolved HCV infected patients or healthy individuals [265, 267, 310, 311]. Moreover, T_{regs} derived from chronically infected patients were significantly more suppressive than those from spontaneously resolved ones [268]. Together, this causes a shift in the ratio between T-cell sustaining cytokines (e.g. IL-2) and suppressive cytokines (e.g. IL-10 and TGFβ), consequently dampening the HCV-specific effector T-cell response [267, 312, 313]. Our group has recently shown that failure of CD4 T-cell help during acute HCV is partially attributed to the imbalance between Th17 and T_{regs}, where IL21-producing Th17 limited the exhaustion of HCV-specific T cells, while Galactin 9-producing T_{regs} enhanced this exhaustion [269]. Importantly, the lack of generalized immune suppression in patients with chronic HCV infection argues in favour of an Ag-specific suppression [314].

Finally, the liver as an organ represents a tolerogenic environment [315]. Thus, primary contact of T cells with sinusoidal endothelial cells in the liver rather than lymph nodes typically results in tolerance [316, 317], and this abortive activation could be the reason why HCV-specific T cells with the highest avidity undergo functional silencing [318]. A study dissecting mechanisms of failure of intrahepatic CD8 T cells demonstrated impaired

production of IFNγ that was limited to HCV-specific cells, despite their accumulation in the liver and the presence of CD4 helper T cells [319].

1.4.4.2. Viral evasion strategies

The majority of HCV infections lead to chronicity, even in people with an intact immune system [320]. This could be attributed to the inhibition of specific steps in the intracellular antiviral signalling pathways by HCV proteins. NS3/4A was shown to specifically disrupt viral sensing pathways at early steps, by splicing mitochondrial antiviral signaling protein (MAVS) and TIR domain-containing adapter inducing IFN-β (TRIF) downstream of the two major sensors of HCV-RNA, RIG-I and TLR3, respectively (Figure 11-A). Other HCV-proteins (e.g. the core, E2 and NS5A) disrupt various early and late steps along the JAK-STAT signaling pathway which is induced by type I and III IFNs (Figure 11-B) [321-323].


A
CD81
SR-BI
LDLR
GAG
HCV PAMP
CLDN1
NS3-NS4A
HCV IRES
MAVS
Peroxisome
14-3-3ε
MAM
MAVS
OCLN
E2
TRIM25
NS3-NS4A
PKR
NS5A
eIF2α
P
MAVS
ER
3
1
2
MAVS cleavage from the MAM blocks RIG-I signaling and IFN-β induction
Block PKR (kinase-independent) induction of antiviral signaling
Relieve PKR (kinase-dependent) control of host factors to maintain cell growth
IFN-β
NS3-NS4A
NF-κβ
Innate antiviral genes
5
TRIF
Viral-mediated regulation?
TLR3
4
Control of TLR3 signaling to prevent excess inflammation
IRF-3
IFN-λ
IFN-λ
IFN-λ
IFN-λ

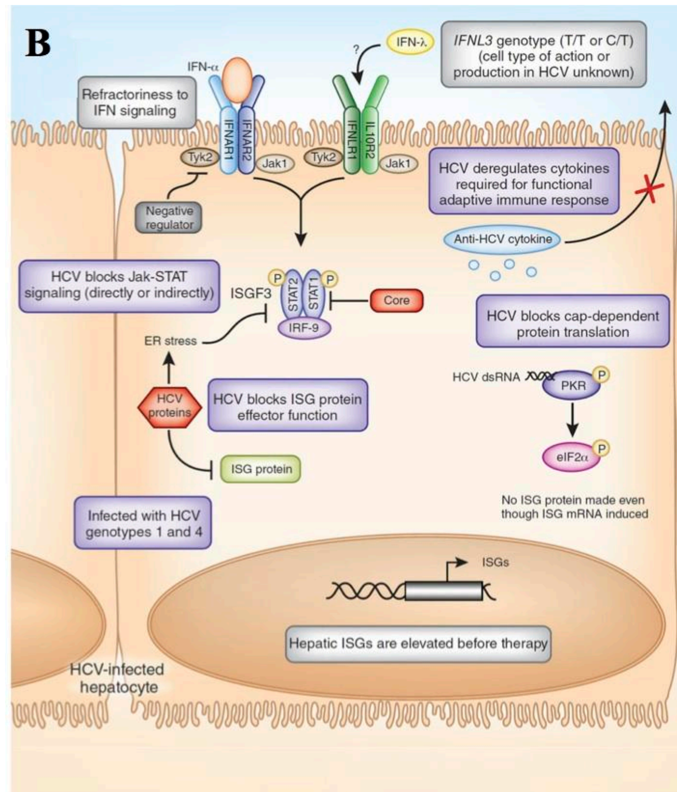

B
IFN-λ
IFNL3 genotype (T/T or C/T) (cell type of action or production in HCV unknown)
IFN-α
Refractoriness to IFN signaling
IFNAR1
IFNAR2
IFNLR1
IL10R2
Tyk2
Jak1
Tyk2
Jak1
HCV deregulates cytokines required for functional adaptive immune response
Negative regulator
Anti-HCV cytokine
HCV blocks Jak-STAT signaling (directly or indirectly)
ISGF3
STAT2
STAT1
Core
IRF-9
HCV blocks cap-dependent protein translation
ER stress
HCV dsRNA
PKR
HCV proteins
HCV blocks ISG protein effector function
eIF2α
ISG protein
No ISG protein made even though ISG mRNA induced
Infected with HCV genotypes 1 and 4
ISGs
Hepatic ISGs are elevated before therapy
HCV-infected hepatocyte

Figure 11: HCV proteins subvert intracellular signaling.

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Legend: (A) Immune evasion by HCV in the hepatocyte occurs at several points during viral infection. HCV NS3-NS4A protease cleaves the signaling adaptors MAVS and TRIF to inactivate PKR (1), RIG-I (3) and TLR3 (4) signaling pathways to prevent induction of innate antiviral genes and IFN-β. HCV E2 and NS5A proteins inactivate PKR-dependent activation of the host translation factor eIF2α to reactivate protein translation during infection (2). **(B)** Upon binding of IFN-α to receptors in neighbouring cells, HCV proteins could prevent IFN actions by directly or indirectly blocking Jak-STAT signaling. Tyk2, tyrosine kinase 2; Jak1, Janus kinase 1.

Inhibition of functions of innate immune cells (NKs and DCs) by HCV proteins has also been reported. HCV recombinant E2 protein was reported to directly inhibit NK cytotoxicity and IFN-γ production [324, 325] (Figure 12). The hindrance of DC functions by uptake of HCV proteins has been demonstrated; where core and NS3 proteins inhibited DC differentiation and maturation, causing inhibition of *in vivo* induction of cellular immune responses [326-328]. The NS3/4A encoded serine protease alters the transfer of glycoproteins, suggesting interference with MHC-I presentation of viral epitopes, which could affect T-cell priming [329]. However, the *in vivo* importance for the majority of these findings has not been established, since these results were obtained *in vitro* using overexpression of the corresponding proteins [224].

Mutational escape within targeted epitopes is the most common strategy used by RNA viruses to evade the adaptive immune response. Mutations of HCV genome occur early and become fixed in the viral quasispecies [330, 331]. CD8 T cells exerting sub-optimal immune pressure on their corresponding viral epitopes select for resistant variants. Specific HLA alleles produce selective pressure on certain epitopes [332]. The rate of mutations in MHC I-restricted epitopes was significantly higher (thirteen-fold more) compared to other regions outside T-cell epitopes, indicating that these mutations were in response to a CD8 T-cell pressure, generating epitope sequences that are less efficiently recognized by CD8 T cells and poorly induce new T-cell responses [330, 333, 334]. In some cases specific mutations even in one epitope were associated with HCV persistence [332]. Luckily, viral fitness limits the variability within some HCV immunological epitopes [335].

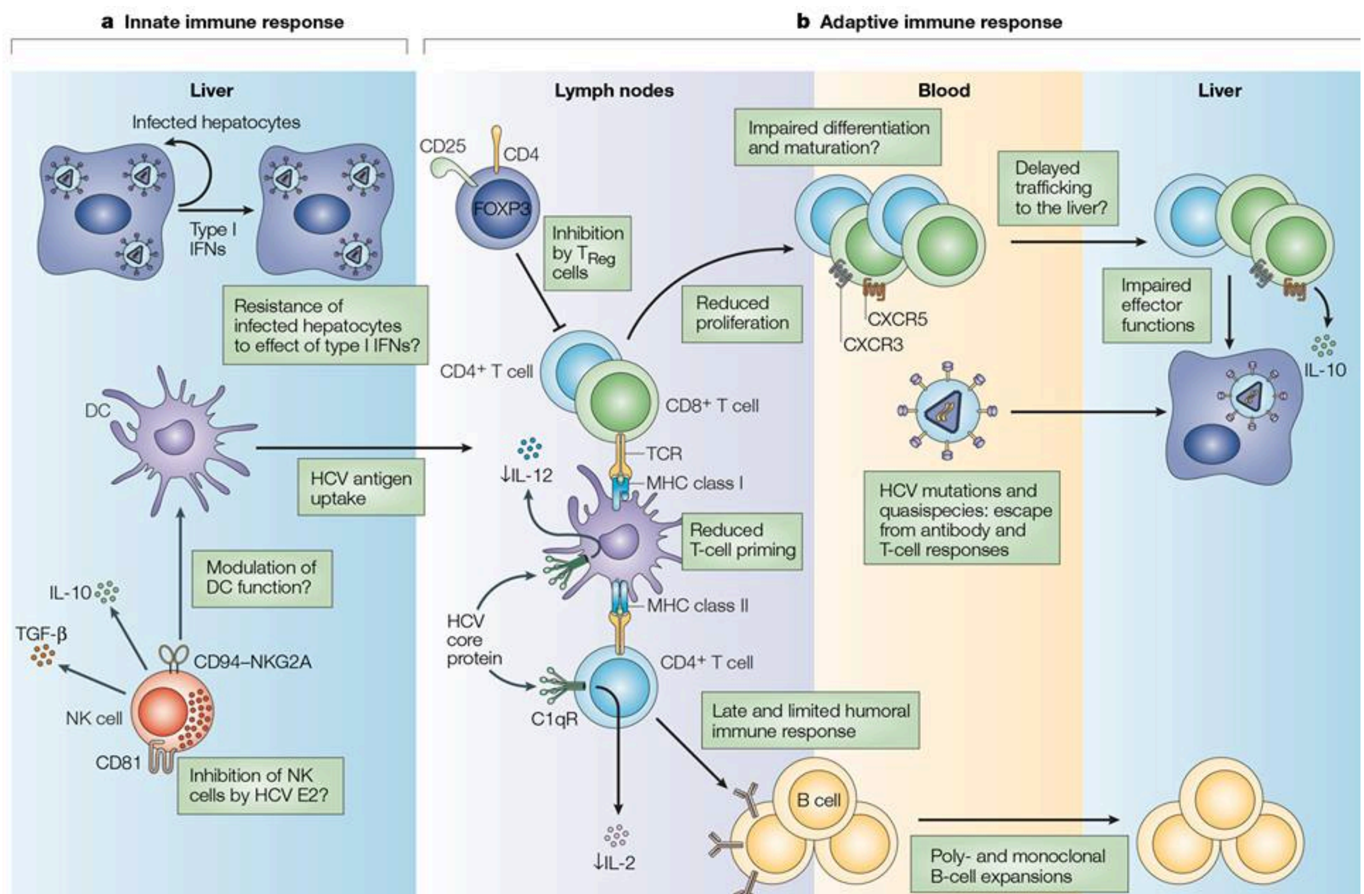

Figure 12: Mechanisms of HCV-protein interference with the immune system.

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Legend: HCV-proteins interfere with the different steps of innate immunity (A) and adaptive immunity (B). The details are specified in the green boxes and the text. CXCR, CXC-chemokine receptor; FOXP3, forkhead box P3 transcription factor; NKG2A, NK group 2 – member A; TGFβ, transforming growth factor beta

1.5. Protective immunity against HCV and vaccine trials

1.5.1. Memory immune responses against HCV

Different chimpanzee studies have demonstrated the generation of memory T cells following clearance of HCV infection. Memory T-cell responses were maintained in the blood of chimpanzees for several years after clearance of a primary HCV infection, whereas the antibody titres against HCV proteins were low or even undetectable. The frequency of HCV-specific T cells was fixed for several years after the resolution of primary infection [239]. The hierarchy of CD4 T-cell responses was preserved during secondary infection years later [57]. Nevertheless, it has been shown that frequent exposure to very low doses of HCV (subinfectious doses) predisposes to T_{regs} expansion, leading to suppression of effector T_{mem} upon subsequent infection. *In vitro* depletion of T_{regs} restored HCV-specific T-cell effector responses [336].

Evidence from rechallenge studies suggest that this memory response might be protective against HCV upon re-exposure, since these studies showed that chimpanzees spontaneously clearing a primary infection had a higher level of protection than naïve ones when rechallenged with homologous but not heterologous viral sequences. This was accompanied by a shorter duration of viremia (~42 versus ~98 days) and lower viral loads (as low as 100-folds) in the cases that cleared the second infection [337, 338]. However, other studies did not observe protection even against homologous rechallenge [339]. A broad CD8 T-cell response with high magnitude was associated with rapid control of the second HCV infection upon rechallenge, even with a poor antibody response, suggesting that CMI could be sufficient for protection from HCV persistence [239, 240]. Control of the reinfection was temporally and kinetically linked to the appearance of HCV-specific CD8 T cells with cytolytic activity in the liver and blood. This was associated with the expansion of memory T-helper cells [90, 238]. IFN-γ producing CD4 and CD8 T cells were detected at a higher frequency and as early as day fourteen into the second infection compared to eight weeks into the primary infection [340-343]. A recent study demonstrated that control upon HCV challenge following vaccination was associated with CD127+ $PD1^{lo}$ CD8+ T cells that persisted at high levels for long times and were bifunctional (IFNγ+ TNFα+) [344]. Another

recent study showed that clearance of HCV reinfection upon heterologous rechallenge depends on the activation of both intrahepatic innate and adaptive immune responses (NKs and CD8 markers, respectively), as well as enhanced production of IFNs type I and type II, and elevated ISGs [338]. The protective role of memory T cells was confirmed by using antibody-mediated depletion of either CD4 or CD8 T cells. CD4 T-cell depletion was associated with low level viremia, but memory CD8 T cells partially controlled viremia [238], whereas memory CD8 T cell depletion led to a significant delay in control of viremia, and this control coincided with the re-detection of the cells [239].

Together, these data demonstrate a protective role for memory T cells upon repeated HCV challenge in chimpanzees without correlating with the development of nAbs, and show that it mainly reduces the level of viremia and the duration of infection (protective immunity) rather than conferring sterilizing immunity [224, 238, 239, 338, 341].

Nevertheless, these findings need to be interpreted cautiously. Despite being the closest primate to humans, there are several discrepancies between chimpanzees and humans in some gene clusters, some of which are involved in the immune response [345]. It has indeed been shown that chimpanzees respond differently from humans to chronic HCV infection in terms of pathology, disease progression and kinetics of viral clearance. In addition, the conditions for chimpanzee experiments are much more controlled, where the chimpanzees are maintained in a restricted environment and the viral sequences used for the challenging are laboratory reference sequences representing a single clone from one genotype [346]. In addition, the number of chimpanzees available for the studies is very limited. Medical Research on chimpanzees is no longer possible except in very limited situations [347].

Human studies also demonstrated the presence of HCV-specific memory was also demonstrated in humans. A group of women infected with HCV from a common source were followed for several years after the initial infection. It was shown that spontaneous resolvers possessed HCV-specific CD4 and CD8 T_{mem} that could be detected in their peripheral blood 20 years post-clearance of the virus, whereas antibodies were barely detectable [348]. Phenotypic characterization of HCV-specific T cells in spontaneously resolved cases showed they were expressing the lymphoid homing marker CCR7 and CD45RO, both characteristics of memory T cells [73]. Our group and others previously demonstrated that spontaneous

resolution from a primary HCV infection was associated with the generation of an HCV-specific memory population with a $CD127^{hi}$ $Bcl2^{hi}$ profile [251, 258, 259]. The increased expression of both markers has been correlated with the generation of long-lived memory CD8 T cells [37].

There is a lack of thorough studies dissecting the determinants of a protective immune response upon re-exposure to HCV. Nevertheless, some studies shed light on the rates of reinfection in populations at risk and on the possible role of a T_{mem} immune response in the protection against persistence upon HCV reinfection. Epidemiological studies in populations at high risk of getting infected with HCV, especially IDUs, observed that the incidence of reinfection in patients who have cleared a primary HCV infection is less than those observed for infection in naïve patients belonging to the same cohorts under the same risk factors [118, 119, 349]. Unfortunately, there is a great discrepancy in the results of the epidemiological studies, since other studies showed that the incidence rates were not significantly different or were even higher in spontaneous resolvers of a previous HCV infection [350-352]. One of the major factors causing this discrepancy is the variation in the testing intervals for HCV-RNA to determine the frequency of occurrence of infection or reinfection. Other factors include differences in the characteristics of individual subjects, as age, ethnicity and risk behaviours [353]. Nevertheless, all studies that measured clearance rate in both groups showed that the rate of clearance of reinfection was higher than that for primary infection [118, 351, 354]. This could be due to the fact that individuals who spontaneously clear HCV acute infection generate a robust T_{mem} population and it was already demonstrated that resolution of reinfection correlated with T-cell responses [355, 356]. This provided proof-of-concept that the human immune system is capable of conferring protective immunity against HCV.

What we know about the signature of a protective immune response is far less defined than our knowledge about successful responses against primary HCV infection, especially in terms of the phenotype and functionality of HCV-specific populations that might be protective upon HCV infection in humans. Some general features of the HCV protective response were drawn from the few cohorts that have been established in the last few years with the potential of having enough patients to study the characteristics of a protective immune response [357]. Albeit, it remains challenging to gather enough samples longitudinally from patients with

differential outcomes of reinfection to thoroughly dissect the correlates of protection. The most elaborate human study, to date, was performed by Osburn *et al.* [354]. Using enzyme-linked immunospot (ELISPOT) technique this study showed that spontaneous clearance of reinfection is associated with broadened T-cell responses. It also showed the generation of cross-reactive humoral responses in patients who spontaneously resolved the second infection episode. However, phenotypic and polyfunctional characterisation was not performed. Another limitation to this study is that 10/11 subjects examined resolved the second infection, thus a group of chronic patients served as the control group. To date, there are no studies defining the exact correlates of protection against HCV infection.

1.5.1. HCV vaccine trials

HCV has many attributes that makes it a candidate for eradication through prophylactic vaccination; it is a blood-borne infection (much easier to control than pathogens transmitted by aerosol or food), it has no natural non-human reservoir, it can be cleared spontaneously by the immune system and people who clear a primary infection have a higher clearance rate (i.e. more protected) upon re-exposure [358].

An effective HCV vaccine would have a great impact on reducing the future disease burden worldwide, since it would break the transmission cycle in endemic developing countries where public health efforts have failed over decades to reduce HCV transmission [359]. Also, new HCV treatments have a very high cost and lower efficacy against genotypes other than genotype 1, thus they are not expected to have an impact on the disease burden worldwide [360].

The plethora of data suggesting that CMI is the major arm clearing primary HCV infection led to the assumption that it should be the target for vaccine development. Nevertheless, the unclear role for Abs in resolution of primary infection does not rule out a possible contribution of humoral immunity in protective immunity.

There are major challenges and biological barriers facing the development of HCV vaccines. The first is the genetic diversity of HCV sequences and its high ability to undergo mutations. A successful prophylactic vaccine would need to induce an effective immune response capable of surmounting this high mutational capacity. Second, the ability of HCV

proteins to distort the different arms of the immune system necessitates the design of an effective vaccine that generates memory immune cells capable of mounting a prompt recall response upon re-exposure in a fashion that outpaces the virus and prohibits it from producing enough viral proteins that are able to antagonise the immune system.

All present vaccine trials could be considered empirical, since the exact correlates that need to be induced and subsequently measured to verify the efficacy of protection conferred by candidate vaccines remain unknown. Three major approaches have been adopted for vaccine design against HCV. The traditional approach uses recombinant envelope proteins to induce nAbs. It has gained more potential recently with the discovery of broadly nAbs generated in chronic patients against HCV. Vaccination with recombinant E1/E2 proteins was shown to induce cross-reactive nAbs that might contribute to protective immunity upon exposure to HCV [361-363]. The second approach uses virus-like particles (VLPs) that express HCV structural proteins to try to induce both humoral immunity and CMI, but it mainly induced CMI and did not provide protection upon homologous rechallenge except in two out of four chimpanzees [364]. The third and most promising approach is designing an HCV vaccine that would induce a potent T-cell immune response. Replication-defective recombinant viral vectors were used to deliver HCV Ags to induce T-cell responses [e.g. adenovirus (Ad), vaccinia virus (VV), modified vaccinia Ankara (MVA)]. HCV proteins could also be delivered using DNA vaccines, where recombinant plasmids expressing various proteins could be injected into the host. The two candidate vaccines that showed promising preliminary results and are at an advanced stage of development are the vaccines developed by Okairos (Rome, Italy) [acquired by Glaxo-Smith-Kline Inc.] using viral vectors for priming and viral vectors or DNA vaccines for boosting [365].

The first vaccine uses heterologous prime/boost regimens with chimpanzee adenovirus Ad3Ch3 and a rare strain of human adenovirus (Ad6) expressing the entire non-structural region of genotype 1b BK strain (NS3-5B). It has already been tested in a phase I clinical trial in humans (ClinicalTrials.gov NCT01436357). Both vectors primed broad CD4 and CD8 T cell responses that were capable of responding to heterologous strains of HCV genotypes 1a and 3a. Polyfunctional HCV-specific T cells (IL-2+ IFNγ+ TNFα+) could be sustained for at least a year after boosting with the heterologous vector [366]. The second vaccine uses a

regimen of priming with Ad6 encoding NS3-5B of genotype 1b BK strain and boosting with NS3-5B-encoding plasmid DNA. A study in chimpanzees that received this vaccine then challenged with HCV demonstrated that control upon HCV challenge following vaccination was associated with CD127+ PD1^{lo} CD8+ T cells that persisted at high levels for long times and were bifunctional (IFNγ+ TNFα+). Nevertheless, three out of five chimpanzees were not protected [344].

An important lesson learned from HIV vaccine trials is that the presence of a memory immune response did not necessarily correlate with the clinical protection upon reexposure [107, 108]. To date, the lack of a clear definition of the signatures that would constitute a protective immune response upon exposure to HIV hinders the rational design of an effective vaccine. The same limitation faces HCV vaccine trials, since the correlates of protective immunity against HCV need to be clearly defined first, so that they could be monitored to verify the degree of protection conferred by vaccines in humans.

CHAPTER 2:

HYPOTHESIS AND OBJECTIVES

As with many chronic viral infections, the development of an effective prophylactic vaccine is hindered by the lack of knowledge about the determinants of a protective memory immune response in humans. HCV represents a unique model to study protective immunity in chronic viruses upon natural exposure, since ~30% of the patients are able to clear the primary infection spontaneously. The objective of this thesis is to study protective immunity against HCV in a cohort of IDUs who cleared a primary HCV infection and are continuously at risk of reinfection. We performed an exhaustive longitudinal analysis at time points before, during and after the HCV reinfection episode to dissect the general characteristics of HCV-specific T cells.

The presence of HCV-specific memory T cells has already been demonstrated in both chimpanzees and humans [341, 348]. Evidence from rechallenge studies in chimpanzees suggest that this memory response might be protective against HCV upon re-exposure, since these studies showed that chimpanzees spontaneously clearing a primary infection had a higher level of protection than naïve ones when rechallenged with homologous viral sequences. This was accompanied by a shorter duration of viremia and lower viral loads in the cases that cleared the second infection [337, 338]. However, other studies did not observe protection even against homologous rechallenge [339]. A protective role for memory T cells was confirmed by using antibody-mediated depletion of either CD4 or CD8 T cells [238, 239]. In humans, the majority of studies in populations at risk of HCV infection and reinfection (e.g. IDUs) showed that the incidence of reinfection in patients who have cleared a primary HCV infection is less than those observed for infection in naïve patients belonging to the same cohorts under the same risk factors. All the studies that measured the clearance rate in both groups showed that the clearance of reinfection was higher than that for primary infection, supporting the notion that the memory immune response is protective in most cases [118, 351, 354]. It is necessary to understand why individuals who spontaneously resolved a primary HCV infection would not be able to clear a second infection episode, versus others who successfully clear consecutive episodes of infection. To date, there is a lack of thorough studies dissecting the determinants of a protective immune response upon re-exposure to HCV or defining the correlates of protection against chronic viruses in humans.

Our hypothesis is that most subjects who spontaneously resolved a primary HCV infection would be protected against chronicity of infection upon re-exposure, thus achieving protective immunity rather than sterilizing immunity (i.e. the ability to clear HCV rapidly rather than not developing an infection at all). Protection against reinfection would likely be associated with enhanced breadth, frequency, polyfunctionality and functional avidity of HCV-specific CD4 and CD8 memory T cells. HCV-specific memory T cells associated with protection would likely be distinguished by a characteristic phenotype similar to that observed upon spontaneous resolution of primary HCV infection. The protective capacity of memory T cells upon reinfection will depend on possessing a T-cell repertoire characterized by high diversity of its clonotypic composition and flexibility enabling the generation of *de novo* T cell responses against different viral variants. The efficacy of this memory immune response would be –at least partially– dependent on the degree of homology of the sequences of the HCV-genome in the different episodes. On the other hand, subjects who fail to clear HCV reinfection (despite clearing a primary infection) would suffer from defects in the generation of an effective memory T-cell population capable of mounting a recall response upon reinfection, especially against different viral variants.

We were able to recruit nine patients who resolved a primary HCV infection, but had a differential outcome of the second infection, and for whom longitudinal blood samples during the multiple episodes of HCV-infection were available. This represented a great advantage by allowing us to compare the different characteristics of the immune response under the same conditions. This rare cohort enabled us to perform an exhaustive longitudinal phenotypic and functional characterization of the HCV-specific memory immune responses, and also enabled us to analyse the dynamics of epitope-specific CD8 T-cell repertoire upon reinfection with different viral variants and correlate these dynamics with their avidity. More importantly, examining two groups of patients with differential outcomes of reinfection enabled us to determine which characteristics of the recall response were specifically associated with protection. Despite the limited number of patients (a challenge common when working with such cohorts), but still this study would provide primary insight for the first time into the characteristics of a protective immune response against HCV in a real-life infection setting. This would provide a road-map for the following steps needed to fully understand protective immunity against HCV at the cellular, molecular and clonotypic levels.

We had two specific aims for the project; the first aim was to define and dissect the general characteristics HCV-specific T cells that confer protective immunity upon reinfection. The second aim was to analyse the dynamics of the TCR repertoire of HCV-specific CD8 T-cell in protected versus unprotected patients and in relation to infection with variant viral sequences.

For the first specific aim we intended to **define the correlation between protective immunity upon HCV reinfection and several characteristics of the adaptive immune response.** These characteristics included; 1) the total magnitude and breadth of T-cell responses upon stimulation with peptide pools representing the entire HCV genome, 2) the proliferative capacity, functionality and polyfunctionality of CD4 and CD8 T-cells upon stimulation with immunodominant peptide pools and minimum peptides to which the patient possessed specific tetramer+ CD8+ T cells, 3) the frequency and phenotype of tetramer+ CD8+ T cells and 4) the effect of variation in the viral sequences of the targeted epitopes on the generation of an effective recall response.

For the second specific aim we intended to **define the dynamics of the clonotypic composition of the TCR-repertoire of HCV-specific CD8 T cells associated with protective immunity upon HCV reinfection.** We examined the correlation between protective immunity upon HCV reinfection and several characteristics of the TCR repertoire. These characteristics included; 1) the diversity of the repertoire composition, 2) role of specific clonotypes in protective immunity and 3) the recruitment of new clonotypes that were not mobilized during the primary infection. We also examined the dynamics of the CD8 T-cell repertoire and its functional avidity and polyfunctionality in response to different variants of targeted epitopes.

CHAPTER 3:

MANUSCRIPT 1:

Signatures of Protective Memory Immune Responses during HCV Reinfection

(*Gastroeneterology*, 2014)

Signatures of Protective Memory Immune Responses during HCV Reinfection

Short Title: Protective Immunity during HCV reinfection

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Abbreviations used in this paper: ELISPOT: Enzyme-linked immunospot assay; HCV: Hepatitis C virus; ICS: Intracellular cytokine staining; IDUs: Injection drug users; p.ri: Post reinfection; SFC: spot forming cell; TNFα: Tumor necrosis factor alpha

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ABSTRACT

Background & Aims: Vaccine development against hepatitis C virus (HCV) is hindered by our limited understanding of immune correlates of protection during real life exposures. Our aim was to define such correlates during HCV reinfection.

Methods: We monitored the phenotypic and functional dynamics of HCV-specific T cell memory responses in HCV-resolved injection drug users (IDUs) during natural re-exposure and re-infection.

Results: Individuals who spontaneously resolved two successive infections (n=5), demonstrated expansion of HCV-specific polyfunctional memory CD4 and CD8 T cells. We detected CD127^{hi} HCV-specific memory CD8 T cells prior to reinfection regardless of the ability to clear the subsequent infections. Protection against viral persistence was associated with the expansion of a CD127^{neg}, PD-1^{lo} effector memory T cells at the peak of the response. In addition, we observed broadening of the T cell response suggesting generation of *de novo* T cell responses. Individuals who failed to clear their subsequent infection (n=4) demonstrated limited expansion of HCV-specific CD4 and CD8 memory T cells and variable levels of expression of the exhaustion marker PD-1 on HCV-specific CD8 T cells. Finally, viral sequencing of dominant HCV epitope regions from patients who developed viral persistence upon reinfection demonstrated variations in the infecting viral sequence that were not recognized by the pre-existing memory T cells.

Conclusions: Our results suggest that protection from persistence upon HCV reinfection is dependent on the magnitude, breadth and quality of the HCV-specific memory T cell response. The sequence homology and cross recognition between the infecting viral strains remain critical determinants of protective memory.

Keywords: hepatitis C virus; reinfection; protective immunity

INTRODUCTION

Despite the recent introduction of potent antivirals against HCV, there is an urgent need for an effective prophylactic vaccine. A first step is defining correlates of protective immunity during real life exposure among high risk populations like IDUs[1]. CD4 and CD8 HCV-specific T cell responses are induced during acute HCV and mediate spontaneous resolution. An effective response leading to viral clearance is typically of high magnitude, broad, polyfunctional (i.e. producing more than one cytokine or function) and sustained[2]. Individuals who spontaneously resolve acute HCV develop long-lived memory T cells[3, 4]. Chimpanzees who have resolved one HCV infection were protected from chronic infection upon re-exposure but protection was less efficient upon heterologous viral re-challenge (reviewed in [5]). Accelerated viral clearance was associated with rapid recall of memory T cell responses[4] and CD4 T cell help was critical to maintain an efficient memory T cell response[6]. In humans, high risk IDUs who have already resolved one HCV infection were less likely to be re-infected than HCV-naïve individuals[1, 7]. Osburn et al. demonstrated that reinfections were characterized by reduced peak and duration of viremia as compared to primary infection and associated with broadened cellular immune responses that facilitated viral clearance[8]. However, the detailed phenotype and function of HCV-specific T cells during reinfection in a real-life exposure setting remain poorly defined.

Here, we examined longitudinally the breadth, phenotype and effector functions of the HCV-specific memory T cell response and variations in viral sequence during HCV reinfection in a group of IDUs who have previously resolved a primary HCV infection.

MATERIALS AND METHODS

HCV RNA, Genotype and HCV-Antibody Testing

Qualitative HCV-RNA was tested using COBAS Ampliprep/COBAS Amplicor HCV Test, version 2.0 (Roche Molecular Systems, Branchburg, NJ) (). HCV genotyping was performed as previously described[9]. Anti-HCV antibodies were assessed by the AxSym HCV Assay (Abbott GMBH & CO, K.G.).

Peptides and HLA Class I Tetramers

Peptides were synthesized by Sheldon Biotechnology Centre, McGill University (Montreal, QC, Canada). MHC class I tetramers were synthesized by the National Immune Monitoring Laboratory (NIML), (Montréal, QC, Canada) or the NIH Tetramer Core Facility (Emory University, Atlanta, GA) and are as follows: HLA-A1 restricted HCV NS3 peptide amino acids (aa) 1436–1444 (ATDALMTGY) [A1/NS3-1436], HLA-A2 restricted HCV NS3 peptide aa 1073–1081 (CINGVCWTV) [A2/NS3-1073], HLA-B27 restricted HCV peptide NS5B peptide aa 2841-2849 (ARMILMTHF) [B27/NS5B-2841]..

Flow cytometry based assays

All assays were performed on frozen PBMCs using a standard BD LSR II instrument with FACSDiva software version 6.1.3 (BD Biosciences). Data files were analyzed using FlowJo software version 9.5 for Mac (Tree Star, Inc., Ashland, OR). Tetramer staining coupled with phenotypic analysis, intracellular cytokine staining (ICS) and CD107a degranulation assay were performed as previously described[10] in response to HCV peptide pools (1 μg/ml) or HCV minimum peptide (10 μg/ml). Polyfunctionality was assessed by exporting flow cytometry standard (FCS) data as Boolean gates using FlowJo and SPICE softwares[11]. Carboxyfluorescein succinimidyl ester (CFSE) 6 days proliferation assays were performed as previously described[10] with or without HCV minimum peptide or peptide pool.

HCV Epitope Sequencing

HCV RNA was extracted from EDTA plasma using AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, Union City, CA), reverse transcribed, PCR amplified, cloned and

sequenced as previously described[10] at the Molecular Biology and Functional Genomics service of the Institut de Recherches Cliniques de Montréal (Montreal, QC).

RESULTS

Identification of HCV reinfection cases

This study was conducted among participants of the Montreal Acute Hep C IDU Cohort Study (HEPCO)[12] and approved by the institutional ethics committee (Protocol SL05.014). Primary HCV infection was identified in cohort participants who were initially negative for both HCV RNA and anti-HCV antibodies for at least 6 months of follow-up, then had a positive HCV RNA and/or antibody test as previously described[10, 13]. Participants who have resolved their primary HCV infection or participants who tested HCV RNA negative and HCV antibody positive at recruitment were enrolled in the reinfection study and followed every 3 months. HCV reinfection was defined by an HCV-RNA positive test following two negative tests, at least 60 days apart. The day of the first positive RNA test was defined as day zero post detection of reinfection (p.ri.). This study includes nine cases of reinfection identified between 2009 and 2012 for whom clinical data documenting their primary infection and longitudinal blood samples during the reinfection episode were available. Samples before the reinfection episode were available for 6 patients. Samples from time points during or right after clearance of the primary infection were available for 4 patients. Five patients spontaneously resolved their second infection while four patients became chronically infected, referred to hereafter as the SR/SR group and the SR/CI group, respectively. Patients' demographics, clinical characteristics and infection history are listed in Supplementary Table S1.

Spontaneous resolution of HCV reinfection is associated with an increase in the magnitude and breadth of the HCV-specific T cells

We examined the magnitude and breadth of the HCV-specific T cells using an IFNγ enzyme-linked immunospot (ELISPOT) assay against overlapping peptide pools representing the HCV genotype 1a H77 polyprotein. We chose this reference sequence for the preliminary screening since all second infections were of genotype 1 and subtype 1a is the most common subtype in our cohort. The genotype of the primary infecting HCV viral strain could not be determined in many subjects because of rapid viral clearance, low viral loads or unavailability of samples. Wherever samples were available, we monitored the ELISPOT response before and during the second infection in the SR/SR (Figure 1A) and SR/CI (Figure 1B) patients. The

magnitude of the HCV-specific T cell response during reinfection was higher in the SR/SR group as compared to the SR/CI group. The average frequency at the earliest time point tested following reinfection (Mean: 8 weeks) was 7440 spot forming cells (SFC)/million PBMCs in the SR/SR group versus 1760 SFC/million PBMCs in the SR/CI group. The response to pools representing the structural and non-structural regions of HCV was higher for the SR/SR group (Mean: 1470 and 5970 SFC/million PBMCs, respectively) as compared to the SR/CI group (Mean: 160 and 1600 SFC/million PBMCs) (Figure 1C). The breadth of the immune response, measured by the number of peptide pools targeted by the immune response, was also higher in the SR/SR group at the earliest time point during reinfection. An average of 10 (out of 11) peptide pools were targeted in the SR/SR group, versus 7 pools for the SR/CI group (Figures 1A, 1B and data not shown).

Expansion of the HCV-specific memory T cells was associated with viral clearance upon reinfection in the SR/SR group. One exception was patient SR/SR-3, who successfully cleared his reinfection within 4 weeks despite no change in the magnitude and breadth of the response against the HCV genotype 1a peptides (Supplementary Figure S1A). Since the infecting subtype could not be determined, we tested his response to a panel of peptides corresponding to genotype 1b J4 reference sequence and observed an increase in the overall frequency and breadth of HCV-specific T cell responses upon reinfection (Supplementary Figure S1B). We also retested patients where the reinfection subtype was undetermined using genotype 1b peptides (Supplementary Figure S1 C, D and E) and demonstrated that the response was slightly higher against the 1a peptide panel as compared to 1b at the same time points suggesting that they were probably infected with a genotype 1a virus.

Spontaneous resolution of HCV reinfection is associated with enhanced proliferation of HCV-specific CD4 and CD8 T cells

Next, we examined the proliferative capacity of HCV-specific CD4 and CD8 T cells in a CFSE dilution assay against peptide pools corresponding to the immunodominant regions of HCV (NS3 and NS5B) and/or the peptide pools showing the highest response in ELISPOT. Representative FACS data are presented in Supplementary Figure S2. Proliferation data for CD4 and CD8 T cells from two patients of each group are presented as stimulation index (SI) in Figure 2. Patient SR/SR-1 for whom samples prior to reinfection were available

demonstrated high proliferation of CD4 T cells against the peptide pools tested (NS3-1 and NS5B-1) at baseline (Figure 2A). This proliferative capacity increased 3 folds at 6 weeks p.ri. Similarly, patient SR/SR-4 demonstrated some CD4 T cell proliferation at week 6 p.ri. against several pools that increased 3-5 fold by 32 weeks p.ri. (Figure 2A). In contrast, we observed very limited increase in CD4 T cell proliferation for patient SR/CI-1 and the existing proliferative response became undetectable by week 57 p.ri and patient SR/CI-3 demonstrated very limited proliferation (Figure 2B).

For CD8 T cell proliferation, patient SR/SR-1 demonstrated high proliferative response prior to reinfection that increased to an SI of 560 in response to NS3 pool 1 at 6 weeks p.ri. (Figure 2C). In patient SR/SR-4 very high levels of proliferation were observed at 6 weeks against all pools tested and although they partially declined at week 14, they recovered and were sustained at week 51 p.ri. (Figure 2C). In contrast, no CD8 proliferative responses were detected in the two SR/CI patients against the peptide pools tested (Figure 2D). In summary, spontaneous resolution of second HCV infection was associated with an increase in HCV-specific CD4 and CD8 T cell proliferative responses.

Spontaneous resolution of HCV reinfection is associated with enhanced cytokine production by HCV-specific T cells

Polyfunctional CD8 T cells producing several effector cytokines and molecules are associated with spontaneous resolution of primary acute HCV[10]. We thus examined the polyfunctionality of HCV-specific T cells longitudinally during reinfection in two SR/SR and two SR/CI patients against peptide pools corresponding to the immunodominant regions of HCV and/or pools showing the highest response in ELISPOT. We assessed the production of the antiviral cytokines IFNγ, tumor necrosis factor alpha (TNFα) and the T cell growth factor IL-2 for CD4 and CD8 T cells. For CD8 T cells we also monitored the expression of the degranulation marker CD107a. Representative FACS plots are presented in Supplementary Figure S3.

The production of the individual cytokines by CD4 T cells was higher in the two SR/SR patients (Figures 3A and 3B) when compared to the SR/CI patients (Figures 3C and 3D). IFNγ was the major cytokine produced by $CD3^+CD4^+$ T cells from the SR/SR patients

reaching up to 3.33% followed by TNFα reaching up to 2.13% (Figures 3A and 3B). In contrast, production of individual cytokines was weak in the two patients from the SR/CI group reaching only 0.69% of $CD3^{+}CD4^{+}$ T cells (Figures 3C and 3D). When polyfunctionality was examined, we observed that 6-33% of the total cytokine producing cells in patient SR/SR-1 produced more than one function at different stages of reinfection as compared to 3-21% in patient SR/CI-2. Cytokines producing cells were strictly monofunctional, producing primarily IFNγ in patient SR/SR-4. In patient SR/CI-4, although 50% of cytokine producing cells produced two cytokines at 23 weeks p.ri, the low frequency of cytokine producing cells may have biased the results. These results suggest an association between the level of individual cytokine production (IFNγ or TNFα) by HCV-specific CD4 T cells rather than polyfunctionality with the protection from chronicity upon reinfection.

HCV-specific CD8 T cells from the SR/SR patients also expressed higher levels of the individual cytokines and CD107a as compared to SR/CI patients (Figure 4). IFNγ was the major cytokine produced by $CD3^{+}CD8^{+}$ T cells from the SR/SR patients reaching up to 5.22% followed by TNFα and the degranulation marker CD107a (Figures 4A and 4B). Individual cytokine production or CD107a expression was weak in the SR/CI group with a maximal production of 0.49% of $CD3^{+}CD8^{+}$ T cells (Figures 4C and 4D). Polyfunctionality was also higher in the SR/SR group, where we observed that ~6% of the total functional cells in the two SR/SR patients produced three functions at different stages of reinfection as compared to none in the two SR/CI patients. Furthermore, 15-52% of total cytokine/CD107a expressing cells expressed two functions in the SR/SR patients as compared to none in the SR/CI group (except the latest time point in patient SR/CI-4 where the overall frequency of functional cells was 0.2%) (Figure 4). In summary, the frequency of individual cytokine producing CD8 T cells was higher in the SR/SR group and within these functional cells the frequency of polyfunctional cells was higher. These results suggest an association between the level of individual cytokine production and polyfunctionality of HCV-specific CD8 T cells and protection from viral persistence upon reinfection.

Spontaneous resolution of HCV reinfection is associated with higher polyfunctionality of HCV-specific CD8 T cells targeting minimal epitopes

Next, we evaluated polyfunctionality of HCV-specific CD8 T cells in response to predicted minimal epitopes restricted by HLA-A2 and showing the highest response (NS5B-2594 and NS3-1073) in two patients from the SR/SR and two patients from the SR/CI group. The production of individual cytokines and/or expression of CD107a was higher in the SR/SR group when the cells were stimulated with the minimum cognate peptide reaching 3.58% (mean 0.54%) of the total $CD3^{+}CD8^{+}$ T cells (Figures 5A and 5B). In contrast, individual cytokine production and/or CD107a expression in the SR/CI group never exceeded 0.22% (mean 0.06%) (Figures 5C and 5D). The frequency of CD3+CD8+ T cells expressing two or three functions was 14% and 7%, respectively of total CD3+CD8+ functional T cells in the SR/SR patients (Figures 5A and 5B) and almost none in the SR/CI patients (Figures 5C and 5D). Similar to previous results using peptide pools, these data suggest an association between the level of individual cytokine production and polyfunctionality of HCV-specific CD8+ T cells targeting dominant epitopes with the protection from persistent viremia upon reinfection with HCV.

Spontaneous Resolution of HCV reinfection is associated with expansion of $CD127^{lo}$ HCV tetramer+ CD8 T cells

Next, we performed longitudinal phenotypic analysis of HCV-specific CD8 T cells to assess their frequency, differentiation and exhaustion status using the predicted MHC class I tetramers detailed in Supplementary Table S1. We monitored markers of T cell maturation and memory (CD27 and CD127) and T-cell activation/exhaustion (PD1, 2B4 and Tim3) on tetramer+ CD8 T cells. Representative FACS results for patient SR/SR-1, using the A2/NS3-1073 tetramer are shown in Figure 6A and Supplementary Figure S4A, and for A2/NS5B-2594 tetramer in Supplementary Figure S5. Representative data for patient SR/CI-1, using the A1/NS3-1436 tetramer, are shown in Figure 6B and Supplementary Figure S4B. Tetramer frequency data during reinfection in all subjects are represented in Figure 6C. We observed an average expansion of 42 folds at the peak of response (<6 weeks p.ri.) in patients of the SR/SR group (Figure 6C). The only exception was patient SR/SR-3, where the reinfecting HCV genotype was likely 1b rather than the 1a sequence used in the tetramer (Supplementary Figure S1A). Very limited expansion was observed in the tetramer+ population upon reinfection in all patients of the SR/CI group (Figure 6C).

The most distinctive phenotypic change was expression of CD127. In one SR/SR and two SR/CI patients where samples were available during the first infection, we observed that approximately half of the HCV tetramer+ CD8 T cells were CD127^{hi}. As patients cleared their primary HCV infection, the majority of tetramer+ CD8 T cells became CD127^{hi} as previously described[10] and consistent with a memory T cell phenotype[14]. A similar phenotype was observed for the tested time points prior to reinfection. At the earliest time point tested during reinfection we observed peak expansion of tetramer+ cells in SR/SR patients and the majority of these expanded cells were CD127^{lo}, consistent with an effector phenotype (Figures 6A and 6D). In the SR/CI patients, tetramer+ cells did not expand or undergo significant changes in the CD127 expression (Figures 6B and 6D). There was no significant variation in the expression of the other markers examined (CD27, Tim3 or 2B4) at the different time points tested for the two groups of patients (Supplementary Figure S4). Finally, We observed higher expression of the activation marker PD1 on HCV tetramer+ CD8+ T cells in most patients (3/4) of the SR/CI group in comparison to SR/SR patients (Figures 6B and 6E). In summary, spontaneous resolution of a second HCV infection was associated with expansion of pre-existing memory T cells into CD127^{lo} effectors and lower levels of PD1.

In patient SR/SR-1 who was positive to two tetramers A2/NS3-1073 and A2/NS5B-2594, we observed different levels of expansion of CD8 T cells specific to the two tetramers. The frequency of the A2/NS5B-2594 tetramer+ T cells was at a much lower level than that for NS3-1073 prior to reinfection (0.082% and 0.458%, respectively) (Figure 5A and Supplementary Figure S5), yet the A2/NS5B-2594 tetramer+ population expanded 120 folds upon reinfection (Supplementary Figure S5) compared to 7 folds only for the A2/NS3-1073 tetramer+ population (Figure 5A) (9.84% and 3.37%, respectively). This suggests a shift in the dominance of the epitopes targeted in this patient during reinfection.

Heterogeneity in targeted epitopes and reduced recognition during reinfection in the SR/CI group

Despite the limited expansion of HCV-tetramer+ CD8 T cells in patients of the SR/CI group upon reinfection, they proliferated in response to their cognate epitope in an *in vitro* CFSE proliferation assay (data not shown) suggesting that they are not inherently defective in proliferation. To determine whether this lack of expansion in the SR/CI patients is due to

reinfection with variant viral sequences that are not recognized by the pre-existing memory T cells, and to determine whether the change existed since the beginning or arose during reinfection, we sequenced the NS3-1436 and NS3-1073 epitopes targeted by the four patients at the earliest and the latest time points available during reinfection (Figure 7A). We detected a mismatch between the amino acid sequence of the reference peptide used in the tetramer and the autologous HLA-A1 restricted NS3-1436 epitope in patients SR/CI-1 and SR/CI-3 at the early and late time points. This ^{1444}Y → F substitution was previously shown to be recognized less efficiently by specific T cells[15]. An amino acid substitution ^{1077}V → A was observed in the NS3-1073 for patients SR/CI-2 and SR/CI-4 at the early time point. This variant was also previously suggested to be less efficient in inducing CD8 T cell expansion and IFNγ production[16, 17]. This variant remained fixed in patient SR/CI-4 at the late time point but underwent three additional changes in patient SR/CI-2: ^{1077}V → A; ^{1081}V → I and ^{1078}W→ L substitution.

To determine how well the pre-existing memory T cells recognized the reference versus the autologous sequences, we tested their functional avidity by measuring dose-dependent production of IFNγ in response to stimulation by the different epitope variants during the reinfection episode in SR/SR and SR/CI patients. We observed higher functional avidity of HCV-specific T cells from SR/SR patients in response to the NS3-1073 reference epitope sequence when compared to SR/CI patients (Figure 7B). Patient SR/SR-4 had higher functional avidity than patient SR/SR-1 in response to the reference sequence and the response was >10-folds than patient SR/CI-2. Patient SR/CI-4 poorly recognized all variants. Interestingly, Patient SR/SR-4 also responded to the intermediate variant sequence of the NS3-1073 epitope indicating better cross-recognition of this variant. The reference sequence of the NS3-1436 was also better recognized than the autologous variant by patients SR/CI-1 and SR/CI-4. Altogether, these results suggest that variations in the targeted epitope could be a possible mechanism underlying the functional differences or lack of recognition in HCV-specific CD8 T cells observed between the two groups of patients.

DISCUSSION

We have performed an exhaustive phenotypic and functional characterization of the virus-specific T cell response during HCV reinfection. Although all nine patients in this study had successfully eliminated an earlier HCV infection, only five of them were able to spontaneously resolve their subsequent infection. Spontaneous resolution of reinfection was associated with an increase in both the magnitude and breadth of the total HCV-specific T cell response suggesting generation of *de novo* T cells responses. In addition, we observed expansion of HCV-specific memory T cells and the transient appearance of $CD127^{low}$ population indicative of an effector T cell phenotype. Patients who failed to clear their subsequent infection showed very limited expansion of HCV-specific T cells. Variations in the autologous sequence of the targeted epitopes were detected in all patients of this group and not recognized by the pre-existing memory T cells.

Protection from viral persistence upon HCV reinfection was associated with an increase in the magnitude and breadth of the HCV-specific T-cell response as observed by ELISPOT assays and tetramers consistent with the observations of Osburn *et al.* [8]. For the SR/SR group the increase in the magnitude and the *de novo* responses mostly targeted the non-structural proteins underscoring the immune dominance of this region[18, 19]. Furthermore, in patient SR/SR-1, we observed a shift in dominance between the two epitopes targeted. Further research with additional patients is required to elucidate whether shifting dominance may occur upon reinfection with variant viral strains or may reflect differential expansion capacity of memory T cells.

Proliferation of CD4 and CD8 T cells is predictive of the outcome of primary HCV infection[20, 21]. We demonstrate that protective immunity upon reinfection was associated with higher proliferative capacity for both CD4 and CD8 T cells when stimulated with peptide pools representing immunodominant regions. Nevertheless, proliferation assays using cognate epitopes demonstrated that HCV tetramer+ memory CD8 T cells in the SR/CI unprotected group proliferated to similar levels as those of the SR/SR protected group (data not shown). This suggests they are not inherently defective, and despite being present at low frequency, they could expand if stimulated with the right peptide or antigen.

Spontaneous resolution of primary acute HCV and response to vaccination were associated with generation of polyfunctional HCV-specific CD127+ CD8 T cells[10, 22]. Here,

we demonstrate that polyfunctionality is also an important determinant of viral clearance upon reinfection. Furthermore, we extend this polyfunctionality to CD8 T cells targeting entire regions of HCV, as well as, minimal epitopes. Data from the HIV model suggest that such polyfunctional T cells are cells of the highest functional avidity[23]. It is also tempting to speculate that repeated reinfections with the same viral variant would selectively expand and enrich such cells. On the other hand, rechallenge with a slightly different antigenic variant may favor expansion of T cells carrying a flexible T cell receptor that can recognize these new variants. Flexibility in the T cell repertoire correlated with control of viral escape mutants during HCV and HIV infections[24-26] and would require further investigation in the reinfection setting.

Our results suggest that pre-existing variations in the viral sequence of the epitopes targeted by the memory immune response could be a possible reason for persistence upon reinfection. We demonstrated higher functional avidity in response to the reference sequence of the epitope in the SR/SR group. In addition, three SR/CI patients had higher response to the reference sequence than the autologous sequence. No mutations were observed during the course of the reinfection, except for the NS3-1073 epitope in patient SR/CI-2 where the epitope underwent three changes during the reinfection episode, suggesting that it may have been under immune selection pressure. The fact that this intermediate variant was recognized by patient SR/SR-4 suggests that this variant can be recognized by some individuals. However, those changes in the targeted epitopes do not explain the general decrease in the frequency of the immune response observed in ELISPOT analysis. Thus, the association between the magnitude of the T cells and the degree of homology in the entire HCV genome between the infecting viruses in both episodes of infection remains to be assessed.

The capacity to detect HCV reinfection is dependent on the testing interval, so we cannot rule out that we have missed shorter low level infections that may have contributed to exhaustion of HCV-specific T cells or expansion of HCV-specific regulatory T cells (Tregs) that may suppresses T cell responses against subsequent primary acute infection[27]. Since the patients studied here have all cleared a previous primary acute HCV infection with systemic viremia and that the memory T cells responded efficiently to *in vitro* stimulation with their cognate peptides, it is unlikely that inhibition by Tregs was a major player. These issues need to be addressed by closer follow-up intervals and improved patient questionnaires that can

identify high risk exposures, with tools to detect low level viremia or potential subclinical infections. Similarly, the contribution of other CD4 T cell subsets like Th17 cells in the maintenance of memory CD8 T cells, as well as the cross regulation between Tregs and Th17 will require additional investigations[13].

Osbourn *et al.* demonstrated that spontaneous resolution upon HCV reinfection correlates with generation of neutralizing antibodies. We attempted to examine the neutralizing capacity of longitudinal plasma samples against a panel of cell-cultured HCV (HCVcc) representing the most common HCV genotypes in Canada (Genotypes 1a, 1b and 3a). However, no distinct difference could be detected between the SR/SR and SR/CI group which may be due to the low number of patients in each group (data not shown).

In conclusion, we demonstrate that protection against HCV persistence upon re-exposure is possible. Although we could not assess whether there was sterilizing immunity or long-term protection in our cohort, our results and results from other groups suggest that protection from viral persistence is higher upon subsequent exposure than primary infection. We also demonstrate that changes in the breadth of the immune response and immune dominance are possible, suggesting generation of *de novo* T cell responses and offering hope for vaccine development. Immunization and boosting with different HCV genotypes may afford a broader and more effective protection.

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"Author names in bold designate shared co-first authorship"

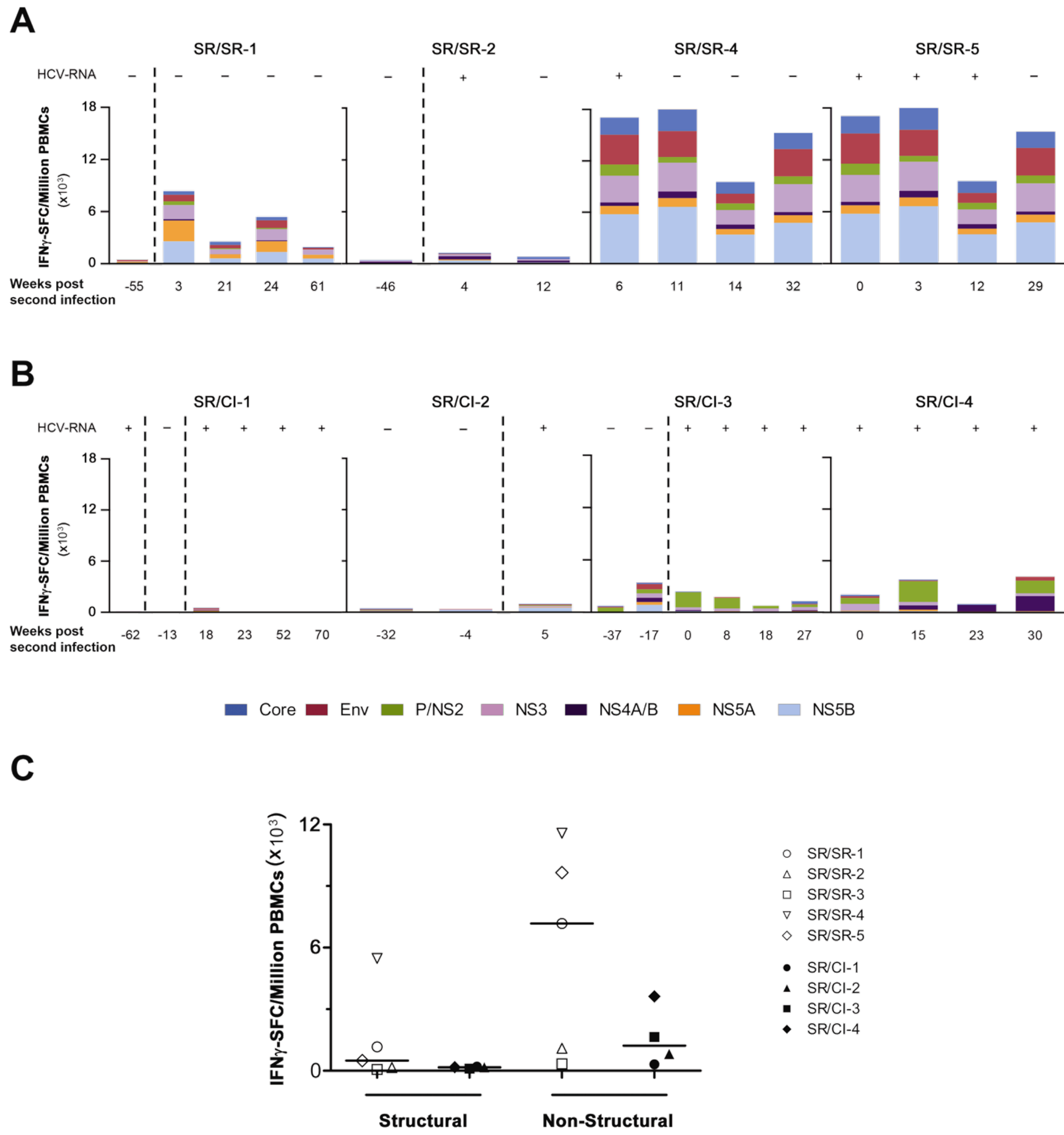


Figure 1

Figure 1: Higher magnitude and breadth of the HCV-specific T cell response in the SR/SR as compared to SR/CI patients. PBMCs from the indicated time points were tested in an IFNγ ELISPOT assay against overlapping peptide pools representing the HCV (H77) polyprotein. The frequencies of IFNγ spot forming cells (SFC) per million PBMCs from four SR/SR patients **(A)** and four SR/CI patients **(B)** are shown. The dashed lines delineate the different infection episodes. **(C)** Frequency of cells targeting the structural versus the non-structural proteins at the earliest time point (mean 8 weeks) during reinfection in the SR/SR (open symbols) and the SR/CI (solid symbols) patients.

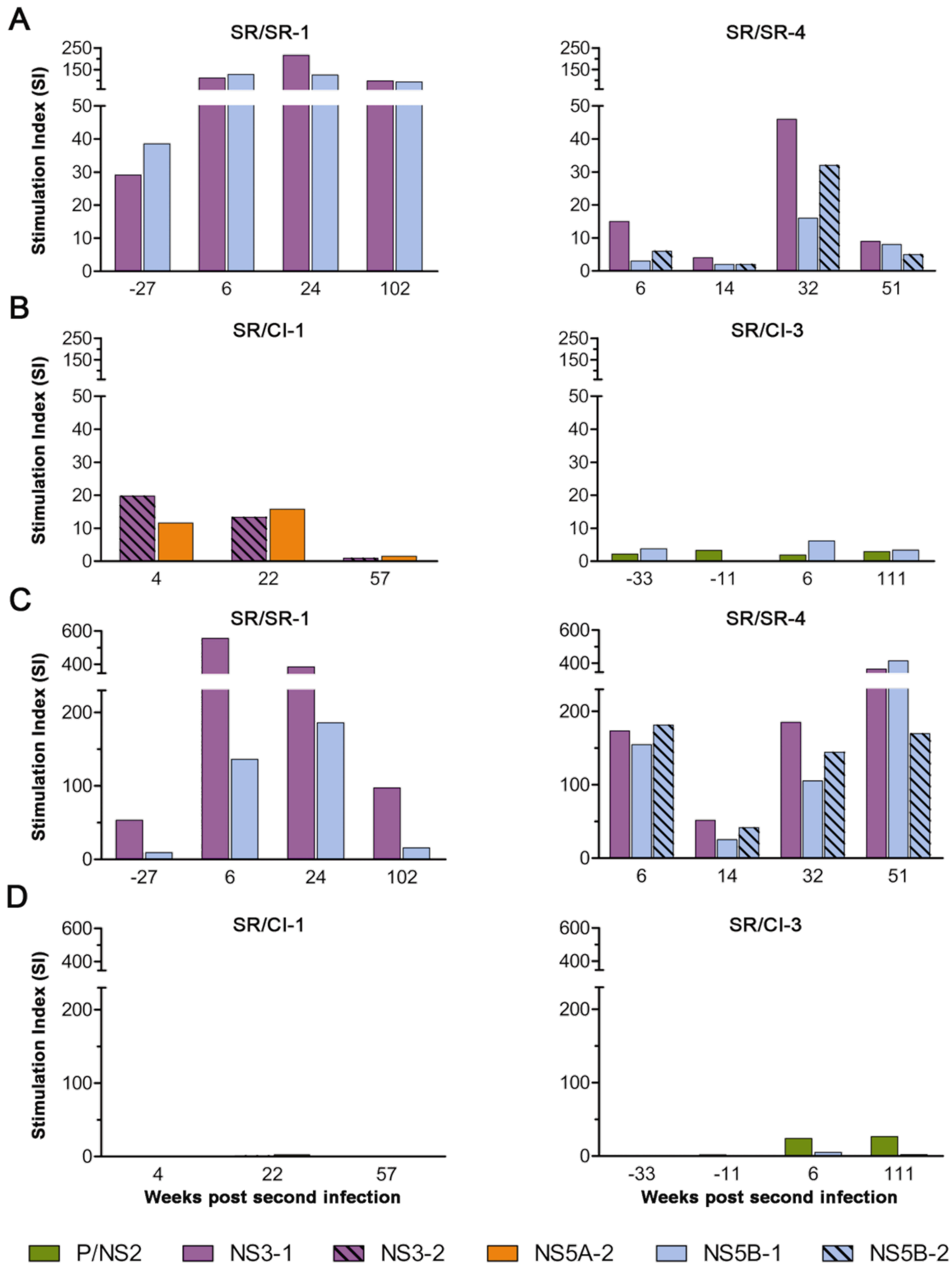


Figure 2

Figure 2: Higher proliferative capacity of HCV-specific CD4 and CD8 T cells from SR/SR patients as compared to SR/CI patients. Proliferation of HCV-specific T cells in a 6-day CFSE proliferation assay against overlapping peptide pools spanning the HCV proteins indicated. Representative proliferation data for CD4 T cells from two SR/SR **(A)** and two SR/CI **(B)** patients. Representative proliferation data for CD8 T cells from two SR/SR **(C)** and two SR/CI **(D)** patients. Proliferating cells were identified as percent viable $CFSE^{lo}$, CD4+ or CD8+, CD3+ T cells and presented as stimulation index (SI). SI is calculated by dividing the percentage of proliferating cells in the stimulated sample by the percentage of proliferating cells in the non-stimulated control under the same conditions.

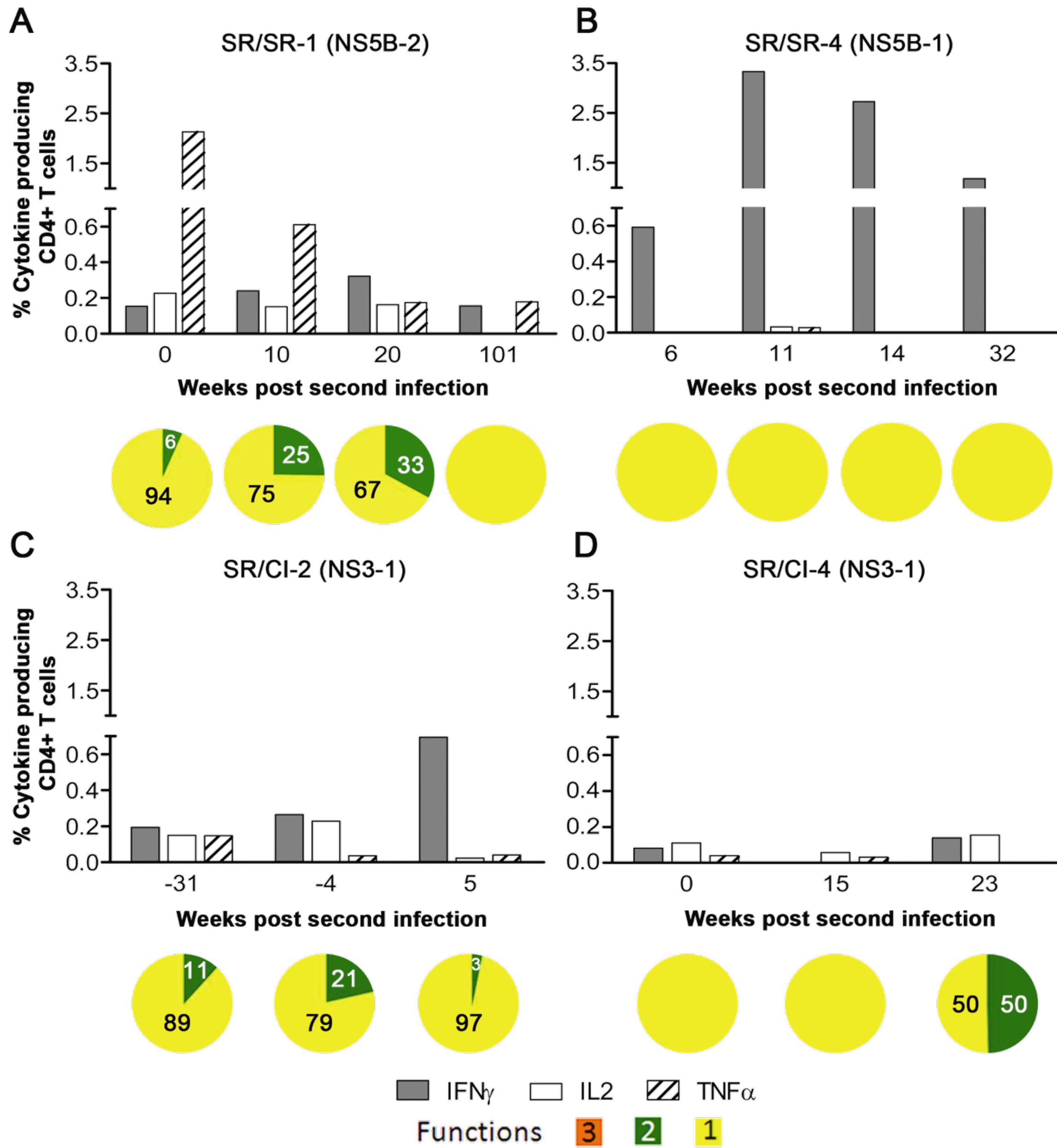


Figure 3

Figure 3: Higher production of cytokines by HCV-specific CD4 T cells from SR/SR patients as compared to SR/CI patients during reinfection. PBMCs were stimulated with overlapping peptide pools representing HCV (NS3 and NS5B) and pools showing the highest response in the IFNγ ELISPOT then ICS was performed to measure the production of IFNγ, IL-2 and TNFα. Representative results from two SR/SR patients **(A, B)** and two SR/CI patients **(C and D)**, gated on CD4+ CD3+ viable T lymphocytes. Peptide pools are indicated between brackets and represent the following regions: NS3-1 (NS3 aa: 1016-1341), NS5B-1 (NS5B; aa: 2416-2720) and NS5B-2 (NS5B; aa: 2710-3014). For polyfunctionality, orange represents triple functional cells, green represents double functional and yellow represents monofunctional cells of any combination.

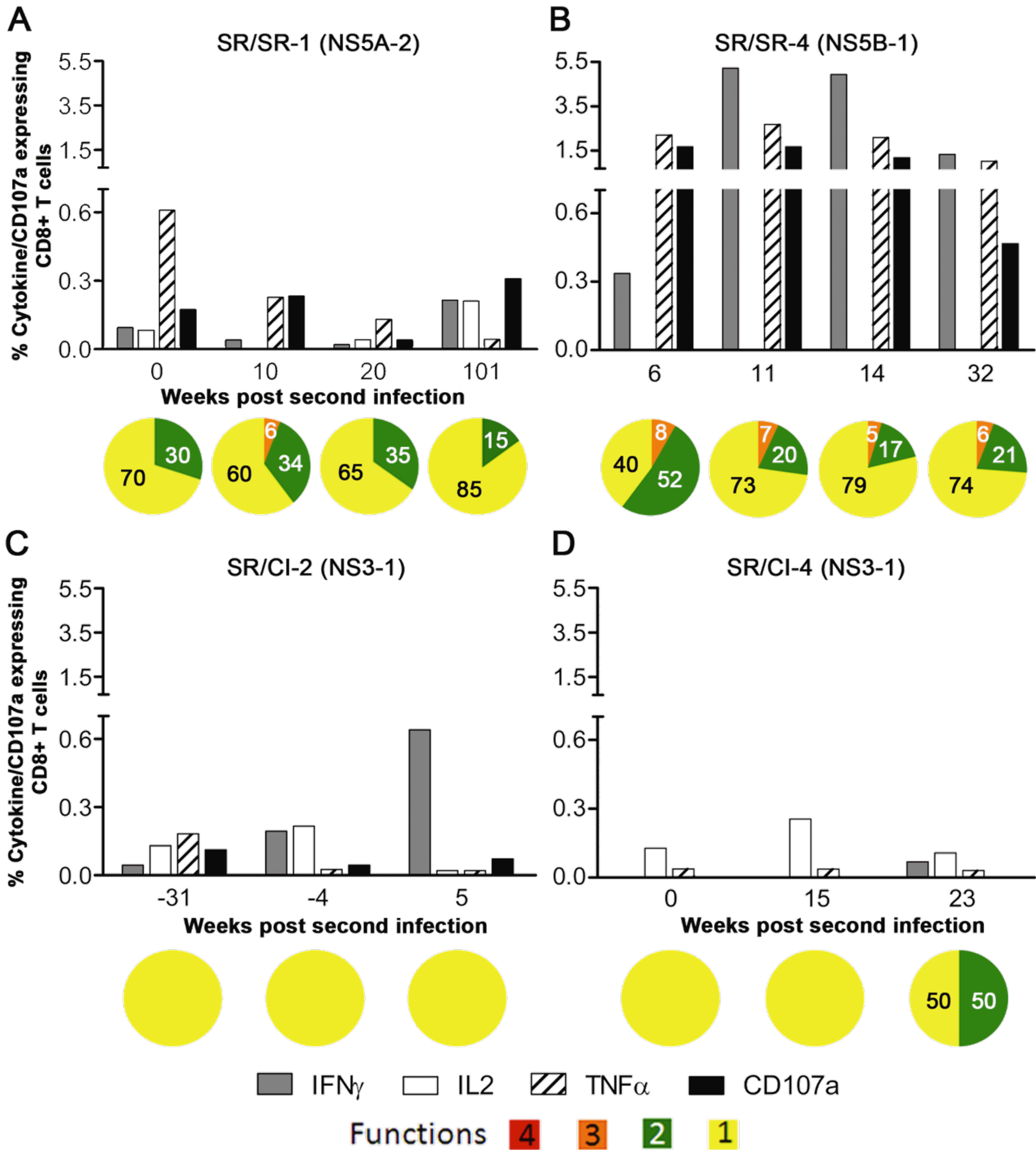


Figure 4

Figure 4: Higher polyfunctionality of HCV-specific CD8 T cells from SR/SR patients in comparison to SR/CI patients during reinfection. PBMCs were stimulated overnight with overlapping peptide pools representing HCV (NS3 and NS5B) and HCV pools showing the highest response in the IFNγ ELISPOT. ICS was performed to measure the production of IFNγ, IL-2 and TNFα and expression of CD107a. Representative results from two SR/SR patients **(A, B)** and two SR/CI patients **(C and D)**, gated on CD8+ CD3+ viable T lymphocytes. Peptide pools are indicated between brackets and represent the following regions: NS3-1 (NS3 aa: 1016-1341), NS5A-2 (NS5A; aa: 2192-2426) and NS5B-1 (NS5B; aa: 2416-2720). For polyfunctionality, orange represents triple functional cells, green represents double functional and yellow represents monofunctional cells of any combination.

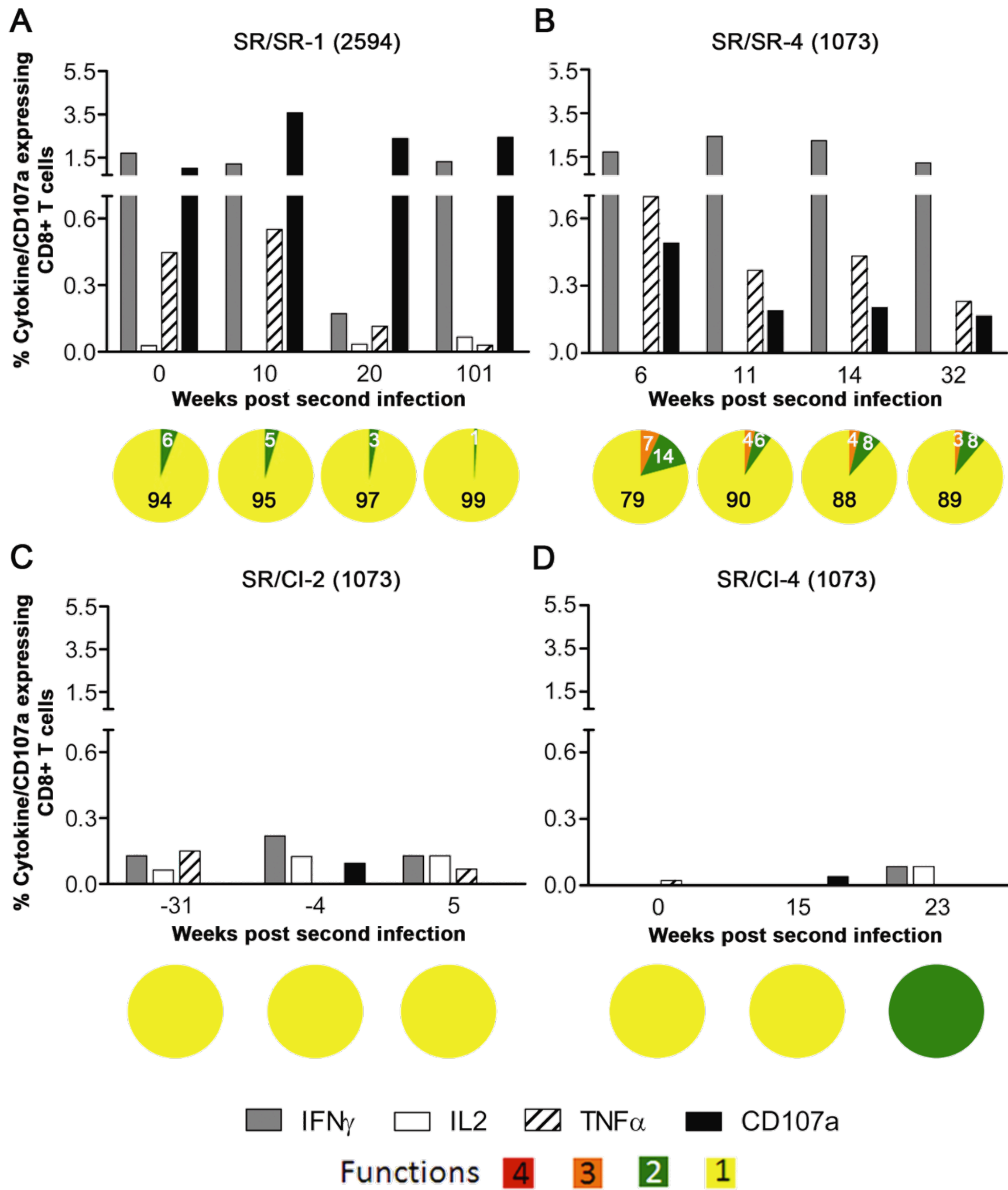


Figure 5

Figure 5: Higher polyfunctionality of HCV-specific CD8 T cells targeting minimal epitopes in SR/SR patients in comparison to SR/CI patients during reinfection. PBMCs from the indicated time points were stimulated overnight with the minimum cognate peptide and ICS/CD107a staining was performed. Representative results from two SR/SR patients **(A B)** and two SR/CI patients **(C and D)**, gated on viable CD3+CD8+ T lymphocytes. Peptides used are: 1073 = A2 restricted NS3-1073 and 2594 = A2 restricted NS5B-2594.

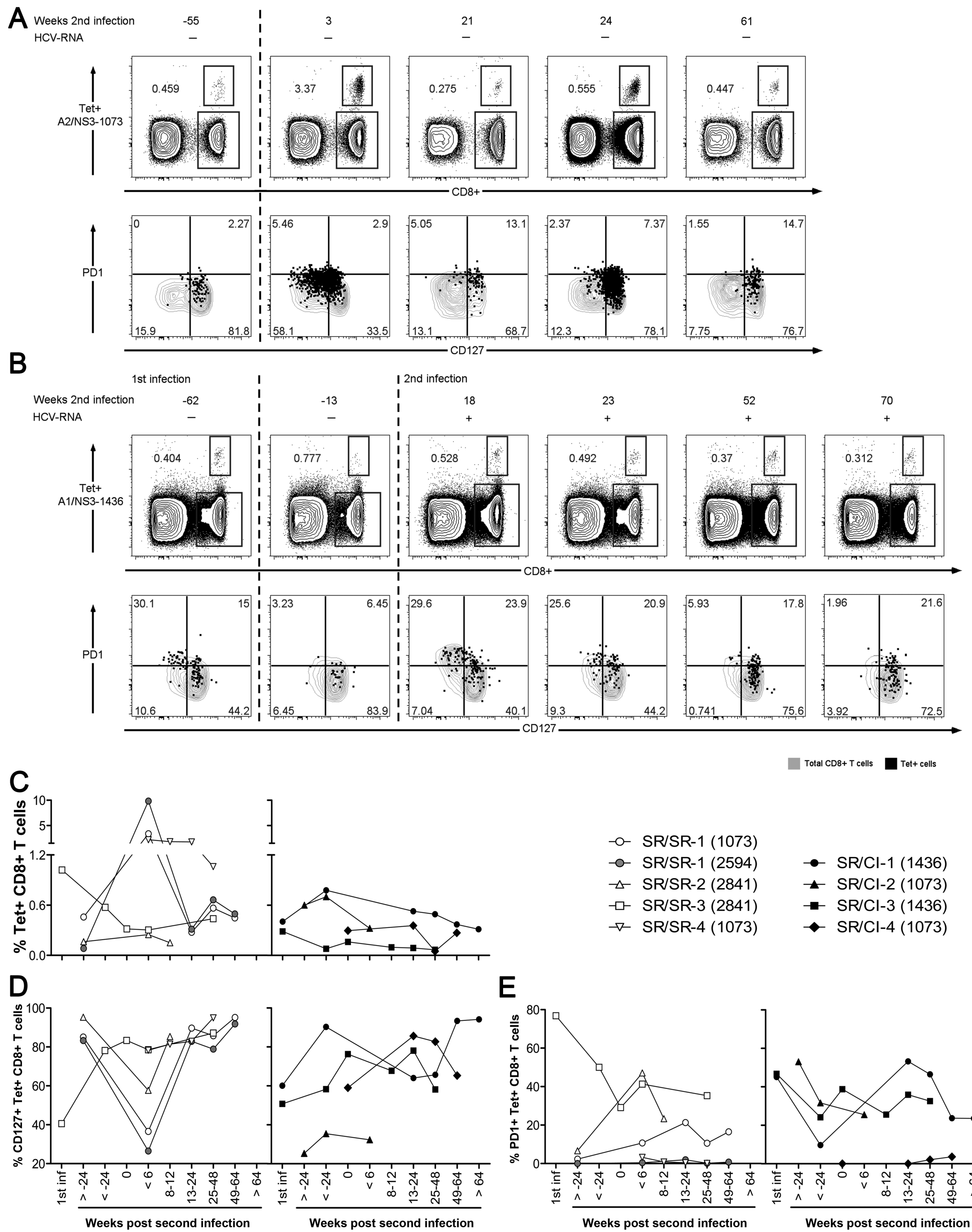


Figure 6

Figure 6: Expansion of HCV-specific tetramer+ CD8 T cells upon reinfection and generation of a CD127^{lo} effector T cell population in SR/SR patients. HCV tetramer staining and phenotyping were performed directly *ex vivo* on PBMCs from the indicated time points. Representative dot plots for longitudinal tetramer staining (upper panels) and phenotyping (lower panels) for **(A)** patient SR/SR-1 with A2/NS3-1073 tetramer and **(B)** patient SR/CI-1 with A1/NS3-1436 tetramer. Top panels are gated on viable CD3+ T lymphocytes. Lower panels are gated on viable CD3+CD8+ T lymphocytes. Tetramer frequency is represented as percent Tetramer+ CD8+ CD3+ T cells. In lower panels, black dots represent the tetramer+ HCV-specific CD8+ T cells overlaid on grey contour plots of CD8+ CD3+ T cells. Numbers in each quadrant represent percent expression on tetramer+ CD8+ CD3+ T cells. The dashed lines delineate the different infection episodes. **(C-E)** Tetramer expansion and phenotyping data from all SR/SR and SR/CI patients; **(C)** Frequency of HCV tetramer+ CD8+ CD3+ T cells, **(D)** Frequency of CD127+ tetramer+ CD8+CD3+ T cells, **(E)** Frequency of PD1+ tetramer+ CD8+CD3+ T cells.

A

Patient	HLA-A1/NS3-1436		HLA-A2/NS3-1073	
	Early	Late	Early	Late
SR/CI-1	ATDALMTGY --------F (6/6)	ATDALMTGY --------F (9/9)		
SR/CI-2			CINGVCWTV ----A---- (10/10)	CINGVCWTV ------L–I (8/8)
SR/CI-3	ATDALMTGY --------F (4/4)	ATDALMTGY --------F (11/11)		
SR/CI-4			CINGVCWTV ----A---- (11/11)	CINGVCWTV ----A---- (5/5)

B

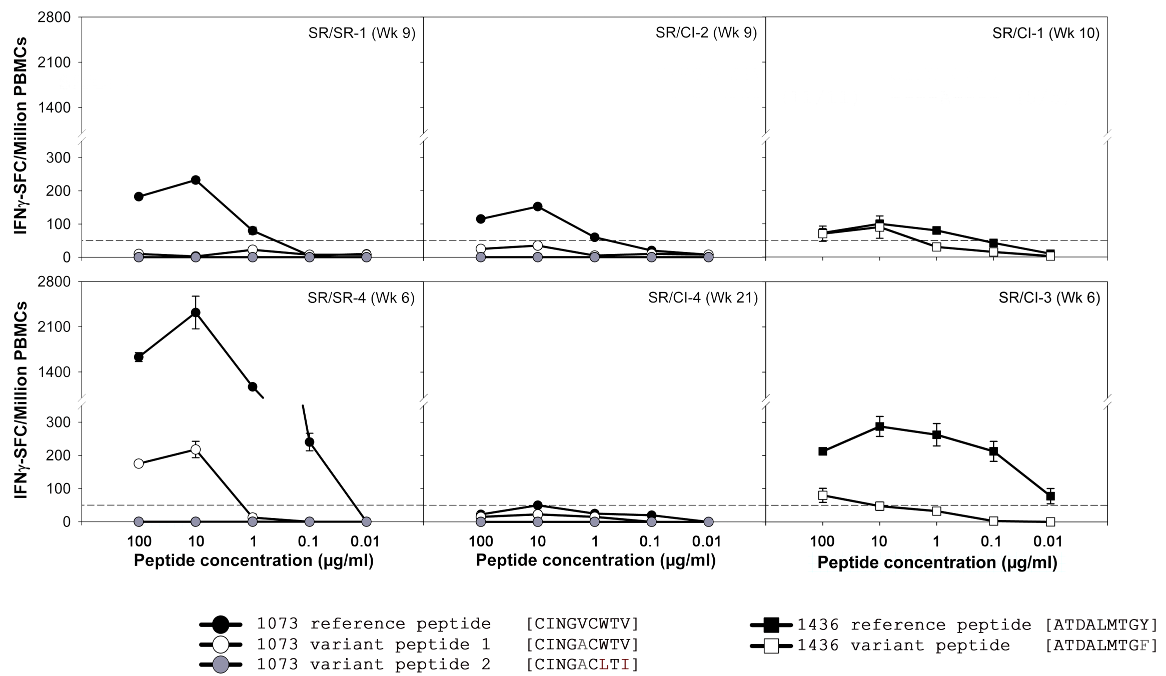


Figure 7

Figure 7: Sequence variation in targeted epitope and reduced recognition by CD8 T cells in SR/CI patients. (A) Sequences of the epitopes targeted by tetramer+ CD8 T cells in SR/CI patients at early and late time points during reinfection. (B) Responses of total PBMCs collected during reinfection in a dose-response IFN-γ ELISPOT assay to the reference sequences versus variant autologous sequences for two SR/SR patients in response to the NS3-1073 epitope (left panels), two SR/CI patients in response to the NS3-1073 epitope (middle panels) and two SR/CI patients in response to the NS3-1436 epitope (right panels).

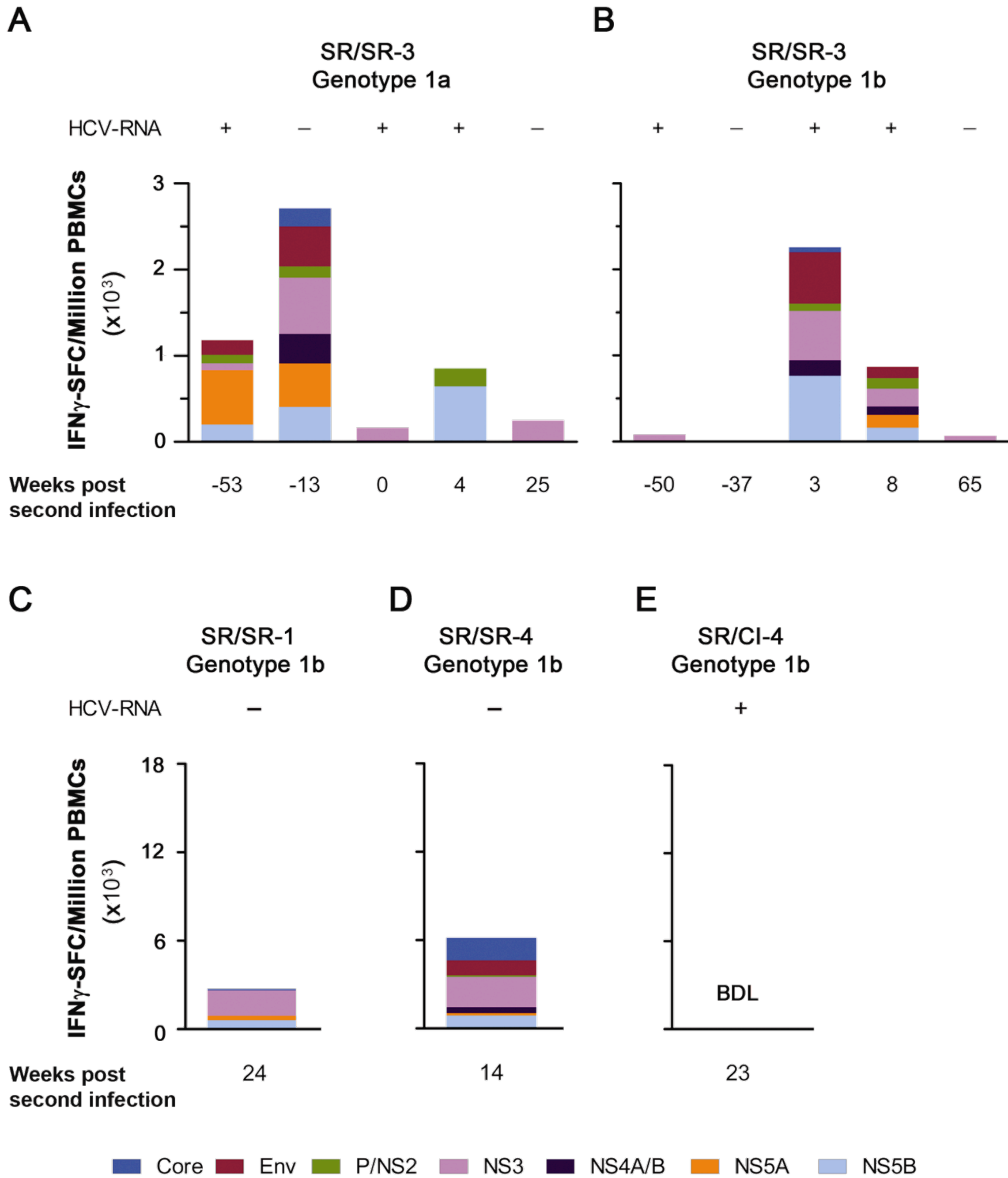
Supplementary Table S1: Patients' characteristics and demographics

Patient code	Status at start of follow-up	Age at reinfection (years)	Gender	Duration of injection (years)	No of Injections during past month[a]	Serum ALT during early reinfection[b] (U/L)	HCV Genotype (First infection/ Second infection)	HLA Class I	Reactive Tetramers
SR/SR-1	HCV Resolver	53	M	13.4	> 30	3090	ND/1	A2, B58	A2/NS3-1073 A2/NS5B-2594
SR/SR-2	HCV Resolver	43	M	15.4	≤ 30	25	ND/1	A24/A26, B27/B49	B27/NS5B-2841
SR/SR-3	HCV Naïve	42	M	14.4	> 30	23	1a/1	A23/A32 B27/B44	B27/NS5B-2841
SR/SR-4	Early reinfection	36	F	12.3	≤ 30	15	ND/1	A2/A24 B7/B39	A2/NS3-1073
SR/SR-5	Early reinfection	44	M	31.5	> 30	183	ND/1a	A2/A23 B18/B49	None
SR/CI-1	HCV Naive	28	M	6.1	> 30	88	ND/1a	A1 /A3 B44/B49	A1/NS3-1436
SR/CI-2	Acute primary HCV	29	M	1.1	≤ 30	17	1a/1a	A2, B39/B44	A2/NS3-1073
SR/CI-3	HCV Resolver	30	F	4.1	≤ 30	42	ND/1a	A1/A29 B35/B44	A1/NS3-1436
SR/CI-4	Early reinfection	33	M	3	> 30	20	ND/1b	A2, B44/B51	A2/NS3-1073

ND = Not determined; SR: Spontaneous Resolution; CI: Chronic Infection

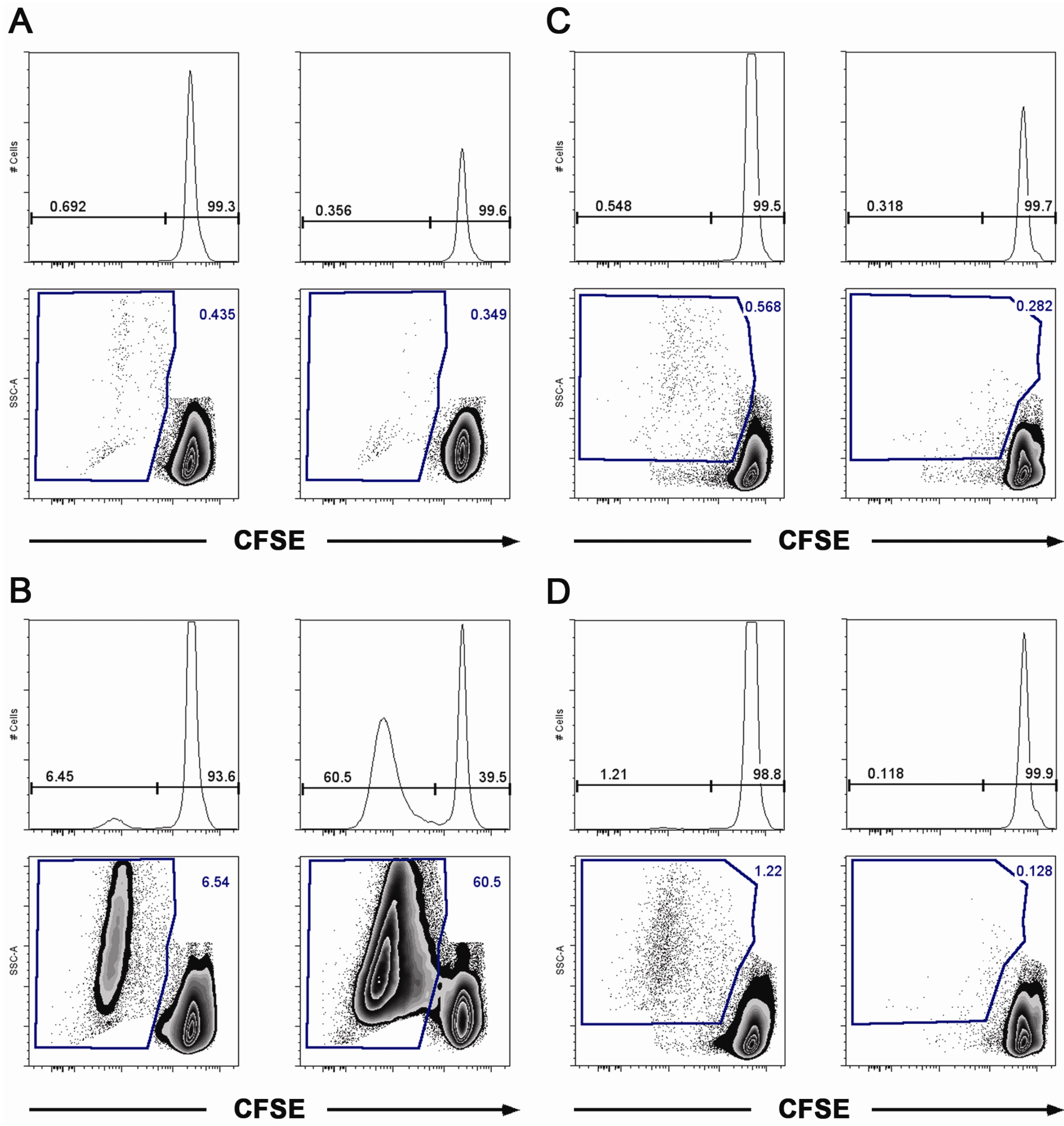
[a] A strong and independent predictor of HCV incidence in this cohort (Bruneau J et al, Addiction. 2012 Jul;107(7):1318-27)

[b] at < 4 wks



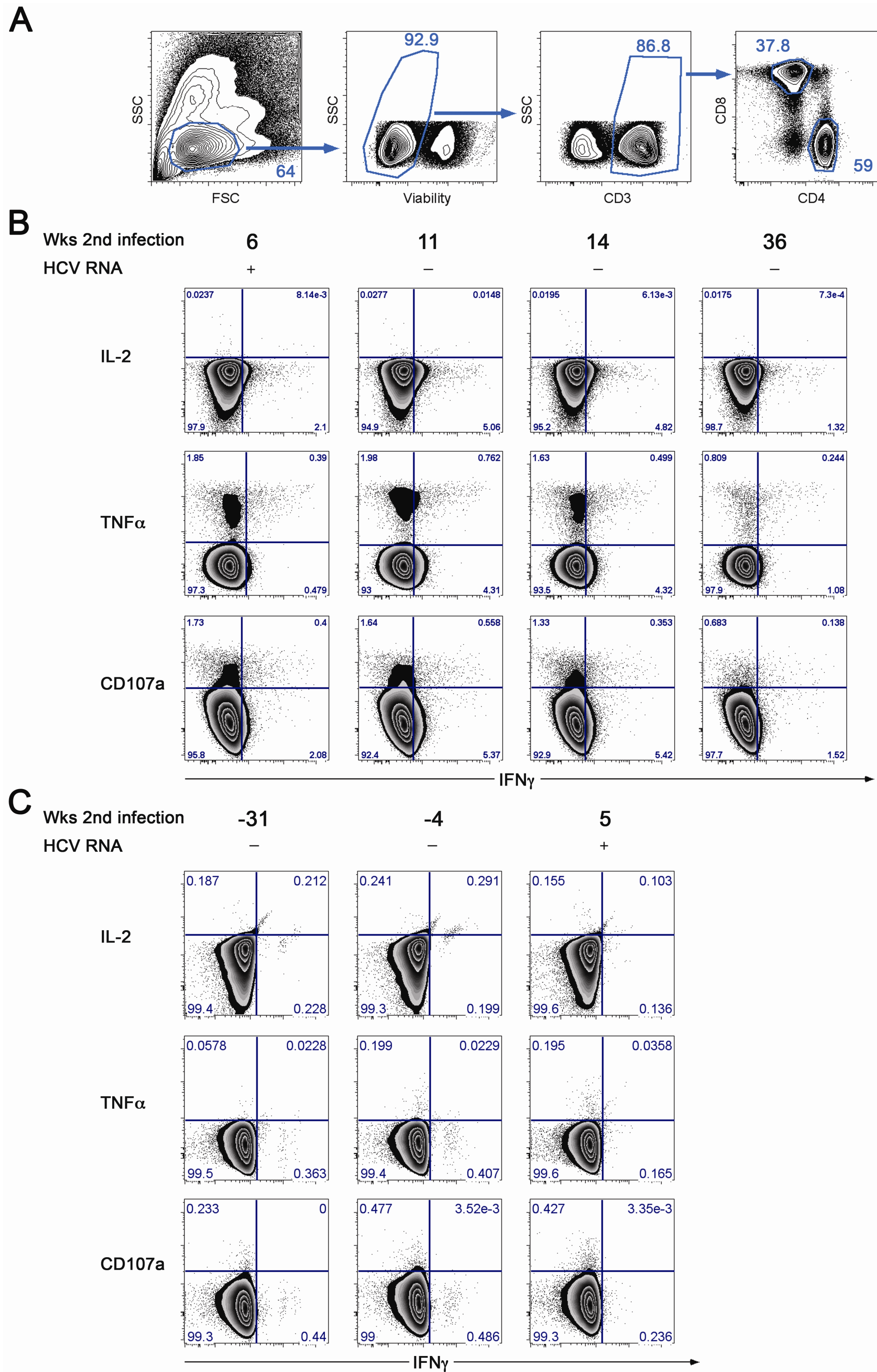
Supplementary Figure S1

Supplementary Figure S1: Lack of expansion of HCV-specific T cells in patient SR/SR-3 upon reinfection, is due to being infected by a different HCV subtype. Results from longitudinal ELISPOT assays for patient SR/SR-3 where PBMCs were stimulated with overlapping peptide pools representing the entire HCV genome for **(A)** genotype 1a and **(B)** genotype 1b. The dashed lines delineate the different infection episodes. Responses to genotype 1b peptide pools during the reinfection episode for patients with genotype 1 and an unknown subtype SR/SR-1 **(C)**, SR/SR-4 **(D)** and SR/CI-4 **(E)**. BDL = Below detection level. The genotype of the infecting virus during primary infection was 1a for patient SR/SR-3, but unknown for the other three patients. The reinfecting virus was genotype 1 for the three SR/SR patients, but the subtype was not defined. For patient SR/CI-4 the reinfecting virus was genotype 1b.



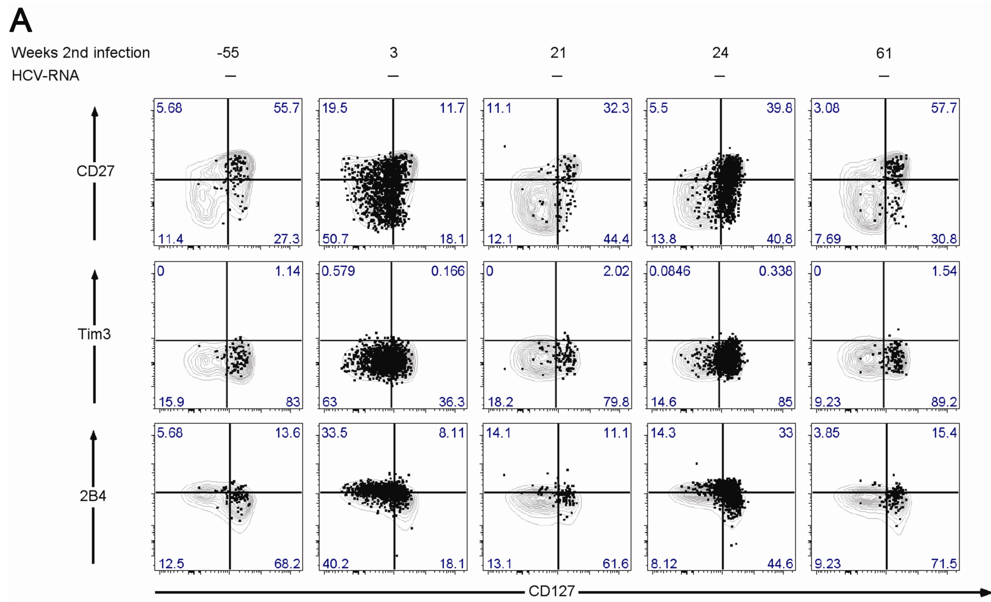
Supplementary Figure S2

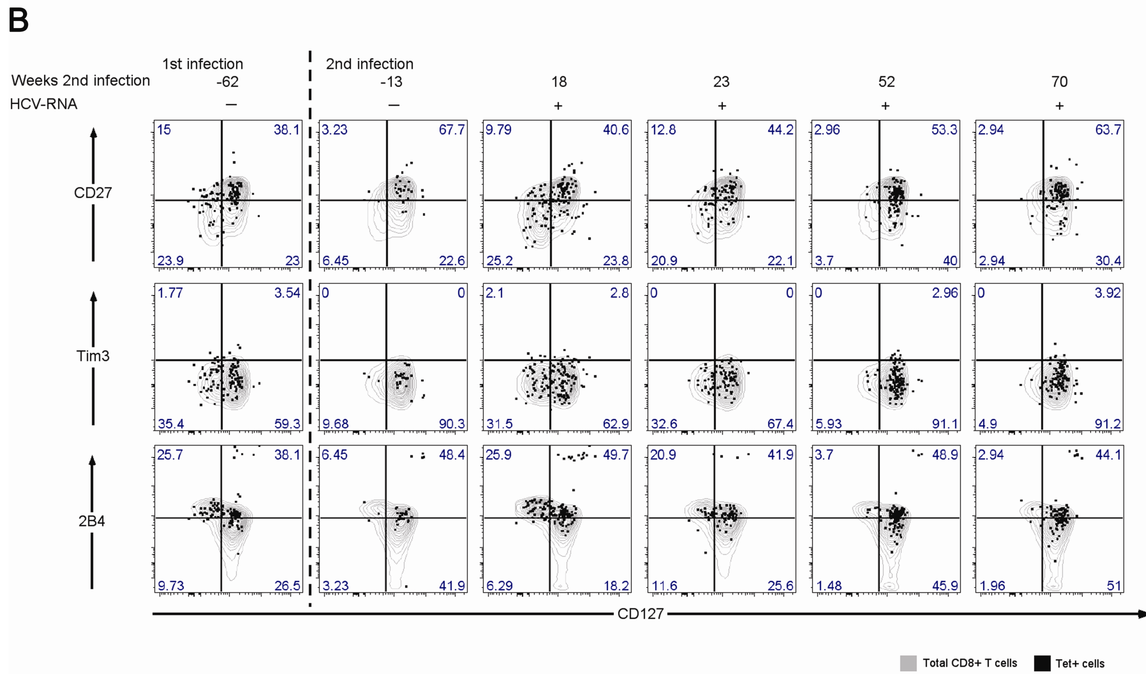
Supplementary Figure S2: Higher proliferative capacity for HCV-specific CD4 and CD8 T cells in SR/SR patients in comparison to SR/CI patients. Proliferation of HCV-specific CD4 and CD8 T cells was assessed in a 6-day CFSE proliferation assay against a panel of overlapping peptide pools representing the immunodominant regions of HCV (NS3 and NS5B) and HCV regions showing the highest response in the IFNγ ELISPOT assay. Representative dot plots showing the proliferation indicated by the percentage of $CFSE^{lo}$ cells from patient SR/SR-4 **(A)** non-stimulated versus **(B)** stimulated with peptide pool NS3-1 (NS3 aa: 1016-1341) and patient SR/CI-3 **(C)** non-stimulated versus **(D)** stimulated with peptide pool P7/NS2 (P7/NS2 aa: 743-1026). The cells were gated on viable CD3+ then CD4+ or CD8+ T lymphocytes. The percentage of proliferating cells is shown on the left of the histograms (upper panels) and on the dot plots (lower panels).



Supplementary Figure S3

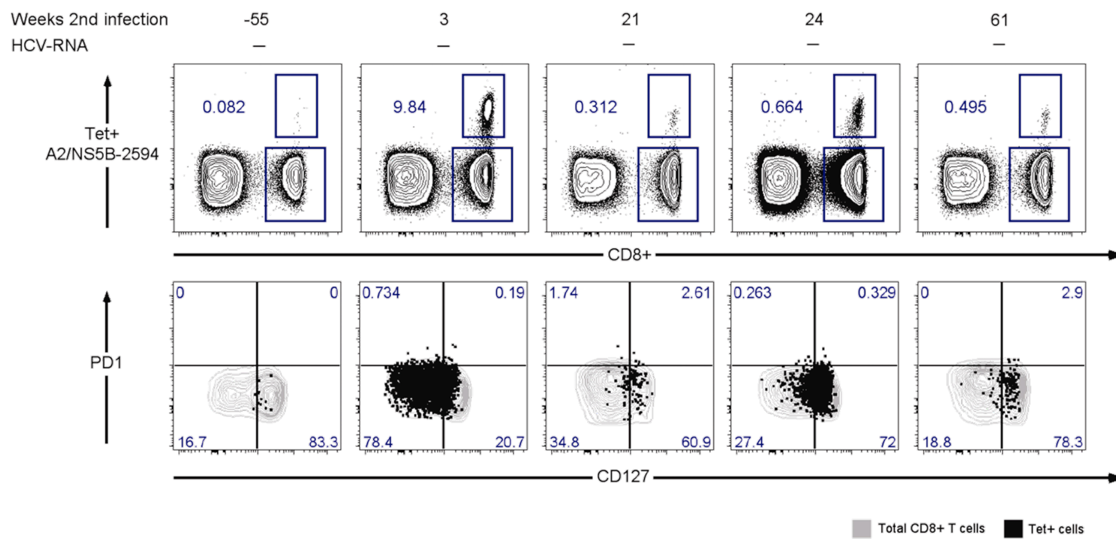
Supplementary Figure S3: Higher production of cytokines/CD107a expression by HCV-specific CD8 T cells in patients from SR/SR group in comparison to patients from SR/CI group. **(A)** Gating strategy. Representative dot plots from **(B)** patient SR/SR-4 and **(C)** patient SR/CI-2 showing the production of cytokines (IFNγ, IL-2 and TNFα) and the expression of CD107a in response to stimulation with peptide pools representing NS5B-1 and NS3-1, respectively, gated on viable CD8+ CD3+ T lymphocytes.





Supplementary Figure S4

Supplementary Figure S4: No significant changes in CD27 or the exhaustion markers Tim3 or CD244 (2B4) in either of the 2 groups of patients. Representative dot plots for phenotyping of **(A)** patient SR/SR-1 with A2/NS3-1073 tetramer and **(B)** patient SR/CI-1 with A1/NS3-1436 tetramer, showing the expression of CD27, Tim3 and 2B4 on tetramer+ cells. The black dots represent the tetramer+ HCV-specific CD8+ T cells overlaid on the grey contour plots representing total CD3+ CD8+ T cells in the same patient. Percentages of expression on tetramer+ cells are shown in each quadrant.



Supplementary Figure S5

Supplementary Figure S5: Shift in immunological dominance of the targeted epitope between the primary infection and the reinfection episode in patient SR/SR-1. Dot plots for longitudinal tetramer staining (upper panel) and phenotyping (lower panel) from patient SR/SR-1 with A2/NS5B-2594 tetramer gated on viable CD3+ T lymphocytes. The A2/NS5B-2594 tetramer+ population expanded higher frequencies than the A2/NS3-1073 tetramer+ cells that were present at a higher frequency post the primary infection **(Figure 5A and 5C)**.

DETATILED MATERIALS AND METHODS

IFNγ ELISPOT assay

HCV-specific T cell responses were measured using an IFNγ ELISPOT assay as previously described[1] and an input of 2×10^5 PBMCs /well against 10 pools of overlapping peptides spanning the entire HCV polyprotein. Peptides were corresponding to the HCV genotype 1a (H77) and genotype 1b (J4) sequences (BEI Resources, Manassas, VA). Specific Spot forming cells (SFC) were calculated as "mean number of spots in test wells − mean number of spots in negative control wells" and normalized to SFC/10^6 PBMCs. A response was scored positive if greater than 50 SFC/10^6 PBMCs.

Flow cytometry Antibodies

Directly conjugated antibodies against the following molecules were used: CD3-Alexa Fluor® 700 or –FITC (clone UCHT1), CD4-Pacific Blue™ or –PE or –FITC (clone RPA-T4), CD8-Alexa Fluor® 700 or –Pacific Blue™ (clone RPA-T8), CD8-APC-H7 (clone SK1), CD27-APC-H7 (clone M-T271), CD279/PD-1-APC (clone MIH4), CD244/2B4-FITC (clone 2-69), IFNγ-APC (clone B27), IL-2-PE (MQ1-17H12), TNFα-Alexa Fluor® 700 (clone MAb11) and CD107a/LAMP-1-PE-Cy™ 5 (clone H4A3) all from BD Biosciences, San Diego, CA; CD3-ECD (clone UCHT1) and CD8-ECD (clone SFCI21Thy2D3) from Beckman Coulter, Marseille, France; CD127/IL-7Ra-eFluor®450 (clone eBioRDR5) from eBioscience, San Diego, CA; Tim3-PerCP (clone 344823) from R&D Systems Inc., San Diego, CA). Live cells were identified using Aqua Live/Dead fixable dead cell Stain Kit (Life Technologies, Burlington, ON). 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,) using FACSDiva software version 6.1.3 (BD Biosciences). Data files were analyzed using FlowJo software version 9.4.11 for Mac (Tree Star, Inc., Ashland, OR).

Phenotypic and functional analysis of HCV-specific T cells

All assays were performed on archived frozen PBMCs. Tetramer staining for CD8-specific T cells was performed as previously described[2] using the listed panel of tetramers. Intracellular

cytokine staining (ICS) and CD107a degranulation assay were performed as previously described[2] in response to HCV peptide pool (1 μg/ml) or HCV minimum peptide (10 μg/ml) or SEB (200 ng/ml) as a positive control. Polyfunctionality was assessed by exporting flow cytometry standard (FCS) data as Boolean gates using FlowJo (version 9.5) and SPICE software[3]. Carboxyfluorescein succinimidyl ester (CFSE) proliferation assays were performed as previously described[2] in a 6 day proliferation assay with or without HCV minimum-peptide or peptide pool (final concentration 1 μg/ml). Recombinant human IL-2 (20 IU/ml) (NIH AIDS Research and Reference Reagent Program, ATCC, Manassas, VA) was added 3 days post stimulation.

Functional avidity assays

Functional avidity of epitope specific CD8 T cells was measured as the dose-dependent production of IFNγ in response to stimulation by the reference and variant epitope sequences in an IFN-γ ELISPOT assay, as previously described [4].

Statistical Analysis

Statistical analysis for ELISPOT results was performed with GraphPad Prism 5 using one-tailed Mann-Whitney test at a confidence interval of 90%.

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CHAPTER 4:

MANUSCRIPT 2:

Dynamics of Virus-Specific CD8 T-cell Repertoire during HCV Reinfection

(Manuscript prepared for submission to ***Blood***)

Dynamics of Virus-Specific CD8 T-cell Repertoire during HCV Reinfection

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Abbreviations used in this paper: CDRs: Complementarity-determining regions; ELISPOT: Enzyme-linked immunospot assay; HCV: Hepatitis C virus; HIV: Human immune deficiency virus; p-MHC: peptide-MHC; TCR: T-cell receptor.

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Disclosures: None

ABSTRACT

We have previously demonstrated that protection upon HCV reinfection correlates with increased breadth of the immune response and shifting epitope dominance (observed in one patient), suggesting the generation of *de novo* T-cell responses. Protection was also associated with HCV-specific T cells possessing higher polyfunctionality and functional avidity in response to reference epitopes and higher flexibility in response to epitope variants than T cells in the non-protected group. Here, we performed longitudinal analysis of the HCV-specific T cell repertoire during HCV reinfection in the same two groups of patients; protected patients, who spontaneously resolved two consecutive infections (SR/SR group, n=3) and non-protected patients, who resolved primary infection but became persistently infected upon reinfection (SR/CI group, n=2). Our results demonstrate that effector CD8 T-cell clonotypes associated with protection upon reinfection were recruited from the pre-existing memory population, but with a change in dominance and hierarchy of the clonotypes. The TCR repertoire was narrow in SR/SR patients pre-reinfection, with increased focusing upon reinfection. These results suggest that effector HCV-specific CD8 T-cell clonotypes associated with protection upon reinfection are recruited from the memory population, with focusing of the repertoire on specific clonotypes that exhibit superior functional avidity and polyfunctionality.

INTRODUCTION

Definition of the correlates of protective immunity is crucial for designing prophylactic vaccines against chronic viruses such as HIV and HCV. We have previously demonstrated that protection from chronicity upon reinfection with HCV correlated with expansion of HCV-specific polyfunctional effector T cells and increased breadth of T-cell responses, suggesting the generation of *de novo* T cell responses. In contrast, viral persistence was associated with limited expansion of virus-specific T cells and infection with variant viral strains, which were not recognized by the pre-existing virus-specific memory T cells [1].

In this previous study we showed higher levels of functionality and polyfunctionality in protected (SR/SR) patients when compared to non-protected (SR/CI) patients in response to cognate minimal epitope [1]. Autologous HCV-epitopes sequenced during reinfection were different from the reference epitopes in all SR/CI patients. Dose-responses demonstrated 2 to 10-folds higher avidity of HCV-specific T cells in SR/SR patients to the reference sequence when compared to SR/CI patients, and one SR/SR patient had a good response to one of the epitope variants, suggesting higher flexibility of the TCR-repertoire. SR/CI patients had very low/no avidity for the autologous epitope sequence of the reinfecting virus [1].

The adaptive immune system generates a large number of T-cell clonotypes, each expressing a unique T-cell receptor (TCR). The TCR is formed of a heterodimer of the α and β chains. The variable region of both chains is generated by somatic gene recombination encoded by the variable (V), junction (J) and diversity (D) gene segments. Hypervariable complementarity-determining regions (CDRs) are encoded within the V gene segments. CDR3 of the β-chain is formed by combinatorial and junctional variation, and has a major role in determining the specificity of the TCR[2]. The interplay between the TCR-repertoire and chronic viruses has been previously studied for different viral infections. For HIV, specific clonotypes dominated the response against an HIV-epitope in patients who controlled viral replication. These specific clonotypes showed higher avidity and polyfunctionality, and both characteristics were interrelated [3, 4]. Certain clonotypes were observed in several controllers, thus termed “public” [5, 6], whereas other studies did not observe preferential use of particular clonotypes [7, 8]. Mutations occurred in targeted epitopes to avoid recognition by these highly

effective clonotypes, driving expansion of alternative clonotypes with dual reactivity against both the original and mutated epitopes [9]. For HCV, rechallenging chimpanzees with HCV following clearance of a primary infection demonstrated that resolution temporally coincided with the expansion of the dominant clonotype that was associated with the clearance of the primary infection [10]. Higher diversity within the CDR3 region of TCR correlated with viral clearance and better control of the evolution of escape mutations within the targeted epitope, with temporary narrowing of the repertoire at the peak of the recall response [11].

Here, we examined dynamics of the TCR repertoire relative to changes in HCV sequences during reinfection. Our objectives were to distinguish the role of pre-existing memory versus *de novo* T-cell responses in protection and to determine the importance of flexibility and diversity in the T-cell repertoire in controlling infection with variant HCV strains. Our results suggest that HCV-specific tetramer+ CD8+ T-cell clonotypes mobilized during the reinfection episode were exclusively recruited from the pre-existing memory population and that protective immunity in SR/SR patients is associated with a narrower TCR-repertoire that becomes more focused upon reinfection.

MATERILAS AND METHODS

Study population and identification of HCV reinfection cases

This study was conducted among participants of the Montreal Acute Hep C IDU Cohort Study (HEPCO)[12] and approved by the institutional ethics committee (Protocol SL05.014). Primary HCV infection was identified in cohort participants who were initially negative for both HCV RNA and anti-HCV antibodies for at least 6 months of follow-up, then had a positive HCV RNA and/or antibody test as previously described[13, 14]. Participants who have resolved their primary HCV infection or participants who tested HCV RNA negative and HCV antibody positive at recruitment were enrolled in the reinfection study and followed every 3 months. HCV reinfection was defined by an HCV-RNA positive test following two negative tests, at least 60 days apart. The day of the first positive RNA test was defined as day zero post detection of reinfection (p.ri.). Nine cases of reinfection identified between 2009 and 2012. This study includes five patients for whom samples before the reinfection episode were available. Samples from time points during or right after clearance of the primary infection were available for three patients. These five patients belonged to two groups; three patients spontaneously resolved their second infection while two patients became chronically infected, referred to hereafter as the SR/SR group and the SR/CI group, respectively. Patients' demographics, clinical characteristics and infection history are listed in Table 1.

HLA Class I Tetramers

MHC class I tetramers were synthesized by the National Immune Monitoring Laboratory (NIML), (Montréal, QC, Canada) or the NIH Tetramer Core Facility (Emory University, Atlanta, GA) and are as follows: HLA-A1 restricted HCV NS3 peptide amino acids (aa) 1436–1444 (ATDALMTGY) [A1/NS3-1436], HLA-A2 restricted HCV NS3 peptide aa 1073–1081 (CINGVCWTV) [A2/NS3-1073], HLA-B27 restricted HCV peptide NS5B peptide aa 2841-2849 (ARMILMTHF) [B27/NS5B-2841].

Flow cytometry Antibodies

Directly conjugated antibodies against the following molecules were used: CD3–FITC (clone UCHT1), CD8–Pacific Blue™ (clone RPA-T8) and CD45RO–Alexa Fluor® 700 (clone

UCHL1) from BD Biosciences, San Diego, CA; CD127/IL-7Ra–Alexa Fluor® 647 (clone HIL-7R-M21) from eBioscience, San Diego, CA. Live cells were identified using Aqua Live/Dead fixable dead cell Stain Kit (Life Technologies, Burlington, ON). Multiparameter flow cytometry and sorting was performed using a BD Aria II u instrument equipped with blue (488 nm), red (633 nm), and violet (405 nm) lasers (BD Biosciences,) using FACSDiva software version 6.1.3 (BD Biosciences). Data files were analyzed using FlowJo software version 9.4.11 for Mac (Tree Star, Inc., Ashland, OR).

Purification and sorting of tetramer+ CD8+ T cells

Frozen PBMCs were thawed and the CD8+ T cells were purified using the negative selection MACS CD8+ T cell Isolation Kit (Miltenyi Biotec Inc, Auburn, CA). Tetramer staining for CD8-specific T cells was performed as previously described[13] using the listed panel of tetramers. Surface staining for CD3, CD8, CD45RO and CD127 was performed as previously described[13] using the listed panel of antibodies.

Sequencing the Vβ-chain of the TCR

Genomic DNA was extracted from the sorted cells and the variable β-chain (Vβ) of TCR was sequenced using the high-throughput sequencing technology provided by Adaptive Biotechnology (Seattle, USA). The obtained data were analyzed using ImmunoSEQ™ software. Clonotypes with a percentage of less than 0.01% of the total repertoire were excluded from the analysis.

RESULTS

Clonotypes expanding at the peak of reinfection are recruited from the pre-existing memory population rather than being *de novo* responses

We performed longitudinal analysis of the HCV-epitope-specific T-cell repertoire during HCV reinfection in five patients with different outcomes of the reinfection episode; three patients who resolved two successive HCV infections (SR/SR group), and two patients who became persistently infected upon reinfection (SR/CI group) (Table 1). Epitope-specific CD8 T cells identified by MHC class I tetramer (as detailed in Table 1) were sorted at various time points; pre-reinfection, at the peak of reinfection and late/post the reinfection episode. The gating strategy used for sorting is explained in Supplementary Figure 1. The TCRBV gene and TCRBJ gene usage, as well as the CDR3 chain amino acid sequence for all Vβ chains for the different T-cell clonotypes were analysed (Supplementary Tables S1 to S5). This analysis led to several observations about the TCR-repertoire of epitope-specific CD8 T cell populations.

We and others have reported that reinfection was associated with a significant increase in the breadth of T-cell responses suggesting the generation of *de novo* T-cell responses [1, 15]. This raised the possibility that within the epitope-specific CD8 T-cell population, the effector cells appearing at the peak of the immune response during reinfection in the SR/SR group could be due to the recruitment of new clonotypes from the naïve T-cell pool. We distinguished the effector versus memory HCV-specific T cells according to the expression of the IL7-receptor α chain (CD127), and analyzed the repertoire of HCV-specific T-cell populations at the peak of immune response against reinfection to determine whether effector T cells were recruited from the memory pool or generated *de novo*.

Our longitudinal analysis of the Vβ-chain of TCR did show a conserved pattern of Vβ usage in all of the tested patients, where the dominant and sub-dominant clonotypes forming the population at the peak of immune response during reinfection were recruited from the memory population generated following the clearance of the primary infection, with lack of appearance of new clonotypes (Figure 1 and Supplementary Tables S1 to S5). Nevertheless, the frequency of the different clonotypes changed upon reinfection causing a change in the

hierarchy of the utilized clonotypes. These results suggest that the effector CD8 T cells were effector memory cells (T_{EM}) recruited from the central memory pool rather than being recruited from the naïve repertoire.

Protective immunity in SR/SR patients is associated with a narrower TCR-repertoire that becomes more focused upon reinfection

The interplay between the TCR-repertoire of epitope-specific CD8 T cells and chronic viruses has been studied in the context of the LCMV model, as well as HIV and HCV infections. The results from these studies were controversial concerning the diversity of the repertoire and its correlation with control of infection and reinfection, where some studies showed control was associated with a more diverse TCR-repertoire[11], while other studies demonstrated it was associated with a more focused repertoire[16, 17].

Our analysis revealed that the clonotype-composition of the repertoire in the tested SR/SR patients was less diverse than that observed for SR/CI patients at pre-reinfection, where 75-83% of the repertoire was attributed to 14-23 TCR-clonotypes, and it became more focused at the peak of the immune response during reinfection for patients SR/SR-1 and SR/SR-2 (92% and 96% of the repertoire was attributed to 15 and 2 TCR-clonotypes, respectively) (Figure 2A, 2B and 2C). In those two patients, the repertoire retained a more focused status post reinfection when compared to the pre-reinfection time point, with 77% and 93% of the repertoire attributed to 16 and 4 clonotypes, respectively (Figure 2A and 2B). Patient SR/SR-3 showed no focusing of the repertoire upon reinfection (Figure 2C), and this comes in accordance with our previous results, where there was no expansion of the tetramer+ population in this patient upon reinfection, and ELISPOT data suggested the patient was reinfected with a different HCV subtype [1]. In contrast, the repertoire in the tested SR/CI patients was more diverse; where the dominant clonotypes (present at ≥1%) represented 37% and 55% of the repertoire and were attributed to 18 and 23 TCR-clonotypes, respectively. At the peak of the immune response during reinfection the repertoire was still more diverse than in the SR/SR patients (the dominant category represented 28-64% of the repertoire) (Figure 3A and 3B). The remaining ~40-60% of the repertoire was represented by 130-300 clonotypes, whereas in the SR/SR patient the number of minor clonotypes never exceeded 116 unique

clonotypes (except at the time point post infection for patient SR/SR-1, where the sample was a leukaphoresis sample, and it had ~10-40 fold the number of sequences available for other time points in the same patient and other patients (indicated by an asterisk in the top row of Figure 2A)). Late during reinfection, we did not observe increased focusing of the repertoire in SR/CI patients, as compared to the pre-reinfection time point.

A narrower repertoire that becomes more focused upon reinfection in the protected group was further supported by the observation that the top three clonotypes and the top two clonotypes in patients SR/SR-1 and SR/SR-2 represented 28% and 59% of the repertoire pre-reinfection, respectively (Figure 2A and 2B, the dissected pie-charts in the lower rows). Upon reinfection they became more dominant, representing 51% and 96% of the repertoire, respectively. Post reinfection they were more dominat when compared to the pre-reinfection time point, forming 39% and 90% of the repertoire, respectively. On the other hand, the top three clonotypes in the tested SR/CI patients never represented more than 27% of the repertoire, and were sometimes as low as 9% (Figure 3A and 3B, the dissected pie-charts in the lower rows).

Similarly, the Jβ usage was less diverse for SR/SR patients in comparison to SR/CI patients; where the top three TCRBJ alleles utilized at the different time points had an average of 81% in the former group versus 55% in the latter (Figure 4). The top Jβ-gene allele in the SR/SR group was TCRBJ 2.7, and it accounted for 43-97% of the total usage within the TCRβ chains in all clonotypes, versus 23-28% for the top Jβ-gene allele in SR/CI patients.

Distinct clonotypes mobilized against the same epitope in different patients

A study in HIV patients with a similar HLA allele background showed that infection controllers targeted an immunodominant epitope with T-cell clonotypes distinct from those observed in progressors. These clonotypes were characterized by superior control of viral-replication *in vitro* and enhanced cross-reactivity to different variants of the epitope [8]. Some of these clonotypes were recurrently observed in several patients of the controllers' group (6/19) [5]. Whether protective immunity upon HCV reinfection in a human cohort would be associated with specific clonotypes dominating the TCR-repertoire that are distinct from clonotypes observed in the non-protected group is still an area that needs extensive studying.

Our analysis demonstrate that the dominant and subdominant Vβ-CDR3-Jβ detected in the tested patients showed no overlap, where no clonotypes were found to be common between patients targeting the same epitope, whether they belonged to different groups (SR/SR-1 and SR/CI-2 recognizing the HLA-A2 restricted NS3-1073 epitope) (Supplementary Tables S1 and S4) or even when they belonged to the same group (SR/SR-2 and SR/SR-3 recognizing the HLA-B27 restricted NS5B-2841 epitope) (Supplementary Tables S2 and S3). Sequencing the Vβ-chain from more patients is needed, to verify whether some clonotypes are recurrent in the repertoire of different patients from the same group and thus represent "public clonotypes" associated with the outcome. Also, it would allow us to identify whether distinct clonotypes are found in the SR/SR repertoire versus the SR/CI repertoire.

DISCUSSION

Defining the correlates of protective immunity at the clonotypic level is essential for the fine-tuning of the design of prophylactic vaccines against chronic viruses with highly variable sequences such as HIV and HCV. This study represents an initial insight into the dynamics of the virus-specific CD8 T-cell repertoire during HCV reinfection episodes in a real-life setting. Our results suggest that the effector HCV-specific CD8 T-cell clonotypes associated with protection upon reinfection were recruited from the memory population, rather than being *de novo* responses. The repertoire was narrower in the protected patients in comparison to un-protected patients, and it became more focused upon reinfection. As we previously demonstrated, these HCV-specific CD8 T-cells were associated with higher functional avidity and polyfunctional potential than those from un-protected patients [1].

Our longitudinal analysis of the Vβ-chain of the TCR showed a conserved pattern of Vβ usage, where the dominant and sub-dominant clonotypes forming the effector population at the peak of immune response during reinfection were recruited from the pre-existing memory population generated following the clearance of the primary infection, without the appearance of new clonotypes. It has been demonstrated before in the LCMV model that the TCR repertoire of the primary epitope-specific CD8 T-cell response was conserved in the memory pool and the secondary effector recall response, where there was identity between the clonotypes that formed 60-100% of the primary effector, memory and recall responses [18, 19]. Rapid resolution of HCV infection upon rechallenging chimpanzees also coincided with the expansion of T-cell clonotypes that dominated the memory CD8 T-cell pool. These specific TCR clonotypes were tracked through the different HCV challenges [10, 11].

With regards to the diversity of the repertoire, data from the LCMV mouse model and the HCV chimpanzee model suggested that repeated reinfections would selectively expand and enrich cells of higher functional avidity already recruited during the primary infection provided that the targeted epitope remain unchanged[11, 18]. Indeed, our longitudinal analysis of the TCR-repertoire during HCV reinfection demonstrates that the repertoire composition in the tested SR/SR (protected) patients was narrower and less diverse than the repertoire in the SR/CI (unprotected) patients at the time point pre-reinfection and it became more focused at the peak of the immune response during reinfection, and retained a highly focused

composition post reinfection. In contrast, the repertoire in the tested SR/CI patients was more diverse, even at the peak of immune response during reinfection. It has been demonstrated before in the LCMV mouse model that the repertoire of an epitope-specific CD8 T-cell population was highly focused, where 70% of the response against LCMV was due to three clonotypes [18]. In the cytochrome-c antigen system there was narrowing of the T-cell repertoire upon second exposure relative to the primary repertoire, suggesting selection of the clonotypes with highest affinity [20]. Analysis of the TCR repertoire of epitope-specific CD8 T cells in HIV patients also demonstrated narrower and more focused TCR-repertoires of high-avidity populations in patients who were able to control HIV-1 infection compared to progressors [21]. On the other hand, a previous study of an HCV-epitope (NS3-1406) suggested that higher diversity of the repertoire would be more advantageous, otherwise the virus would undergo mutation of the epitope to exploit gaps present in the repertoire [22]. A chimpanzee rechallenge study suggested a similar conclusion, since the majority of HCV-epitopes that escaped immune recognition upon reinfection were targeted by a CD8 T-cell repertoire with reduced CDR3 amino acid diversity. This suggested that it is more advantageous to generate and maintain a diverse TCR repertoire against individual epitopes, since limited TCR diversity would facilitate CTL escape mutations in HCV infected chimpanzees [11]. Unfortunately, there are no previous studies analyzing the TCR-repertoire for HCV-specific T-cells in humans, and results from chimpanzee studies should be taken cautiously, since it was shown that some TCRBV genes in the chimpanzee have been lost in humans. This could be a probable cause of discrepancy of findings between chimpanzee and human studies for responses against the same HCV-epitopes [23]. Another important factor that might cause discrepancy is that the chimpanzees in these studies were rechallenged with a specific and homologous viral sequence and therefore exposed to a less diverse viral infection, which is completely different from natural human infections where the viral sequences circulating within an individual patient and infecting other humans are a mosaic of highly diverse “quasispecies” [24].

Particular clonotypes were found to be preferentially selected in several HIV-infected individuals who controlled viral-replication. These public clonotypes exhibiting higher avidity imposed selective pressure causing early mutation in the epitope that enables the virus to escape their recognition [5, 9]. However, recent data demonstrated that even variant epitopes

could be recognized by highly efficient T-cell clonotypes expressing particular TCRs that are more flexible and thus recognize multiple epitope variants and possess higher functional avidity [5, 25]. Our longitudinal analysis of the dominant and subdominant Vβ-CDR3-Jβ clonotypes in the tested patients showed no overlap, where no clonotypes were found to be common between patients with the same HLA background and targeting the same epitope, even when they belonged to the same group (patients SR/SR-2 and SR/SR-3). It is very early to draw a definitive conclusion about whether or not specific clonotypes could correlate with protection, taking into consideration the limited number of patients included in this study. In HIV studies, up to 19 patients needed to be recruited to show that some public clonotypes were present in 6 patients [5].

We previously demonstrated that protected patients (the SR/SR group) showed higher avidity of HCV-specific T cells to the reference sequence of the targeted epitopes. Unprotected (SR/CI) patients had moderate/low avidity for the reference epitope sequence, but no avidity for the autologous epitope sequence of the reinfecting virus [1] which could be attributed to "original antigenic sin" [26]. One SR/SR patient had a moderate response to one of the variant epitopes, suggesting higher flexibility of the TCR-repertoire. Flexibility in the T cell repertoire correlated with better control of viral escape mutants in both the HCV and HIV field [5, 8, 9, 11] and it would be interesting to validate such results in a reinfection model. We also observed in our previous study that polyfunctionality upon stimulation with minimum peptides correlated with avidity, where patients with highest avidity had the highest functionality and polyfunctionality, and vice versa [1]. This has already been shown within the context of HIV infection, where superior functional avidity of epitope-specific CD8 T cells was interlinked with their high polyfunctional capacity [3].

These results for the TCR-repertoire from peripheral blood mononuclear cells (PBMCs) are expected to reflect what is happening in the liver, since chimpanzees studies showed that the peripheral epitope-specific CD8 T-cell repertoire reflected the intra-hepatic response, where identical clonotypes were present in both the liver and the peripheral circulation, differing only in having a higher magnitude in the liver [10].

In conclusion, our results show that epitope-specific CD8 T-cell clonotypes expanding at the peak of reinfection episodes are recruited from the memory pool, rather than being *de*

novo clonotypes mobilized from the naïve pool. The repertoire is narrower in the protected patients in comparison to unprotected patients, and it becomes more focused upon reinfection, suggesting that distinct TCRs would provide superior control of HCV-infection due to having higher functional avidity and polyfunctionality. These results argue in favour of designing vaccines that focus on immunodominant epitopes that induce distinct public clonotypes possessing superior functional avidity and polyfunctionality. One major constraint of vaccine development for HIV and HCV is the highly heterologous nature of these viruses. It is possible that a vaccination regimen rechallenging humans with different antigenic variants of the epitope may favour "stretching" of the repertoire by mobilizing T-cell clonotypes bearing flexible TCRs that can recognize different variants of the epitope. Analysis of the T-cell repertoire for more patients and for populations targeting other HCV-epitopes would be needed to confirm these results and to verify the presence of public clonotypes conferring protection against HCV persistence. Generating cell-lines from individual clonotypes would enable testing their individual avidity, functionality and antiviral efficacy.

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Table 1: Patients' demographics and sequencing results for the epitopes during reinfection

Patient code	Status at start of follow-up	Age at reinfection (years)	Gender	HCV Genotype (First infection/ reinfection)	HLA Class I	Tetramers	Autologous epitope sequence during reinfection compared to reference sequence*	
							Early	Late
SR/SR-1	HCV Resolver	53	M	ND/1	A2, B58	A2/NS3-1073 A2/NS5B-2594		
SR/SR-2	HCV Resolver	43	M	ND/1	A24/A26, B27/B49	B27/NS5B-2841		
SR/SR-3	HCV Naïve	42	M	1a/1	A23/A32 B27/B44	B27/NS5B-2841		
SR/CI-2	Acute primary HCV	29	M	1a/1a	A2, B39/B44	A2/NS3-1073	CINGVCWTV ----A---- (10/10)	CINGVCWTV ------L–I (8/8)
SR/CI-3	HCV Resolver	30	F	ND/1a	A1/A29 B35/B44	A1/NS3-1436	ATDALMTGY --------F (4/4) **	ATDALMTGY --------F (11/11)

ND = Not determined; SR: Spontaneous Resolution; CI: Chronic Infection

* Dashes indicate identity with the reference sequence of the peptide used in the tetramer

** No. of positive clones/Total no. of clones

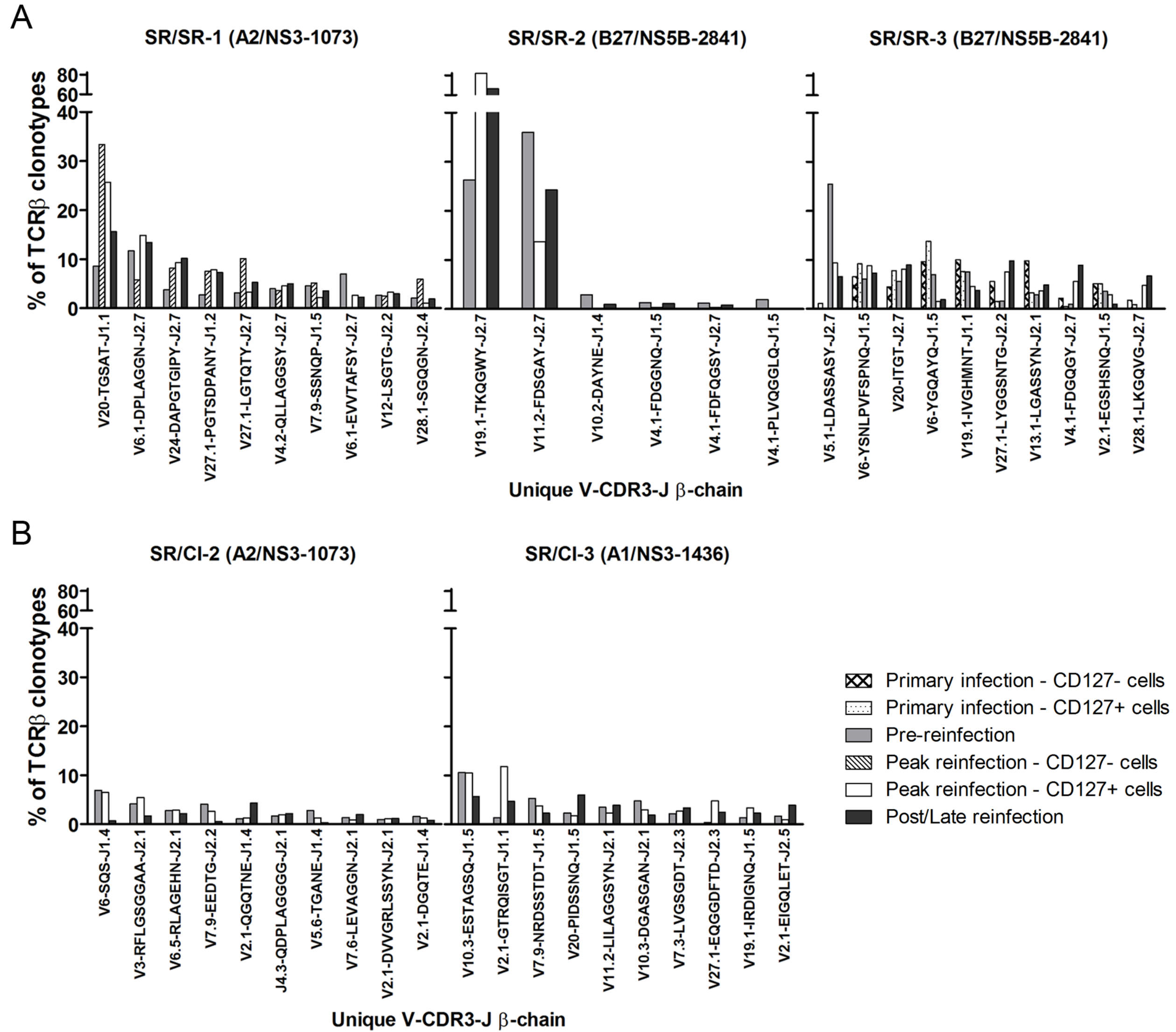
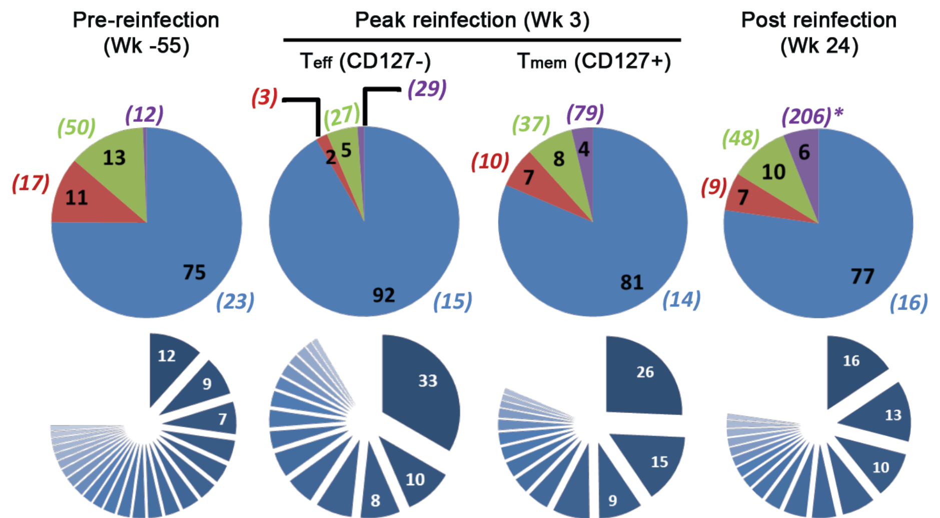


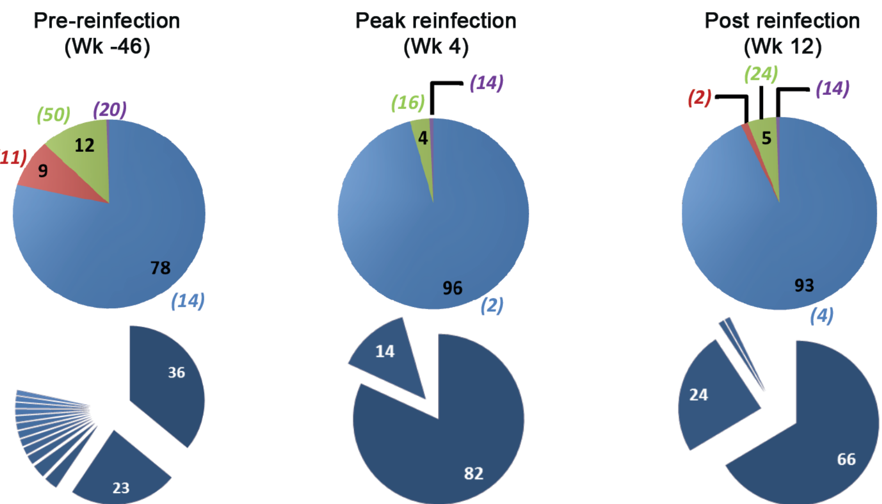
Figure 1

Figure 1: HCV-specific tetramer+ CD8 T-cell clonotypes mobilized during the reinfection episode were exclusively recruited from the pre-existing memory population. The top ten dominant clonotypes (frequency ≥1%) isolated directly *ex vivo* from (A) three SR/SR patients and (B) two SR/CI patients followed-up longitudinally during primary HCV infection (patient SR/SR-3) and reinfection episode at pre-reinfection, peak expansion and post reinfection (all five patients). The numbers between brackets beside the patient's number indicates the epitope for which the T-cell population is specific.

A SR/SR-1



B SR/SR-2



C SR/SR-3

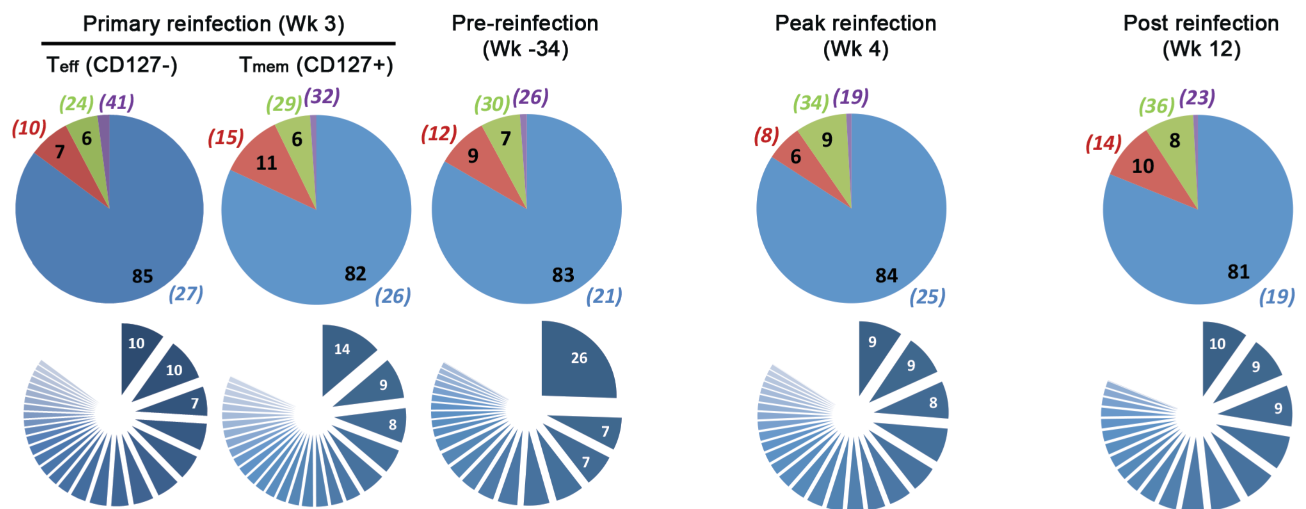
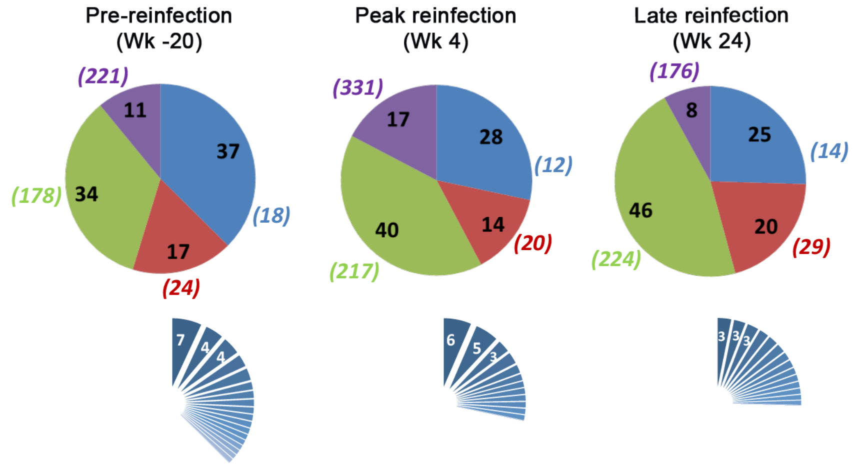
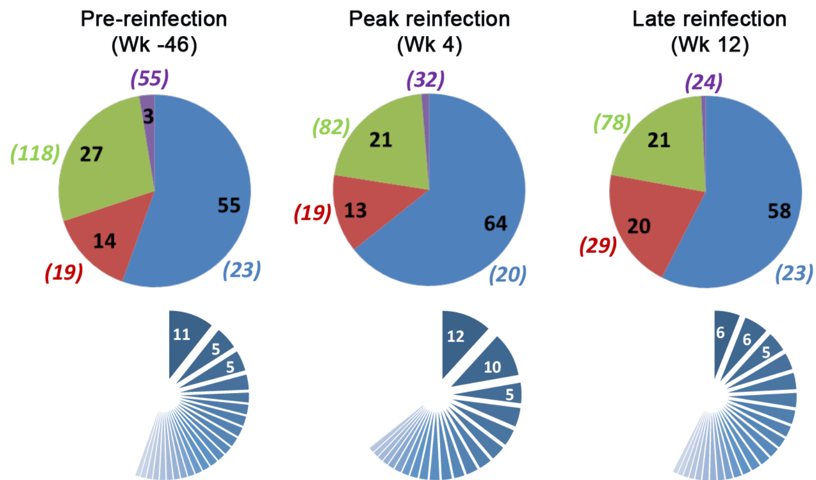


Figure 2

Figure 2: Narrowly focused epitope-specific CD8 T cell repertoire in SR/SR patients. Distribution of the different categories of clonotypes forming the epitope-specific CD8 T cell repertoire for three SR/SR patients at pre-reinfection, peak and post reinfection episode (A) patient SR/SR-1 (B) SR/SR-2 and (C) SR/SR-3. For patient SR/SR-3 there was follow-up during primary HCV infection, as well. The pie charts in the upper rows show the percentage of each category with respect to the total repertoire (dominant, sub-dominant, low-abundance and lowest abundance clonotypes in blue, red, green and violet, respectively). The percentages are represented by black numbers inside the pie. The numbers between brackets around the pie charts represent the number of unique clonotypes forming each category (indicated in the corresponding color). The sliced pie charts in blue at the lower rows represent the dissection of the individual clonotypes forming the dominant category, with the frequency of the most dominant 3 clonotypes written in white inside the corresponding slice.





Dominant (>1%)

Sub-dominant (0.5-0.99%)

Low abundance (0.1-0.49%)

Lowest abundance (<0.1%)

Figure 3

Figure 3: More diverse epitope-specific CD8 T cell repertoire in SR/CI patients. Pie charts showing the distribution of the different categories of clonotypes forming the epitope-specific CD8 T cell repertoire for two SR/CI patients pre-, at the peak and late during the reinfection episode (A) patient SR/CI-2 and (B) SR/CI-3. The pie charts in the upper rows show the percentage of each category (dominant, sub-dominant, low-abundant and lowest abundant clonotypes in blue, red, green and violet, respectively) with respect to the total clonotypes. The percentages are represented by black numbers inside the pie. The numbers between brackets around the pie charts represent the number of unique clonotypes forming each category (indicated in the corresponding color). The sliced pie charts in blue at the lower rows represent the dissection of the individual clonotypes forming the dominant category, with the frequency of the most dominant 3 clonotypes written in white inside the corresponding slice.

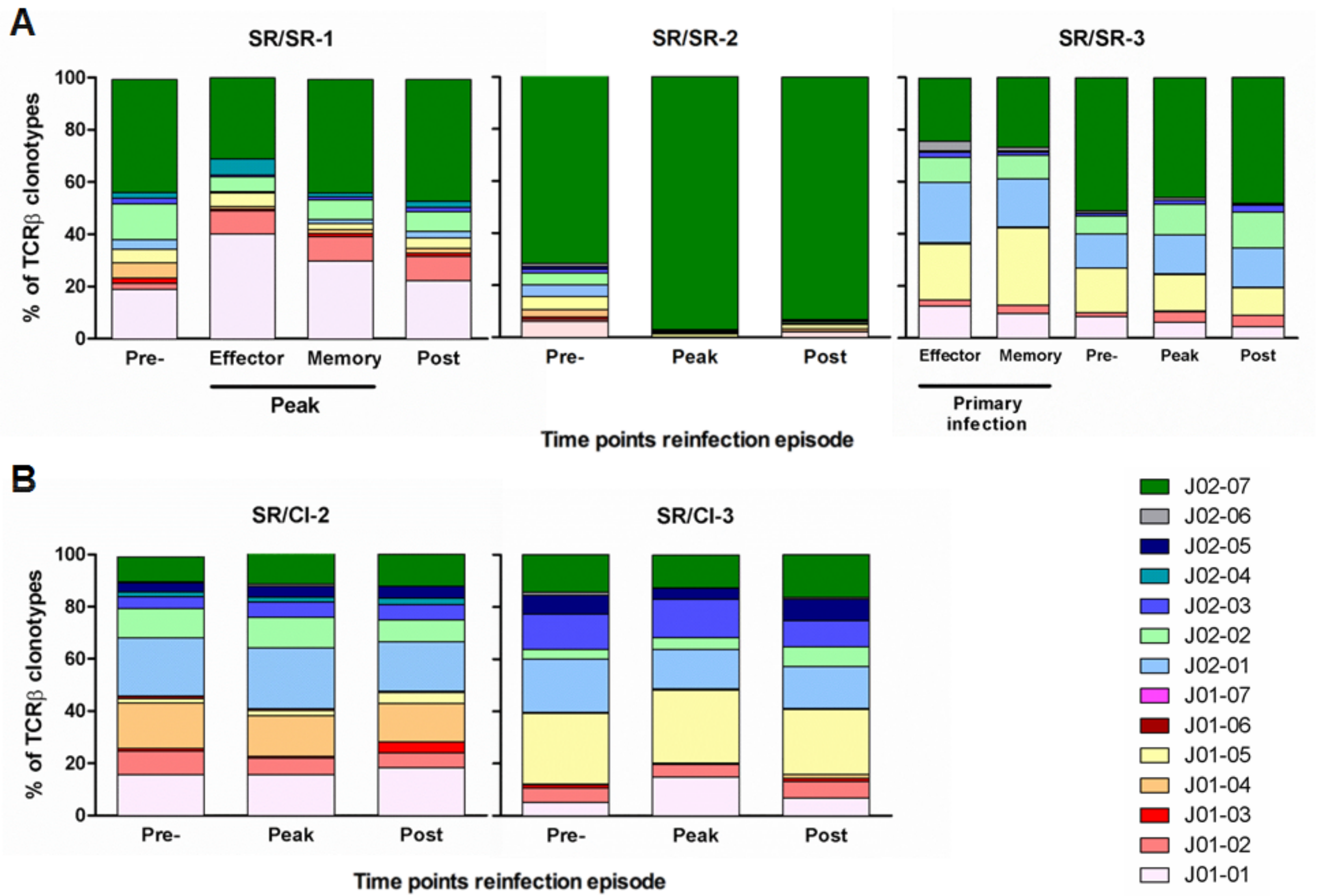


Figure 4

Figure 4: Less diverse TRBV-J usage for epitope-specific CD8 T cell repertoire in SR/SR patients as compared to SR/CI patients. Shown are the percentages of specific J-chain usage for unique clonotypes for (A) three SR/SR patients and (B) two SR/CI patients pre-, at the peak and post/late the reinfection episode.

Supplementary Table 1: Clonotype usage in epitope-specific CD8 T cells for patient SR/SR-1 during an HCV reinfection episode. TRBV and TRBJ usage, CDR3 amino acid sequence, clonotype frequency for the dominant clonotypes (clonotypes present at frequency ≥1%) and the count of TCRβ sequences corresponding to the percentages. The epitope-specific CD8 T cells were isolated directly *ex vivo* from patient SR/SR-1 at time points (A) pre-reinfection, (B) peak reinfection CD127- cells, (C) peak reinfection CD127+ cells and (D) post reinfection, and were analyzed for the TCR-repertoire of epitope-specific CD8 T cells.

(A) Patient SR/SR-1 at pre-reinfection (Wk -55)

TRBV	CDR3	TRBJ	Freq. (%)	Count
06-01	CASSDPLAGGNEQYF	02-07	11.68	7771
20	CSAWTGSATEAFF	01-01	8.55	5686
06-01	CASSEVVTAFSYEQYF	02-07	7	4656
07-09	CASISSNQPQHF	01-05	4.56	3036
04-02	CASSQLLAGGSYEQYF	02-07	4	2662
24	CATSDAPGTGIPYEQYF	02-07	3.78	2517
07-08	CASSLASLGLALSSYEQYF	02-07	3.71	2469
27-01	CASSLGTQTYEQYF	02-07	3.16	2099
03	CASSQDLGLLRDTGELFF	02-02	2.99	1986
27-01	CASSPGTSDPANYGYTF	01-02	2.69	1788
12	CASSLSGTGELFF	02-02	2.66	1769
20	CSAPGPGVSVEKLFF	01-04	2.5	1660
28-01	CASSSGQGNIQYF	02-04	2.05	1366
03	CASSQEPSGSWGEQYF	02-07	2.04	1360
04-01	CASSQAAGVGYPTEAFF	01-01	2.03	1350
10-03	CAISRDSIQFGNTIYF	01-03	1.99	1322
04-03	CASSQEQGAPGELFF	02-02	1.77	1174
14-01	CASSRGPDTEAFF	01-01	1.59	1055
12	CASSFGDQQYF	02-07	1.4	933
24	CATSDQSREQYF	02-07	1.34	890
06	CASSYLFGDANTGELFF	02-02	1.31	872
07-03	CASSLTCDRTHSVYGYTF	01-02	1.16	769
30-01	CAWRLGSGEKLFF	01-04	1.12	743

(B) Patient SR/SR-1 at peak reinfection - CD127- Tet+ Cells (Wk 3)

TRBV	CDR3	TRBJ	Freq. (%)	Count
20	CSAWTGSATEAFF	01-01	33.37	67293
27-01	CASSLGTQTYEQYF	02-07	10.12	20402
24	CATSDAPGTGIPYEQYF	02-07	8.19	16511
27-01	CASSPGTSDPANYGYTF	01-02	7.54	15209
06-01	CASSDPLAGGNEQYF	02-07	5.8	11692
07-09	CASISSNQPQHF	01-05	5.16	10411
28-01	CASSSGQGNIQYF	02-04	3.57	7208
04-02	CASSQLLAGGSYEQYF	02-07	3.57	7197
14-01	CASSRGPDTEAFF	01-01	3.16	6376
unresolved	CASSLSGTGELFF	02-02	2.45	4947
28-01	CASSSGQGNIQYF	02-04	2.37	4785
03	CASSQDLGLLRDTGELFF	02-02	2.02	4069
20	CSAQTGRDTEAFF	01-01	1.94	3909
05-06	CASSQGGDRGDPGDGYTF	01-02	1.23	2488
07-08	CASSLASLGLALSSYEQYF	02-07	1.09	2193

(C) Patient SR/SR-1 at peak reinfection - CD127+ Tet+ Cells (Wk 3)

TRBV	CDR3	TRBJ	Freq. (%)	Count
20	CSAWTGSATEAFF	01-01	25.68	57007
06-01	CASSDPLAGGNEQYF	02-07	14.86	32989
04	CATSDAPGTGIPYEQYF	02-07	9.26	20554
27-01	CASSPGTSDPANYGYTF	01-02	7.86	17448
04-02	CASSQLLAGGSYEQYF	02-07	4.54	10070
27-01	CASSLGTQTYEQYF	02-07	3.28	7280
12	CASSLSGTGELFF	02-02	3.27	7249
06-01	CASSEVVTAFSYEQYF	02-07	2.63	5831
03	CASSQDLGLLRDTGELFF	02-02	2.53	5614
07-09	CASISSNQPQHF	01-05	2.2	4875
03	CASSQEPSGSWGEQYF	02-07	1.86	4133
07-08	CASSLASLGLALSSYEQYF	02-07	1.26	2787
14-01	CASSRGPDTEAFF	01-01	1.14	2540
28-01	CASSSGQGNIQYF	02-04	1.07	2366

(D) Patient SR/SR-1 at post reinfection (Wk 24)

TRBV	CDR3	TRBJ	Freq. (%)	Count
20	CSAWTGSATEAFF	01-01	15.62	78987
06-01	CASSDPLAGGNEQYF	02-07	13.45	68019
24	CATSDAPGTGIPYEQYF	02-07	10.14	51281
27-01	CASSPGTSDPANYGYTF	01-02	7.25	36680
27-01	CASSLGTQTYEQYF	02-07	5.25	26560
04-02	CASSQLLAGGSYEQYF	02-07	4.98	25169
07-09	CASISSNQPQHF	01-05	3.56	17975
12	CASSLSGTGELFF	02-02	2.94	14843
06-01	CASSEVVTAFSYEQYF	02-07	2.28	11524
07-08	CASSLASLGLALSSYEQYF	02-07	2.19	11083
28-01	CASSSGQGNIQYF	02-04	1.89	9543
03	CASSQDLGLLRDTGELFF	02-02	1.74	8775
14-01	CASSRGPDTEAFF	01-01	1.69	8569
04-01	CASSQAAGVGYPTEAFF	01-01	1.62	8186
03	CASSQEPSGSWGEQYF	02-07	1.51	7629
05-06	CASSQGGDRGDPGDGYTF	01-02	1.15	5800

Supplementary Table 2: Clonotype usage in epitope-specific CD8 T cells for patient SR/SR-2 during an HCV reinfection episode. TRBV and TRBJ usage, CDR3 amino acid sequence, clonotype frequency for the dominant clonotypes (clonotypes present at frequency ≥1%) and the count of TCRβ sequences corresponding to the percentages. The epitope-specific CD8 T cells were isolated directly *ex vivo* from patient SR/SR-2 at time points (A) pre-reinfection, (B) peak reinfection and (C) post reinfection, and were analyzed for the TCR-repertoire of epitope-specific CD8 T cells.

(A) Patient SR/SR-2 at pre-reinfection (Wk -46)

TRBV	CDR3	TRBJ	Freq. (%)	Count
11-02	CASSFDSGAYEQYF	02-07	35.97	13631
19-01	CASSTKQGWYEQYF	02-07	23.46	8889
10-02	CASSDAYNEKLFF	01-04	2.9	1098
19-01	CASSTKQGWYEQYF	02-07	2.81	1065
04-01	CASSPLVQGGLQPQHF	01-05	1.9	720
02-01	CASSEGTTNTGELFF	02-02	1.52	575
07-03	CASSWDSGAEAFF	01-01	1.45	549
05-06	CASSLKAVVTGELFF	02-02	1.44	544
04-01	CASSFDGGNQPQHF	01-05	1.3	493
06	CASSIDKLNTEAFF	01-01	1.22	464
04-01	CASSFDFQGSYEQYF	02-07	1.16	439
04-01	CASSQVHLGQSTLNTEAFF	01-01	1.12	425
06	CASALRGANVLTF	02-06	1.07	404
27-01	CASSPYGGSNTGELFF	02-02	1.05	397

(B) Patient SR/SR-2 at peak reinfection (Wk 4)

TRBV	CDR3	TRBJ	Freq. (%)	Count
19-01	CASSTKQGWYEQYF	02-07	81.88	68836
11-02	CASSFDSGAYEQYF	02-07	13.7	11514

(C) Patient SR/SR-2 at post reinfection (Wk 12)

TRBV	CDR3	TRBJ	Freq. (%)	Count
19-01	CASSTKQGWYEQYF	02-07	66.44	40660
11-02	CASSFDSGAYEQYF	02-07	24.29	14863
04-01	CASSFDGGNQPQHF	01-05	1.07	656
10-02	CASSDAYNEKLFF	01-04	1.02	625

Supplementary Table 3: Clonotype usage in epitope-specific CD8 T cells for patient SR/SR-3 during an HCV reinfection episode. TRBV and TRBJ usage, CDR3 amino acid sequence, clonotype frequency for the dominant clonotypes (clonotypes present at frequency ≥1%) and the count of TCRβ sequences corresponding to the percentages. The epitope-specific CD8 T cells were isolated directly *ex vivo* from patient SR/SR-3 at time points at (A) primary infection CD127- cells, (B) primary infection CD127+ cells (C) pre-reinfection, (D) peak reinfection and (E) post reinfection, and were analyzed for the TCR-repertoire of epitope-specific CD8 T cells.

(A) Patient SR/SR-3 at primary infection - CD127- Tet+ Cells (Wk -53)

TRBV	CDR3	TRBJ	Freq. (%)	Count
13-01	CASRLGASSYNEQFF	02-01	9.83	17131
06	CASSYGQAYQPQHF	01-05	9.56	16650
06	CASSYSNLPVFSPNQPQHF	01-05	6.58	11465
19-01	CASSIVGHMNTEAFF	01-01	6.06	10549
27-01	CASSLYGGSNTGELFF	02-02	5.62	9791
02-01	CASSEGSHSNQPQHF	01-05	5.15	8966
20	CSASITGTEQYF	02-07	4.51	7852
19-01	CASSIVGHLNTEAFF	01-01	3.87	6737
04-03	CASSRARGSTEVREQFF	02-01	3.8	6619
12-05	CASGYAGANVLTF	02-06	3.47	6042
05-01	CASSFDRGGYEQYF	02-07	2.56	4451
04-01	CASSFDGQGYEQYF	02-07	2.2	3833
18-01	CASSPATGTEAFF	01-01	2.18	3799
05-06	CASSLNLGAGNNEQFF	02-01	2.1	3657
29-01	CSVGGTGTYGYTF	01-02	2	3486
28-01	CASSLKGQVGEQYF	02-07	1.77	3079
09-01	CASSVSPDNTDTQYF	02-03	1.48	2571
28-01	CASSLEGSAGELFF	02-02	1.42	2469
10-03	CAISESGQALITYEQYF	02-07	1.42	2468
05-01	CASSPGTTNTGELFF	02-02	1.33	2323
11-02	CASSLASNEQFF	02-01	1.33	2312
04-01	CASSHDTGGYEQYF	02-07	1.26	2202
11-02	CASSFLGGLDEQFF	02-01	1.21	2113
04-03	CASSFDRGGYEQYF	02-07	1.21	2110
05-01	CASSLDASSASYEQYF	02-07	1.13	1974
06-05	CASSSGLALVFEQFF	02-01	1.1	1910
04-01	CASSFSSGGYEQYF	02-07	1.04	1813

(B) Patient SR/SR-3 at primary infection - CD127+ Tet+ Cells (Wk -53)

TRBV	CDR3	TRBJ	Freq. (%)	Count
06	CASSYGQAYQPQHF	01-05	13.78	16767
06	CASSYSNLPVFSPNQPQHF	01-05	9.21	11203
20	CSASITGTEQYF	02-07	7.78	9466
19-01	CASSIVGHLNTEAFF	01-01	5.46	6638
02-01	CASSEGSHSNQPQHF	01-05	5.08	6184
13-01	CASRLGASSYNEQFF	02-01	3.3	4013
04-01	CASTAGSNTGELFF	02-02	2.7	3288
29-01	CSVGGTGTYGYTF	01-02	2.64	3210
05-06	CASSLNLGAGNNEQFF	02-01	2.49	3030
11-02	CASSFLGGLDEQFF	02-01	2.4	2922
28-01	CASSLEGSAGELFF	02-02	2.2	2678
05-01	CASSFDRGGYEQYF	02-07	2.08	2531
11-02	CASSLAVSSEQFF	02-01	2.07	2524
19-01	CASSIVGHMNTEAFF	01-01	2.07	2516
05-06	CASSLGLPGLAGAYEQYF	02-07	2.03	2475
04-01	CASSFDTGGYEQYF	02-07	1.93	2345
06	CASSWTAIEQYF	02-07	1.77	2159
18-01	CASSPATGTEAFF	01-01	1.77	2148
05-01	CASSPGTTNTGELFF	02-02	1.7	2074
06-05	CASSSGLALVFEQFF	02-01	1.61	1953
11-02	CASSLASNEQFF	02-01	1.55	1890
27-01	CASSLYGGSNTGELFF	02-02	1.47	1792
12-05	CASGYAGANVLTF	02-06	1.35	1644
10-03	CAISESGQALITYEQYF	02-07	1.25	1519
04-03	CASSRARGSTEVREQFF	02-01	1.17	1427
04-03	CASSFDRGGYEQYF	02-07	1.08	1316

(C) Patient SR/SR-3 at pre-reinfection (Wk -34)

TRBV	CDR3	TRBJ	Freq. (%)	Count
05-01	CASSLDASSASYEQYF	02-07	25.51	23533
05-01	CASREDGGGYEQYF	02-07	7.03	6486
06	CASSYGQAYQPQHF	01-05	6.96	6418
06	CASSYSNLPVFSPNQPQHF	01-05	6.07	5597
20	CSASITGTEQYF	02-07	5.55	5122
19-01	CASSIVGHLNTEAFF	01-01	4.23	3898
02-01	CASSEGSHSNQPQHF	01-05	3.56	3279
19-01	CASSIVGHMNTEAFF	01-01	3.25	3002
13-01	CASRLGASSYNEQFF	02-01	2.86	2637
05-06	CASSLNLGAGNNEQFF	02-01	2.69	2478
28-01	CASSLEGSAGELFF	02-02	2.61	2411
27-01	CASSLYGGSNTGELFF	02-02	1.62	1496
29-01	CSVGGTGTYGYTF	01-02	1.51	1391
27-01	CASSLYRVGYNEQFF	02-01	1.48	1366
11-02	CASSFLGGLDEQFF	02-01	1.44	1328
05-01	CASSPGTTNTGELFF	02-02	1.36	1251
05-01	CASSFDRGGYEQYF	02-07	1.32	1218
05-06	CASSLGLPGLAGAYEQYF	02-07	1.21	1113
10-02	CASCDNTGYEQYF	02-07	1.06	974
11-02	CASSLAVSSEQFF	02-01	1.03	953
04-01	CASSFDGQGYEQYF	02-07	1.03	952

(D) Patient SR/SR-3 at peak reinfection (Wk 4)

TRBV	CDR3	TRBJ	Freq. (%)	Count
05-01	CASSLDASSASYEQYF	02-07	9.43	7774
06	CASSYSNLPVFSPNQPQHF	01-05	8.81	7265
20	CSASITGTEQYF	02-07	8.08	6662
27-01	CASSLYGGSNTGELFF	02-02	7.46	6150
04-01	CASSFDGQGYEQYF	02-07	5.63	4644
28-01	CASSLKGQVGEQYF	02-07	4.77	3931
13-01	CASRLGASSYNEQFF	02-01	3.7	3049
05-01	CASSFDRGGYEQYF	02-07	3.56	2933
19-01	CASSIVGHLNTEAFF	01-01	3.49	2878
05-01	CASREDGGGYEQYF	02-07	3.44	2840
28-01	CASSLEGSAGELFF	02-02	3.16	2606
02-01	CASSEGSHSNQPQHF	01-05	2.88	2373
04-03	CASSRARGSTEVREQFF	02-01	2.31	1905
27-01	CASSLYRVGYNEQFF	02-01	2.07	1711
29-01	CSVGGTGTYGYTF	01-02	1.86	1533
11-02	CASSLAVSSEQFF	02-01	1.83	1513
11-02	CASSLASNEQFF	02-01	1.81	1494
04-03	CASSQSSMQDPYGYTF	01-02	1.55	1278
06	CASSYGQAYQPQHF	01-05	1.5	1235
04-03	CASSFDRGGYEQYF	02-07	1.39	1144
06	CASSWTAIEQYF	02-07	1.19	982
19-01	CASSIVGHMNTEAFF	01-01	1.1	909
02-01	CASNFDKGGYEQYF	02-07	1.07	885
09-01	CASSVSPDNTDTQYF	02-03	1.06	871
29-01	CSVAHTGTEQYF	02-07	1.03	847

(E) Patient SR/SR-3 at post reinfection (Wk 12)

TRBV	CDR3	TRBJ	Freq. (%)	Count
27-01	CASSLYGGSNTGELFF	02-02	9.75	7078
20	CSASITGTEQYF	02-07	9.04	6562
04-01	CASSFDGQGYEQYF	02-07	8.87	6438
V06	CASSYSNLPVFSPNQPQHF	01-05	7.31	5309
28-01	CASSLKGQVGEQYF	02-07	6.71	4870
05-01	CASSLDASSASYEQYF	02-07	6.56	4762
13-01	CASRLGASSYNEQFF	02-01	4.86	3525
05-01	CASSFDRGGYEQYF	02-07	3.85	2792
05-01	CASREDGGGYEQYF	02-07	3.51	2546
28-01	CASSLEGSAGELFF	02-02	2.87	2085
19-01	CASSIVGHLNTEAFF	01-01	2.5	1813
04-03	CASSQSSMQDPYGYTF	01-02	2.41	1750
27-01	CASSLYRVGYNEQFF	02-01	2.29	1663
11-02	CASSLASNEQFF	02-01	2.22	1614
09-01	CASSVSPDNTDTQYF	02-03	2.01	1461
06	CASSYGQAYQPQHF	01-05	1.89	1374
11-02	CASSLAVSSEQFF	02-01	1.72	1252
29-01	CSVGGTGTYGYTF	01-02	1.47	1068
19-01	CASSIVGHMNTEAFF	01-01	1.26	917

Supplementary Table 4: Clonotype usage in epitope-specific CD8 T cells for patient SR/CI-2 during an HCV reinfection episode. TRBV and TRBJ usage, CDR3 amino acid sequence, clonotype frequency for the dominant clonotypes (clonotypes present at frequency ≥1%) and the count of TCRβ sequences corresponding to the percentages. The epitope-specific CD8 T cells were isolated directly *ex vivo* from patient SR/CI-2 at time points (A) pre-reinfection, (B) peak reinfection and (C) late during reinfection, and were analyzed for the TCR-repertoire of epitope-specific CD8 T cells.

(A) Patient SR/CI-2 at pre-reinfection (Wk -20)

TRBV	CDR3	TRBJ	Freq. (%)	Count
06	CASTSQSELFF	01-04	6.91	21786
03	CASSRFLGSGGAAEQFF	02-01	4.22	13302
07-09	CASLEEDTGELFF	02-02	4.13	13023
05-06	CASGTGANEKLFF	01-04	2.85	8983
06-05	CASIRLAGEHNEQFF	02-01	2.77	8742
04-03	CASSQDPLAGGGGEQFF	02-01	1.71	5397
02-01	CASSDGQTEKLFF	01-04	1.62	5105
24	CANRDRGRDEQFF	02-01	1.54	4860
07-06	CASSLEVAGGNEQFF	02-01	1.38	4360
06-05	CASVGGNYGYTF	01-02	1.36	4282
06-06	CASSYSAGTLDYGYTF	01-02	1.32	4149
04-02	CASSQQGEKLFF	01-04	1.24	3912
02-01	CASSQGQTNEKLFF	01-04	1.14	3605
05-06	CASSTGGGTEAFF	01-01	1.11	3505
06-01	CASSELGTGDYEQYF	02-07	1.06	3341
05-04	CASSLPQGRYMNTEAFF	01-01	1.04	3286
02-01	CASRDVVGRLSSYNEQFF	02-01	1.02	3201
06-06	CASTRDTEAFF	01-01	1	3168

(B) Patient SR/CI-2 at peak reinfection (Wk 10)

TRBV	CDR3	TRBJ	Freq. (%)	Count
06	CASTSQSELFF	01-04	6.47	30139
03	CASSRFLGSGGAAEQFF	02-01	5.44	25332
06-05	CASIRLAGEHNEQFF	02-01	2.86	13300
07-09	CASLEEDTGELFF	02-02	2.68	12479
04-03	CASSQDPLAGGGGEQFF	02-01	1.91	8907
04-02	CASSPEQGPLEAFF	01-01	1.42	6618
24	CANRDRGRDEQFF	02-01	1.31	6083
06	CASSPWAVGAMNTEAFF	01-01	1.31	6082
06-01	CASSELGTGDYEQYF	02-07	1.3	6074
05-06	CASGTGANEKLFF	01-04	1.28	5974
02-01	CASSDGQTEKLFF	01-04	1.25	5833
02-01	CASRDVVGRLSSYNEQFF	02-01	1.11	5152

(C) Patient SR/CI-2 at late reinfection (Wk 66)

TRBV	CDR3	TRBJ	Freq. (%)	Count
04-01	CASSQGLPNEKLFF	01-04	3.18	8151
02-01	CASSQGQTNEKLFF	01-04	2.68	6872
05-04	CASSLEQGAVDGNTIYF	01-03	2.63	6745
06-05	CASIRLAGEHNEQFF	02-01	2.18	5584
04-03	CASSQDPLAGGGGEQFF	02-01	2.15	5520
07-06	CASSLEVAGGNEQFF	02-01	1.99	5100
03	CASSRFLGSGGAAEQFF	02-01	1.7	4353
02-01	CASSQGQTNEKLFF	01-04	1.62	4155
05-06	CASSLRSDTEAFF	01-01	1.38	3530
21-01	CASSKQTGTGETKNIQYF	02-04	1.37	3514
19-01	CASSTPPGVNSNQPQHF	01-05	1.29	3313
02-01	CASRDVVGRLSSYNEQFF	02-01	1.22	3123
19-01	CASGWPGGLNSNQPQHF	01-05	1.09	2798
06-06	CASSSAPEGLSYEQYF	02-07	1	2567

Table 5: Clonotype usage in epitope-specific CD8 T cells for patient SR/CI-3 during an HCV reinfection episode. TRBV and TRBJ usage, CDR3 amino acid sequence, clonotype frequency for the dominant clonotypes (clonotypes present at frequency ≥1%) and the count of TCRβ sequences corresponding to the percentages. The epitope-specific CD8 T cells were isolated directly *ex vivo* from patient SR/CI-3 at time points (A) pre-reinfection, (B) peak reinfection and (C) late during reinfection, and were analyzed for the TCR-repertoire of epitope-specific CD8 T cells.

(A) Patient SR/CI-3 at pre-reinfection (Wk -37)

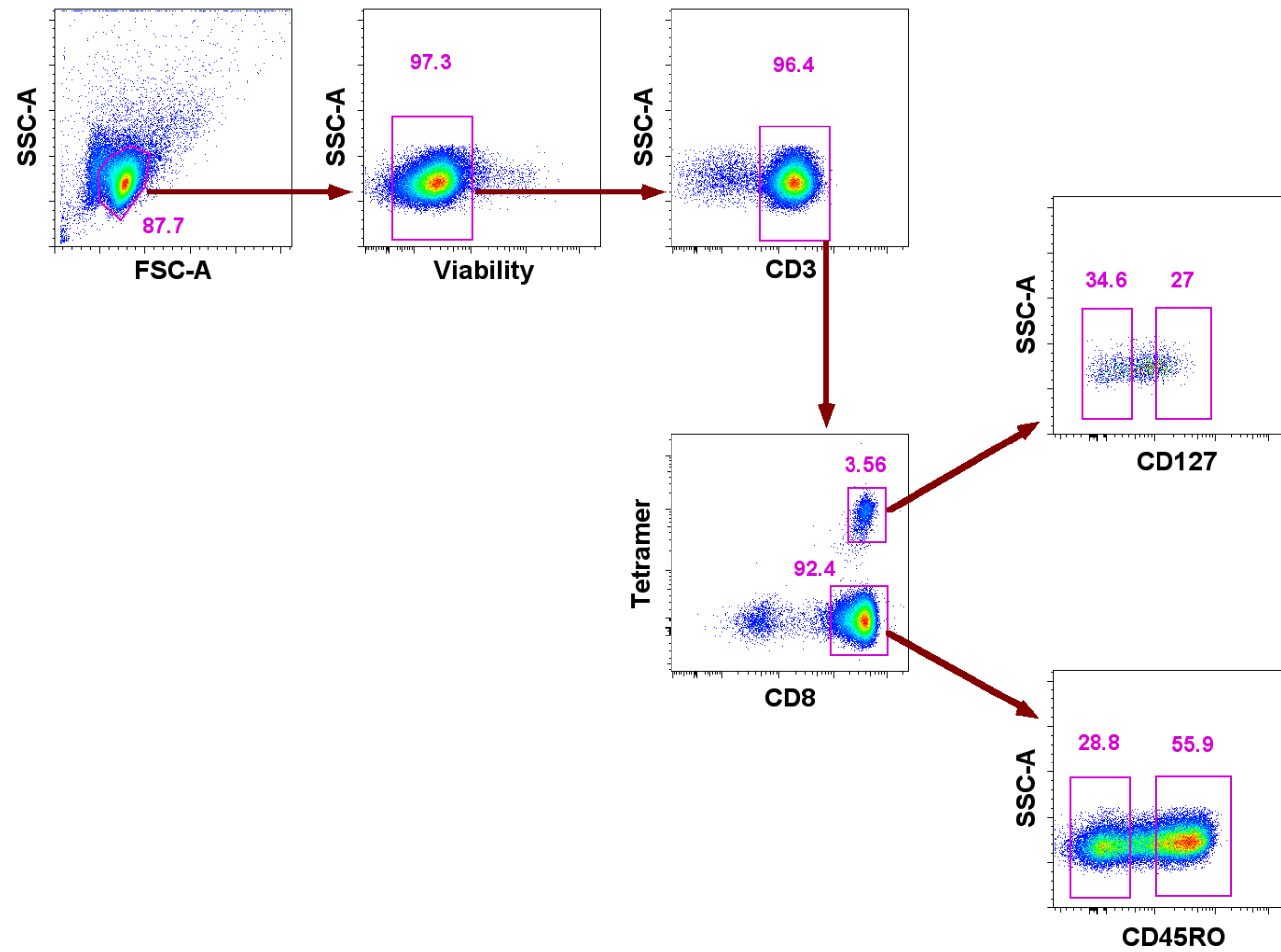
TRBV	CDR3	TRBJ	Freq. (%)	Count
10-03	CAISESTAGSQPQHF	01-05	10.65	4989
07-09	CAGNNRDSSTDTQYF	02-03	5.34	2501
10-03	CAITDGASGANEQFF	02-01	4.83	2263
11-02	CASSLILAGGSYNEQFF	02-01	3.53	1656
10-03	CAISESPQSYEQYF	02-07	2.3	1077
20	CSARPIDSSNQPQHF	01-05	2.25	1056
02-01	CASSEGEINNQPQHF	01-05	2.23	1047
05-01	CASRGDTFYEQYF	02-07	2.17	1019
07-03	CASSLVGSGDTQYF	02-03	2.17	1016
12	CASSIAGPPYNEQFF	02-01	1.83	856
24	CATSDASLSSYNEQFF	02-01	1.77	831
28-01	CASRTPGHLYEQYF	02-07	1.75	818
02-01	CASSEIGQLETQYF	02-05	1.66	780
05-01	CASSWWETSHEQFF	02-01	1.51	706
09-01	CASSPASSSFYEQYF	02-07	1.46	685
19-01	CASSIRDIGNQPQHF	01-05	1.37	644
27-01	CASSLRTAGYNEQFF	02-01	1.35	635
02-01	CASSGTRQISGTEAFF	01-01	1.35	633
21-01	CASSTGLAAQETQYF	02-05	1.32	620
16-01	CASSQSDLTAKQPQHF	01-05	1.29	603
05-01	CASSPWTSGGAYNEQFF	02-01	1.2	561
06	CASRSLLGTRVETEAFF	01-01	1.14	535
05-01	CASSLWGADGYTF	01-02	1	468

(B) Patient SR/CI-3 at Peak reinfection (Wk 5)

TRBV	CDR3	TRBJ	Freq. (%)	Count
02-01	CASSGTRQISGTEAFF	01-01	11.76	4708
10-03	CAISESTAGSQPQHF	01-05	10.46	4187
27-01	CASIEQGGDFTDTQYF	02-03	4.84	1938
06-01	CASSETGQTYEQYF	02-07	4.42	1771
07-09	CAGNNRDSSTDTQYF	02-03	3.85	1541
19-01	CASSIRDIGNQPQHF	01-05	3.42	1369
10-03	CAITDGASGANEQFF	02-01	2.95	1182
07-03	CASSLVGSGDTQYF	02-03	2.71	1086
04-01	CASSQEGSDNQPQHF	01-05	2.44	975
11-02	CASSLILAGGSYNEQFF	02-01	2.33	933
09-01	CASSGGLINTGELFF	02-02	2.29	915
12	CASSIAGPPYNEQFF	02-01	1.92	770
20	CSAREIASSNQPQHF	01-05	1.76	703
06	CASRSLLGTRVETEAFF	01-01	1.59	635
09-01	CASSPASSSFYEQYF	02-07	1.58	631
06	CASSPTGPFYGYTF	01-02	1.37	547
16-01	CASSQSDLTAKQPQHF	01-05	1.19	478
10-03	CAISESAVGYNEQFF	02-01	1.19	477
24	CATSDASLSSYNEQFF	02-01	1.14	458
05-01	CASSPWTSGGAYNEQFF	02-01	1.11	445

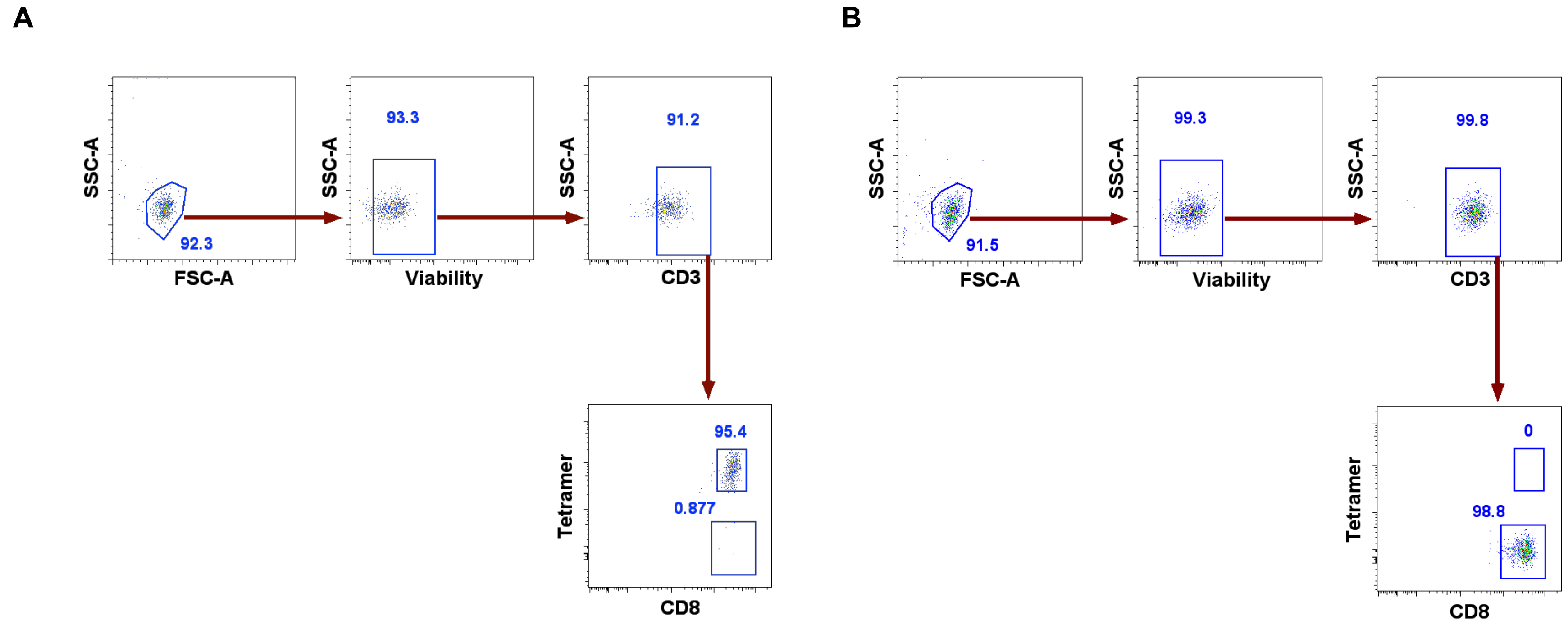
(C) Patient SR/CI-3 at late reinfection (Wk 41)

TRBV	CDR3	TRBJ	Freq. (%)	Count
20	CSARPIDSSNQPQHF	01-05	6	1737
10-03	CAISESTAGSQPQHF	01-05	5.73	1658
02-01	CASSGTRQISGTEAFF	01-01	4.69	1356
11-02	CASSLILAGGSYNEQFF	02-01	3.91	1130
02-01	CASSEIGQLETQYF	02-05	3.85	1114
07-03	CASSLVGSGDTQYF	02-03	3.36	972
09-01	CASSSAVIVPGELFF	02-02	3.19	923
27-01	CASIEQGGDFTDTQYF	02-03	2.53	732
09-01	CASSGGLINTGELFF	02-02	2.34	677
19-01	CASSIRDIGNQPQHF	01-05	2.31	668
07-09	CAGNNRDSSTDTQYF	02-03	2.27	656
12	CASSIAGPPYNEQFF	02-01	2.03	588
10-03	CAITDGASGANEQFF	02-01	1.87	541
10-03	CAISESPQSYEQYF	02-07	1.76	508
02-01	CASSDPLGQGFSYEQYF	02-07	1.56	452
20	CSAREIASSNQPQHF	01-05	1.52	439
05-01	CASSLWGADGYTF	01-02	1.49	430
05-01	CASRGDTFYEQYF	02-07	1.43	415
09-01	CASSPASSSFYEQYF	02-07	1.35	390
10-03	CAISESAVGYNEQFF	02-01	1.14	330
06-01	CASSDTGTHIYEQYF	02-07	1.11	322
07-03	CASSSAPGQLETQYF	02-05	1.07	309
04-01	CASSQEGSDNQPQHF	01-05	1.03	299



Supplementary Figure 1

Supplementary Figure 1: The gating strategy used for the sorting of different epitope-specific tetramer+ CD8+ T-cell populations, as well as naïve CD8+ T cells to be used as a control.



Supplementary Figure 2

Supplementary Figure 2: Verification of the purity of sorted T-cell populations. Dot plots showing the post-sorting purity of the sorted (A) epitope-specific tetramer+ CD8+ T-cell population and (B) naïve CD8+ T cells.

CHAPTER 5:

DISCUSSION AND CONCLUSION

The objective of this study was to define the general characteristics of the adaptive immune responses associated with protection upon reinfection with HCV. HCV represents a unique model that enables studying the correlates of immune protection against chronic viruses, since ~30% of infected patients are able to resolve primary HCV infection spontaneously. It has been shown before that humans and chimpanzees who cleared primary HCV infection or who received trial-vaccines generated long-lived memory T-cells. Nevertheless, their protective potential upon re-exposure to HCV was not consistent in all the studies conducted, where some subjects who successfully cleared a primary HCV-infection developed chronic infection when re-exposed to HCV, especially upon reinfection with heterologous viral sequences [338, 339, 350, 351]. More important, immunological characteristics and signatures associated with protection upon reinfection remain largely undefined. These correlates of protective immunity represent the most crucial factors to be considered and modeled in vaccine design. Even in chimpanzees and humans clearing the reinfecting virus, this occurred after a brief infection period, indicating the absence of sterilizing immunity that was observed for other viruses where the immune response neutralizes the pathogen before establishing an infection. Thus, it is suggested that for complex chronic viruses such as HCV and HIV it is rather protective immunity that could be achieved through vaccination to prevent the establishment of persistent infection upon exposure.

We recruited a group of IDUs who resolved a primary HCV infection and were still at risk of being reinfected with HCV due to sharing needles with HCV-positive individuals. Several subjects among them got reinfected and longitudinal blood samples during the reinfection episode were available for nine patients who had a differential outcome of the second infection. This represented a great advantage by allowing us to compare the different characteristics of the immune response in patients who resolved the second infection (SR/SR group, n= 5) versus a group of patients who became persistently infected upon reinfection under the same conditions (SR/CI group, n= 4) (Manuscript 1 – Supplementary Table S1). This rare cohort of patients and the available longitudinal samples allowed us to address several issues pertaining to protective immunity against chronic viruses in a human setting. Specifically, it enabled us to perform an exhaustive longitudinal phenotypic and functional

characterization of the HCV-specific memory immune responses for both CD4 and CD8 T cells, as well as for epitope specific CD8 T cells during the reinfection episode. This unique group of patients also enabled us to longitudinally analyse the repertoire of epitope-specific CD8 T cells and define its dynamics upon reinfection with different viral variants and the correlation with the avidity and polyfunctionality of these populations, and how these factors are associated with protection. This also allowed us to verify the role of pre-existing memory versus *de novo* T cell responses.

Our results demonstrate that protection from viral-persistence upon reinfection with HCV was associated with higher magnitude and breadth of the cell-mediated immune response, higher functionality/polyfunctionality and proliferative capacity of HCV-specific CD4 and CD8 T cells, and expansion of polyfunctional epitope-specific CD8 T_{EM} cells. We also show that for epitope-specific CD8 T cells, a more focused TCR-repertoire composed of distinct clonotypes with higher functional avidity was associated with protection.

5.1. Correlation between total magnitude and breadth of T-cell responses and protective immunity upon HCV reinfection

Previous studies from our laboratory, as well as other groups have demonstrated an association between the magnitude and breadth of the immune response and spontaneous resolution of primary HCV infection [243, 250]. It was even shown that there was an association between the overall magnitude of response to certain immunodominant non-structural regions of the HCV genome and spontaneous resolution [250]. Another study in an IDU cohort also demonstrated that CD4 T-cell responses predominantly targeting non-structural proteins were associated with resolved HCV infection [254].

We monitored the overall magnitude and breadth of the HCV-specific T-cell responses in incident cases of reinfection before and during the second infection using an IFNγ–ELISPOT assay. Our results showed that the overall magnitude and breadth were higher in the SR/SR group as compared to the SR/CI group (Manuscript 1 - Figure 1). The average total frequency of IFNγ–producing HCV-specific T-cells at the earliest time point tested following reinfection was 5-folds higher in the SR/SR group as compared to the SR/CI group.

The responses to pools representing the structural and non-structural region of HCV were higher for the SR/SR group than the SR/CI group (11-folds and 4-folds, respectively).

The breadth of the immune response, measured by the number of peptide pools targeted by the immune response, was also higher in the SR/SR group at the earliest time point during reinfection. An average of 10 out of 11 peptide pools were targeted in the SR/SR group (representing 9 out of the 10 HCV regions) versus 7 pools for the SR/CI group (Manuscript 1 – Figure 1). In patients for whom samples prior to reinfection were available, a higher increase in the magnitude and breadth of the immune response to HCV-peptides was observed in the SR/SR patients, when compared to their levels prior to reinfection. The *de novo* responses were almost all within the non-structural region (data not shown).

In summary, protection from viral persistence upon HCV reinfection was associated with a higher magnitude and breadth of the total HCV-specific T-cell response as measured by ELISPOT assay. In the SR/SR group, viral clearance upon reinfection was associated with expansion of the HCV-specific memory T cells, in particular cells targeting the non-structural proteins, where the increase in the magnitude and the *de novo* responses were more evident in response to peptide pools of the non-structural region underscoring the immune dominance of this region as previously reported during acute HCV infection [96, 250].

These results suggest that broadening of the HCV-immune response (with the potential generation of *de novo* responses) is associated with protection from chronicity upon reinfection. This is consistent with the observations of Osburn *et al.*, where they observed the generation of *de novo* T-cell responses in patients resolving an HCV reinfection episode, as suggested by the increase in the number of new T-cell responses in comparison to a group of chronically-infected patients [354].

5.2. Correlation between T-cell proliferative capacity and protective immunity

It has been previously shown that the proliferative capacity of CD4 and CD8 T cells is predictive of the outcome of primary HCV infection [243, 246]. Examining the proliferative capacity of HCV-specific CD4 and CD8 T cells against a panel of peptide pools corresponding

to the immunodominant regions of HCV demonstrated higher proliferative capacity in SR/SR patients for both CD4 and CD8 T cells (Manuscript 1 - Figure 2). The proliferative capacity of CD4 T cell increased 3-5 folds from the baseline levels at 6-32 weeks post reinfection (p.ri) in SR/SR patients. In contrast, we observed very limited increase in CD4 T cell proliferation for SR/CI patients and proliferation gradually became undetectable, even if they had a detectable IFNγ-response to the same peptide pool as measured by ELISPOT. The difference in the proliferative response between the two groups was even more striking for CD8 T-cell proliferation. SR/SR patients demonstrated high proliferative responses prior to reinfection that increased at the peak of reinfection to reach very high levels (up to 560-fold of the non-stimulated negative control) and were sustained after viral clearance. In contrast, no CD8 proliferative responses were detected in the SR/CI patients against the peptide pools tested.

In summary, our results demonstrate that protective immunity upon reinfection was associated with higher proliferative capacity for both CD4 and CD8 T cells when stimulated with peptide pools representing immunodominant non-structural regions. This is in accordance with a chimpanzee vaccine study demonstrating that increase in peripheral and intra-hepatic T-cell proliferative responses coincided with the decline in the HCV-RNA titers [364]. Another chimpanzee rechallenge study confirmed the same finding, where vigorous proliferation of CD4 T cells correlated with clearance of the reinfecting virus. It is noteworthy that this vigorous proliferation of CD4 T cells was accompanied by increased frequency, as well as phenotypic and functional changes of tetramer+ CD8+ T cells [341].

This comes in the same line of evidence for the importance of the role of CD4 T cells with helper functions in supporting an effective CD8 T cell recall response upon HCV reinfection. CD4-depletion studies in chimpanzees demonstrated their indispensable role for supporting CD8 T-cell functionality [238]. In human IDU cohorts, this indispensable role for helper CD4 T cells was confirmed, as the reduction of CD4 T-cell count upon HIV infection led to loss of protection against HCV reinfection [118]. The underlying cause(s) of CD4 T-cell failure is an important factor for loss of protective immunity that necessitates further investigation.

5.3. Functionality of HCV-specific T-cells is associated with protective immunity

Our group and others have demonstrated that CD8 T cells simultaneously producing several effector cytokines and effector molecules (i.e. polyfunctional CD8 T cells) are associated with spontaneous resolution of primary HCV infection [251, 367]. This has also been demonstrated for other viruses, as HIV, where control of viral replication was associated with the generation of virus-specific polyfunctional T-cells [88].

Whether polyfunctional CD4 and CD8 T cells were also critical for spontaneous clearance upon reinfection was an important factor that had not been previously studied for chronic viruses in a human setting. We tested the production of the antiviral cytokines IFNγ and TNFα, T-cell growth factor IL-2 and the expression of the degranulation marker CD107a, in response to stimulation with peptide pools corresponding to the immunodominant regions of HCV. We measured the frequency of cells producing individual or multiple cytokines and analyzed the frequency of CD4 or CD8 T cells producing different combinations of the tested cytokines at time points before, during and after the reinfection episode (Manuscript 1 – Figures 3 and 4).

For CD4 T cells, the production of the individual cytokines tested was higher in the SR/SR patients with IFNγ as the major cytokine produced by $CD3^{+}CD4^{+}$ T cells followed by TNFα (Manuscript 1 – Figure 3). In contrast, individual cytokine production was weak in the SR/CI patients. The frequency of polyfunctional cells was low for CD4 T cells in all patients. Nevertheless, when calculated as a percentage of the total cytokine producing cells, we observed that 3-6% of CD4 T cells produced more than one function at baseline in SR/SR patients, reaching 33% at the peak of the reinfection episode. In contrast, cytokine-producing CD4 T cells were strictly monofunctional in SR/CI patients during the reinfection episode, and bifunctional populations appeared to be biased by the low frequency of cytokine producing cells.

For HCV-specific CD8 T cells, SR/SR patients also demonstrated higher levels of production of the individual cytokines/functions tested as compared to SR/CI patients (Manuscript 1 – Figure 4). IFNγ was the major cytokine produced by $CD3^{+}CD8^{+}$ T cells from

the SR/SR group followed by TNFα and the degranulation marker CD107a. Polyfunctionality was also higher in the SR/SR group, where the frequency of bifunctional and triple functional cells was 7 and 16 fold higher in the SR/SR group as compared to the SR/CI group. When results were examined as a percentage of total cytokine producing cells, ~6% of the total cytokine producing cells produced three functions simultaneously at the different stages of reinfection and 15-52% were bifunctional in the SR/SR patients, as compared to none in the SR/CI group (Manuscript 1 – Figure 4, pie charts).

Analyzing the polyfunctionality of CD8+ cells in response to their cognate minimal epitope showed a higher level of production of the individual cytokines in patients from the SR/SR group when compared to patients from the SR/CI group (Manuscript 1 - Figure 5). The production of individual cytokines/expression of CD107a was 9-folds higher and the total frequency of functional cells was 14-folds higher in SR/SR patients. The percentages of bifunctional and triple-functional cells within the total CD3+CD8+ functional T cells in the SR/SR patients was 14% and 7%, respectively. In contrast, cytokine-producing cells were strictly monofunctional in SR/CI patients. These data suggest an association between the level of individual cytokine production and polyfunctionality of HCV-specific CD8+ T cells targeting dominant epitopes with the protection from persistent viremia upon reinfection with HCV.

In summary, the frequency of individual cytokine producing CD8 and CD4 T cells was higher in the SR/SR group and within these functional cells the frequency of polyfunctional cells was higher. These results suggest an association between the level of individual cytokine production and polyfunctionality of HCV-specific CD8 T cells and protection from viral persistence upon reinfection.

Data from chimpanzee and vaccination studies have also confirmed these findings [258, 344]. Where it has been recently shown that protection upon rechallenging chimpanzees that were previously vaccinated was associated with the generation of a population of bifunctional CD8 T cells (IFNγ+TNFα+) [344]. Here, we confirm in a human cohort that polyfunctionality is an important determinant of viral clearance upon reinfection. Furthermore, we extend this polyfunctionality analysis to both CD4 and CD8 T cells targeting entire regions of HCV, as well as minimal epitopes.

The induction of such polyfunctional CD4 and CD8 T cells against HCV immunodominant regions would be very important for successful vaccination. Nevertheless, several points remain to be elucidated; specifically, which combination(s) of cytokines/functions would be the ones consistently correlated with protection? What are the molecular mechanisms underlying the generation of such polyfunctional cells? And what is the contribution of innate immunity to the fine-tuning of these responses? In parallel, it would also be important to define the mechanisms of failure to develop such polyfunctional potential by HCV-specific T cells in SR/CI patients despite their ability to resolve their primary HCV infection.

5.4. Frequency and Phenotype of epitope-specific CD8 T-cells associated with protective immunity

The frequency and phenotype of epitope-specific CD8 T cells are among the important factors that were demonstrated to correlate directly with clearance of viral infection and the generation of a long-lived memory population [251]. These virus-specific memory populations are suggested to confer protection upon reinfection [341].

We used MHC class I tetramers and a nine color antibody panel to detect markers of T-cell maturation and memory (CD27 and CD127), T-cell activation/exhaustion (PD1, 2B4 [CD244] and Tim3) on viable CD3+ CD8+ Tetramer+ cells (Manuscript 1 – Figure 6 and Supplementary Figures S4 and S5). This longitudinal phenotypic analysis of HCV-specific CD8 T cells showed an average expansion of 42 folds in the frequency of tetramer+ CD8+ T cells at the peak of response upon reinfection in SR/SR patients, in contrast to a very limited expansion in all SR/CI patients.

The most distinctive phenotypic changes were in the expression of CD127. For patients where samples were available during the first infection, we observed that approximately half of the HCV tetramer+ CD8 T cells were CD127+ and as the patients cleared their primary infection the majority of tetramer+ CD8 T cells became CD127^{hi}. At the first time point following incidence of reinfection we observed that the majority of expanded cells were CD127^{lo}, consistent with an effector phenotype. In the SR/CI group, there was no significant change in the expression of CD127 in these individuals. This CD127+ CD8+

tetramer+ T population accounted for the polyfunctional population that was observed in response to the cognate minimal peptide. Another important feature observed was the consistently higher expression of the activation marker PD1 on HCV tetramer+ CD8+ T cells in most patients (3/4) of the SR/CI group in comparison to SR/SR patients. Nevertheless, proliferation assays using minimum peptides demonstrated that HCV tetramer+ memory CD8 T cells in the SR/CI unprotected group proliferated at a similar level to those of the protected group (data not shown). This suggests they are not inherently defective, and despite being present at low frequency, they could expand if rechallenged with their cognate peptide with its original amino acid sequence.

Taken together, these results suggest that protective immunity is associated with the generation of long-lived memory $CD127^{hi}$ CD8 T-cell population that gives rise to an effector memory population upon reinfection. Nevertheless, a similar $CD127^{hi}$ CD8 T-cell population was generated following resolution of the primary infection in the SR/CI group, but did not generate an effector recall response during the reinfection episode. Whether variation in the amino acid sequence of targeted epitopes could be the sole reason that accounts for this failure would need further investigation to rule out the possibility of other causes within the signalling pathways and transcriptome of virus-specific memory T-cells.

Our results are concordant with similar observations in chimpanzees that were protected against HCV persistence upon rechallenge after vaccination with an HCV vaccine expressing the NS3-5. The vaccine-induced T-cells appeared early and were maintained at high frequencies for a long time after HCV challenge. These T-cells displayed high levels of CD127 and were bifunctional (IFNγ+TNFα+). PD1 expression was lower than the mock-vaccinated group and seemed to be associated with control viremia [344]. This was also previously described by Shin *et al.* [258] and is consistent with a memory T cell phenotype [37].

The association between the frequency, phenotype and polyfunctionality of certain epitope-specific CD8 T cells and the resolution of primary HCV infection was previously demonstrated [251], and as we show here protective immunity upon reinfection argues for the induction of such immunodominant epitope-specific responses through vaccination. Vaccine design would have to take into consideration the induction of memory immune responses

against immunodominant epitopes associated with the most prevalent HLA alleles in humans to assert the highest probability of protection on the population level. Vaccination strategies should also take into account the different variants of the epitopes to be included in the vaccine to insure an adequate recall response to the different common HCV strains humans are exposed to.

5.5. Effect of viral-epitope variation on protection upon HCV reinfection

HCV is one of the viruses with the highest mutation rates. It replicates rapidly with a half-life of ~3 hours producing an average of 10^{12} viruses/day/patient. With a low fidelity RdRp that lacks a proof-reading function, it has a high mutation rate of 1/1000 bases per year. Continuous mutation during infection is partially driven by the immune pressure exerted by the specific T-cells and antibodies (reviewed in [368]). Importantly, higher mutation rate was observed in the epitope-regions targeted by virus-specific T-cells versus other regions outside the epitope-regions, where the rate of mutation in the former was 13-folds higher than that in the latter [369].

We aimed at determining whether the lack of expansion of HCV-tetramer+ CD8 T cells in patients of the SR/CI group upon reinfection is due to infection with variant viral sequences that are not recognized by the pre-existing memory immune response. For this, we sequenced the region of targeted HCV-epitopes from all four SR/CI patients at early and late time points during reinfection (Manuscript 1 – Figure 7A). We consistently detected a mismatch between the amino acid sequence of the reference peptide used in the tetramer and the autologous epitopes in all patients. The ^{1444} Y→F substitution observed in the NS3-1436 epitope was previously reported [251, 309, 370], and shown to result-in reduced cytokine production, but did not compromise recognition by the T-cell receptor (TCR) [96, 309]. On the other hand, the ^{1077}V→A substitution observed in the NS3-1073 epitope was suggested to affect the recognition by TCR and to be less efficient in the induction of IFNγ production [334, 335, 370]. Surprisingly, this variant underwent three changes in patient SR/CI-2 during the reinfection episode despite the lack of expansion in the tetramer+ population and the lack of specific functionality in response to the minimum peptide (i.e. in the absence of immune

pressure). Specifically, the ^{1077}V→A reverted to the reference sequence and two amino acid substitutions occurred (^{1081}V→I suggested to affect the HLA-A2 binding of the epitope and ^{1078} W→L).

Our results suggest that variations in the amino acid viral sequence of the epitopes targeted by the memory immune response could be a possible mechanism underling the functional differences or lack of recognition in HCV-specific CD8 T cells observed between the two groups of patients. It could also be a possible reason for the phenotypic differences observed between the SR/SR group and SR/CI group. However, those changes in the targeted epitopes do not explain the general decrease in the frequency of the HCV-specific immune response observed in ELISPOT analysis for the SR/CI group. Thus, the association between the total magnitude of the memory immune response and the degree of homology in the entire HCV sequences between the infecting viruses in both episodes of infection remains to be assessed.

These results highlight the major impact of HCV variability on the protective capacity of the immune system upon reinfection, and argue in favour of the hypothesis that protection has a rather narrow spectrum that is effective mainly against closely-related viral sequences. This would translate to the necessity to design vaccination regimens that include various forms of immunodominant epitopes to circumvent the high variability of viral sequences, which is a hallmark of HCV.

5.6. Avidity of HCV-specific T cells and protective immunity

Functional avidity is a measure of the sensitivity of specific TCR to the p-MHC complex, where T-cell clonotypes with higher affinity/avidity would recognize p-MHC at lower concentrations and respond more rapidly with higher functionality. Studies analyzing the TCR-repertoire of epitope-specific CD8 T cells in HIV patients revealed that patients who were able to control HIV-1 infection (controllers) had CD8 T-cells with higher avidity and functionality compared to patients where the infection progressed to AIDS (progressors) [88, 371]. Further investigation showed that functional avidity was a major determinant of the polyfunctionality and HIV-suppressive capacity [89].

We measured the dose-dependent production of IFNγ in response to stimulation by the different epitope sequences in an ELISPOT assay. Results of these dose-response experiments showed higher avidity of HCV-specific T cells in SR/SR patients to the reference sequence when compared to SR/CI patients (Manuscript 1 – Figure 7B). The results of functional avidity were similar to the results of functionality and polyfunctionality; where patient SR/SR-4 who had higher functional avidity than patient SR/SR-1, showed a higher frequency of functional cells for the epitope-specific CD8 T cells. A similar pattern was observed for SR/CI patients, where patient SR/CI-2 who had a relatively higher functionality when compared to SR/CI-2 upon stimulation with the minimum peptide, showed a higher avidity in response to the reference-sequence of the epitope. Both SR/CI patients had higher avidity for the reference epitope sequence than the autologous epitope sequence of the reinfecting virus. We also observed that patient SR/SR-4 had a good response to one of the mutated epitopes, despite not being infected with this amino acid sequence of the epitope in either episodes of infection.

Together with the polyfunctionality data observed upon stimulation with minimum peptides we could observe the obvious correlation, where the patient with the highest avidity (SR/SR-4) had the highest functionality and polyfunctionality upon stimulation by the reference sequence of the cognate peptide. This has already been shown within the context of HIV infection, where superior functional avidity of epitope-specific CD8 T cells was interlinked with their high polyfunctional capacity [88]. It has also been demonstrated in the cytochrome-c protein mouse-model that there was a link between kinetics of the CD4 T-cell repertoire and the affinity of pMHC-TCR interactions [372]. Thus it was necessary to analyze the repertoire of TCRs for epitope-specific CD8 T cells to verify whether it was a decisive factor in the outcome of HCV reinfection and the establishment of protective immunity, especially against different viral variants.

The fact that all four SR/CI patients had higher avidity for the reference epitope sequence than the autologous epitope sequence of the reinfecting virus could be attributed to "original antigenic sin" (Manuscript 1 – Figure 7B). This hypothesis postulates that once a naïve T cell targeting a specific epitope expands and develops into memory, it becomes incapable of recognizing new variants of the same epitope, which facilitates viral persistence

upon generation of escape mutations in targeted epitopes or upon reinfection with an unrecognizable viral variant [373].

Patient SR/SR-4 had a moderate response to one of the mutated epitopes, indicating the possibility of higher flexibility of the TCR-repertoire. A study in HIV patients with similar HLA allele backgrounds showed that infection controllers possessed TCR clonotypes characterized by enhanced cross-reactivity to different variants of the epitope [374]. HIV controllers possessed alternative clonotypes that were mobilized and dominated the response to recognize the mutant form of the epitope. Those newly mobilized clonotypes were shown to recognize both forms of the epitope with similar affinity thus provided high flexibility within the epitope-specific CD8 T-cell repertoire [375, 376]. Indeed, flexibility in the T cell repertoire correlated with better control of viral escape mutants in both the HCV and HIV field [94, 374-376] and it would be interesting to validate such results in a reinfection model.

5.7. Memory versus *de novo* CD8 T-clonotypes in protective immune responses

It has been reported before that reinfection was associated with a significant increase in the breadth of T-cell responses suggesting the generation of *de novo* T-cell responses [354], a finding that we observed with our ELISpot results, as well. This raised the possibility that within the epitope-specific CD8 T-cell population, the effector cells appearing at the peak of immune response during reinfection could be due to the recruitment of new clonotypes from the naïve T-cell pool. Our phenotyping panel was designed to examine the memory and exhaustion status of the epitope-specific CD8 T-cell population, but we still needed to confirm whether the $CD127^{lo}$ T_{eff} cells appearing at the peak of response to the reinfection episode were recruited from the naïve repertoire or if they were T_{EM} recruited from the T_{CM} pool. Clonotypic analysis of TCR could help us verify whether the source of the effector T cells ($CD127^{lo}$) is expansion of pre-existing memory or generation of *de novo* T cell responses [377]. Taking into consideration data from the HIV and HCV models it was tempting to speculate that repeated reinfections would selectively expand and enrich such cells of higher

functional avidity already recruited during the primary infection provided that the targeted epitope remain unchanged [94, 374-376].

We performed longitudinal analysis of the HCV-epitope-specific T-cell repertoire during HCV reinfection in five patients with different outcomes of the reinfection episode; three patients who resolved two successive HCV infections (SR/SR group), and two patients who became persistently infected upon reinfection (SR/CI group) (Manuscript 2 - Table 1). Epitope-specific CD8 T cells identified by MHC class I tetramer (as detailed in the table) were sorted at various time points; pre-, at the peak and late/post the reinfection episode. The gating strategy used for sorting is explained in Supplementary Figure 1 of Manuscript 2. The TCRBV gene and TCRBJ gene usage, as well as the CDR3 chain amino acid sequence for all Vβ chains for the different T-cell clonotypes were analysed (Manuscript 2 - Supplementary Tables S1 and S2).

Our longitudinal analysis of the Vβ-chain of TCR did show a conserved pattern of Vβ usage in all of the tested patients, where the dominant and sub-dominant clonotypes forming the population at the peak of immune response during reinfection were recruited from the memory population generated following the clearance of the primary infection, with lack of appearance of new clonotypes (Figure 1 and Supplementary Tables S1 and S2). Nevertheless, the frequency of the different clonotypes changed upon reinfection causing a change in the hierarchy of the utilized clonotypes. These results suggest that the effector CD8 T cells were T_{EM} recruited from the T_{CM} pool rather than being recruited from the naïve repertoire.

It has been demonstrated before in the LCMV model that the TCR repertoire of the primary epitope-specific CD8 T-cell response was conserved in the memory pool and the secondary effector recall response, where there was identity between the clonotypes that formed 60-100% of the primary effector, memory and recall responses [378, 379]. Rapid resolution of HCV infection upon rechallenging chimpanzees also coincided with the expansion of T-cell clonotypes that dominated the memory CD8 T-cell pool. These specific TCR clonotypes were tracked through the two consecutive HCV challenges [90, 94].

The increase in the breadth of the T-cell responses in SR/SR patients and the narrow TCR-repertoire (that becomes more focused upon reinfection) are seemingly contradictory.

Nevertheless, a possible explanation is that the immune system generates a broader response (i.e. targeting more epitopes) to insure more effective attack on the different viral regions, but without compromising the focusing of the TCR-repertoire of epitope-specific T cells on the fittest clonotypes that would possess higher functional avidity and better cross-reactivity.

5.8. Diversity of the repertoire of HCV-specific CD8 T cells and protective immunity

Studies on the interplay between the TCR-repertoire of epitope-specific CD8 T cells and chronic viruses (LCMV model, as well as HIV and HCV infections) had controversial results concerning the diversity of the repertoire and its correlation with control of infection and reinfection, where some studies showed that control was associated with a more diverse TCR-repertoire, while other studies demonstrated it was associated with a more focused repertoire [94, 371, 378].

Our analysis revealed that the repertoire-composition in the tested SR/SR patients was less diverse than that observed for SR/CI patients at all the time points tested (pre-, peak and post reinfection); where 75-83% of the repertoire was attributed to 14-23 TCR-clonotypes, and it became more focused at the peak of the immune response during reinfection for patients SR/SR-1 and SR/SR-2 (92% and 96% of the repertoire was attributed to 15 and 2 TCR-clonotypes, respectively) (Manuscript 2 - Figure 2A, 2B and 2C). In those two patients, the repertoire retained a more focused status post reinfection when compared to the pre-reinfection time point, with 77% and 93% of the repertoire attributed to 16 and 4 clonotypes, respectively (Manuscript 2 - Figure 2A and 2B). Patient SR/SR-3 showed no focusing of the repertoire upon reinfection (Manuscript 2 - Figure 2C), and this comes in accordance with our previous results, where there was no expansion of the tetramer+ population in this patient upon reinfection, and ELISPOT data suggested the patient was reinfected with a different HCV subtype [380]. In contrast, the repertoire in the tested SR/CI patients was more diverse; where the dominant clonotypes (present at >1%) represented 37% and 55% of the repertoire and were attributed to 18 and 23 TCR-clonotypes, respectively. At the peak of the immune response during reinfection the repertoire was still more diverse than in the SR/SR patients

(the dominant category represented 28-64% of the repertoire) (Manuscript 2 - Figure 3A and 3B). The remaining ~40-60% of the repertoire was represented by >130-300 clonotypes, whereas in the SR/SR patient the number of minor clonotypes never exceeded 116 unique clonotypes (except at the time point post infection for patient SR/SR-1, where the sample was a leukaphoresis sample, and it had ~10-40 fold the number of sequences available for other time points in the same patient and other patients (Manuscript 2 - Figure 2A)). Post reinfection, we did not observe increased focusing of the repertoire in SR/CI patients, as compared to the pre-reinfection time point.

A narrower repertoire that becomes more focused upon reinfection in the protected (SR/SR) group was further supported by the observation that the top three clonotypes and the top two clonotypes in patients SR/SR-1 and SR/SR-2 represented 28% and 59% of the repertoire pre-reinfection, respectively (Manuscript 2 - Figure 2A and 2B, the dissected pie-charts in the lower lines). Upon reinfection they became more dominant, representing 51% and 96% of the repertoire, respectively. Post reinfection they were more dominat when compared to the pre-reinfection time point, forming 39% and 90% of the repertoire, respectively. On the other hand, the top three clones in the tested SR/CI patients never represented more than 27% of the repertoire, and were sometimes as low as 9% (Manuscript 2 - Figure 3A and 3B, the dissected pie-charts in the lower lines).

It has been demonstrated before in the LCMV mouse model that the repertoire of an epitope-specific CD8 T-cell population was highly focused, where 70% of the response against LCMV was due to three clonotypes [378]. In the cytochrome-c antigen system there was narrowing of the repertoire upon second exposure relative to the primary repertoire, suggesting selection of the clonotypes with highest affinity [372]. Analysis of the TCR repertoire of epitope-specific CD8 T cells in HIV patients also demonstrated narrower and more focused TCR-repertoires of high-avidity populations in patients who were able to control HIV-1 infection compared to progressors [371]. On the other hand, a previous study of an HCV-epitope (NS3-1406) suggested that higher diversity of the repertoire would be more advantageous, otherwise the virus would undergo mutation of the epitope to exploit gaps present in the repertoire [93]. A chimpanzee rechallenge study suggested a similar conclusion,

since the majority of HCV-epitopes that escaped immune recognition upon reinfection were targeted by a CD8 T-cell repertoire with reduced CDR3 amino acid diversity. This suggested that it is more advantageous to generate and maintain a diverse TCR repertoire against individual epitopes. In contrast, limited TCR diversity facilitated CTL escape mutations in HCV infected chimpanzees [94]. Interestingly, an earlier study showed that despite some TCRBV sequences being identical in chimapnzees and humans, other TCRBV genes in the chimpanzee have been lost in humans. This could be a probable cause of discrepancy of findings between chimpanzee and human studies for the different HCV-epitopes [381]. Another important factor that might cause discrepancy in the results from chimpanzee studies is that the animals were rechallenged with the same viral sequence and therefore exposed to less diversity on the virus side, which is completely different from natural human infections where individuals are usually infected with a mixture of quasispecies.

5.9. Role of specific CD8 T-clonotypes in protective immunity

A study of epitope-specific CD8 T-cell repertoire in HIV patients suggested the preferential recruitment of particular clonotypes endowed with superior functionality [87]. Another study in HIV patients with a similar HLA-allele background showed that infection controllers targeted an immunodominant epitope with TCR clonotypes distinct from those observed in progressors. These clonotypes were characterized by superior control of viral-replication *in vitro* and enhanced cross-reactivity to different variants of the epitope [374]. Whether protective immunity upon HCV reinfection in a human cohort would be associated with specific clonotypes dominating the TCR-repertoire that are distinct from the clonotypes observed in the non-protected group is still an area that needs extensive studying. Also, particular clonotypes were preferentially selected in several HIV-infected individuals who controlled viral-replication. These public clonotypes exhibiting superior avidity imposed selective pressure causing early mutation in the epitope that enables the virus to escape their recognition [375, 376]. However, the variant epitopes were recognized by specific T-cell clonotypes that were moblilized by the immune system. These newly mobilized clonotypes were more flexible (i.e. recognize multiple epitope variants) and still possessed high functional avidity [375, 382].

The longitudinal sequencing of the Vβ-chain of the TCR showed that the dominant and subdominant Vβ-CDR3-Jβ detected in the tested patients showed no overlap, where no clonotypes were found to be common between patients targeting the same epitope, whether they belonged to different groups (SR/SR-1 and SR/CI-2 recognizing the HLA-A2 restricted NS3-1073 epitope) or even when they belonged to the same group (SR/SR-2 and SR/SR-3 recognizing the HLA-B27 restricted NS5B-2841 epitope) (Supplementary Tables S1 and S2). Sequencing the Vβ-chain from more patients and T cells targeting other HCV-epitopes is needed, to verify whether some clonotypes are recurrent in the repertoire of different patients from the same group and thus represent "public clonotypes" associated with the outcome. Also, it would confirm whether distinct clonotypes are found in the SR/SR repertoire versus the SR/CI repertoire.

It is noteworthy that all of these results for the TCR-repertoire from peripheral blood mononuclear cells (PBMCs) is expected to reflect what is happening in the liver, since rechallenge studies in chimpanzees showed that the peripheral epitope-specific CD8 T-cell repertoire reflected the intra-hepatic response, where identical clonotypes were present in both the liver and the peripheral circulation which differed only in the magnitude (where it was 10-folds higher in the liver) [90].

Collectively, the results from the studies presented in this thesis led us to propose a hypothetical model for the correlates of protection against chronicity upon HCV reinfection (Figure 13).

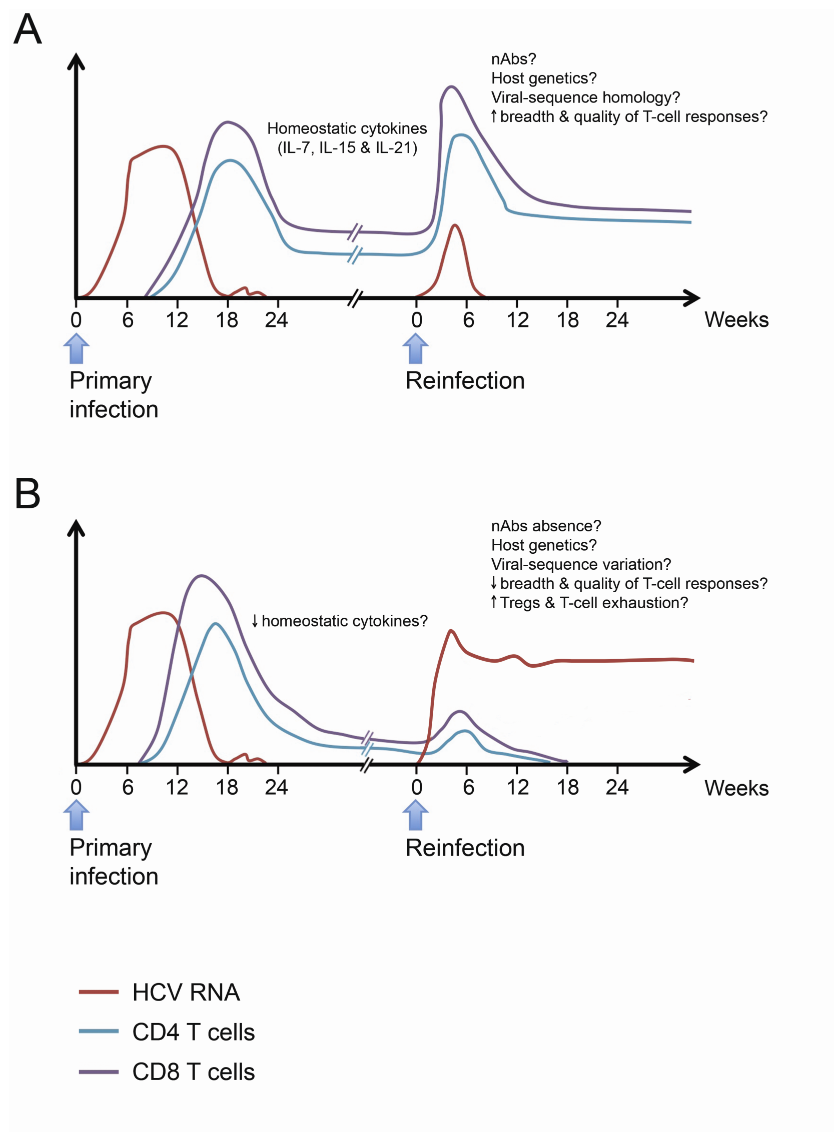


Figure 13: Hypothetical model for protective and non-protective immunity upon HCV reinfection.

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Legend: Individuals who spontaneously clear primary HCV infection develop long-lived virus-specific memory CD4 and CD8 T cells. Such memory T cell populations are maintained by the action of homeostatic cytokines like IL-7, IL-15, and IL-21. Upon HCV reinfection, some individuals will be protected against viral persistence while others will be unprotected. **(A) Spontaneous resolution of primary and secondary HCV infections (SR/SR).** The protected individuals will be able to spontaneously clear the second infection with a shorter period of viremia, as well as reduced peak viremia. Viral clearance will be accompanied by an accelerated memory T cell recall response detectable in both liver and blood characterized by the increased breadth magnitude and functionality/polyfunctionality, together with the expansion of HCV-specific CD8 T cells. The exact characteristics of a protective response are yet to be defined in terms of breadth and quality. **(B) Spontaneous resolution of primary HCV infection followed by chronic infection upon reinfection (SR/CI).** In unprotected individuals, reinfection will be associated with a weak or late recall response and incomplete T cell-mediated control of viremia. The underlying causes of failure to resolve the reinfection despite the ability to spontaneously resolve a prior infection maybe low levels of homeostatic cytokines affecting the maintenance of memory T populations, reduced breadth, or quality of the recall response upon reinfection and increased frequency of Tregs leading to dampening of the immune response, viral persistence, and rapid exhaustion of HCV-specific T cells.

Neutralizing antibodies, host genetics, and homology between the infecting viral sequences at different episodes might also be determinants of protection upon reinfection.

5.10. Shifting epitope dominance upon HCV reinfection

For patient SR/SR-1 who was responsive to two tetramers A2/NS3-1073 and A2/NS5B-2594, we observed different levels of expansion of CD8+ T cells specific to the two tetramers. The frequency of NS5B-2594 tetramer+ population increased by 120 folds upon reinfection compared to 7 folds only for the NS3-1073 tetramer+ population (Manuscript 1 – Figure 6A and Supplementary Figure S5). This occurred despite the fact that the frequency of the tetramer+ cells for the NS5B-2594 was at a much lower level than that for NS3-1073 at the time point before reinfection. This suggests that a shift in the dominance of the targeted epitopes occurred upon reinfection.

This comes in contrast with previous results from rechallenge studies in chimpanzees that showed hierarchy preservation of the frequency of epitope-specific CD4 T cells targeting different epitopes [57]. On the other hand, monitoring the kinetics of three immunodominant epitopes during primary, memory and recall responses in the LCMV model demonstrated changes in the epitope-specific T-cell hierarchies [379].

Nevertheless, we need to examine more patients with multiple tetramer+ populations recognizing different epitopes to verify this assumption, especially patients for whom longitudinal samples from the first episode of infection are available. Also, further investigation is needed to verify whether the change in the hierarchy of these two epitopes is due to differences in the kinetics of epitope processing/presentation or whether it could be attributed to the differential expression kinetics of the two protein regions they are included in (NS3 region versus the NS5B), or both factors.

5.11. Correlation between specific HLA-alleles and protective immunity

Patient SR/SR-3 successfully cleared his reinfection despite the decline in magnitude and breadth of the immune response against the genotype 1a reference peptide panel upon reinfection (Manuscript 1 - Supplementary Figure S1). Being reinfected with HCV of an unknown subtype, we tested his response to a panel of peptides corresponding to genotype 1b reference sequence, and we observed an increase in the overall frequency and breadth of

HCV-specific T cell responses upon reinfection. This represents an interesting case for further study to clarify the mechanisms that enabled this patient to achieve protection against a second infection by HCV even of a different subtype. It is noteworthy that this patient has an HLA allele B*27, which has been previously associated with higher ability to spontaneously clear HCV and control HIV infection [200, 383, 384]. These results suggest that patients bearing this HLA allele might also have superior protection from HCV-persistence upon reexposure. Also, some HLA class II alleles demonstrated a strong association with primary HCV clearance, whereas others were associated with HCV persistence [201-203]. It would be interesting to study these correlations in a reinfection setting.

5.12. Conclusions, limitations and future studies

5.12.1. General conclusions

The rational design of prophylactic vaccines against chronic viral infections necessitates the clear definition and dissection of the correlates of protective immunity, so that they could be monitored during new vaccine trials to verify the degree of protection conferred by these new vaccines. However, several challenges impede the definition of the characteristics of protective immunity for chronic viruses, because in the majority of cases the virus is never eliminated from the body of the host after the primary infection and for viruses where some patients clear the virus spontaneously (e.g. HCV), it has been very challenging to study immune responses in a human reinfection setting. Marginalized populations (as IDUs) represent the major population at risk, with many challenges related to recruitment of patients belonging to these groups. And within these cohorts, it is still more challenging to be able to recruit patients who cleared a primary infection and are at risk of getting reinfected. We were able to recruit patients who resolved a primary HCV infection, but had a differential outcome of the second infection, and for whom longitudinal blood samples during the multiple episodes of HCV-infection were available. This rare cohort enabled us to perform an exhaustive longitudinal phenotypic and functional characterization of the HCV-specific memory immune responses upon reinfection.

A general conclusion from our study is that no sterilizing immunity was observed upon reinfection with HCV in patients who previously resolved a primary infection. This comes in accordance with several rechallenge studies conducted in chimpanzee and epidemiological studies in IDU-cohorts who cleared a primary HCV infection and were still at risk due to continuous exposure to HCV [118, 119, 349]. Our results and results from other groups suggest that protection from viral persistence is higher upon subsequent exposure than resolution of primary infection, offering hope for vaccine development [118, 351, 354]. Spontaneous resolution of reinfection was associated with an increase in both the magnitude and breadth of total HCV-specific T-cell responses in 4/5 protected patients. In addition, we observed expansion of HCV-specific memory T cells and the transient appearance of $CD127^{lo}$ population indicative of an effector T cell phenotype in 3/4 protected patients who recognized specific tetramer. On the other hand, all patients who failed to clear their second infection episode (4/4) showed very limited expansion of HCV-specific T cells, although they did exhibit HCV-specific T cells with a memory phenotype prior to reinfection. Variations in the amino acid sequences of the epitopes targeted by the HCV-specific CD8 memory T cells were detected in all patients of the non-protected group suggesting a role of variation in viral sequence in the failure of recall response. HCV-specific CD4 and CD8 T cells showed higher functionality/polyfunctionalty and proliferative capacity in the protected group. Analysis of the TCR-repertoire of epitope-specific CD8 T cells demonstrated that a more focused repertoire of distinct clonotypes was associated with protection. These T-cell populations showed higher functional avidity and polyfunctionality compared to their counterparts in non-protected patients.

The fact that protection was associated with expansion and broadening in the responses for both CD4 and CD8 T cells upon reinfection, in contrast to non-protected patients who had defective responses for both populations, argues in favour of the assumption that failure to generate a polyfunctional and broad CD8 recall response upon reinfection is –at least partially– a result of CD4 failure. Nevertheless, it is imperative to study other possible causes associated with defects within the signaling and transcriptome networks for the failure of memory CD8 T cells, as well as the inherent causes of failure of memory CD4 T cells to provide the necessary help.

Overall, our results suggest that memory T-cell populations formed following the spontaneous resolution of a primary HCV infection are not all created equal; but rather represent a broad spectrum of cells with different capacities ranging from high-quality memory (capable of resolving future episodes of infection, sometimes of a different genotype/subtype) to low-quality memory (that fails to mount a strong recall response even against closely similar variants). This is supported by similar findings in chimpanzee rechallenge studies [338-340]. What remains crucial to be elucidated, are the factors that contribute to the formation of such high-quality protective memory, and how to tailor vaccines that would induce such a response.

Our results also demonstrated that avidity, diversity and specific clonotypic-composition of the virus-specific T-cell repertoire are important determinants of protective immunity. Our data, together with data from the HIV model suggest that T-cell populations with highest polyfunctionality are the same with highest avidity. It is also tempting to speculate that repeated infections would selectively expand and enrich such cells of higher functional avidity provided that the targeted epitope remain unchanged. Indeed, protection upon reinfection was associated with a more focused TCR repertoire, in contrast to previous findings in chimpanzee studies that associated protection upon HCV reinfection with a more diverse repertoire. Nevertheless, these studies also showed that protection was associated with the expansion of the same TCR-clonotypes that were tracked during the primary HCV challenge [90, 94]. Flexibility in the T-cell repertoire correlated with better control of viral escape mutants in both HCV and HIV infections [94, 374-376], and HIV controllers mobilized alternative cross-reactive clonotypes capable of recognizing the mutant form of the epitope to dominate the response. Thus, exposure to different forms of the epitope could help higher clonal turnover, and those newly mobilized clonotypes were shown to recognize both forms of the epitope with similar affinity thus provided high flexibility within the epitope-specific CD8 T-cell repertoire [375, 376]. These findings can be applicable to many other chronic viral infections, especially those with high variability like HCV. And it is important to verify these findings in the reinfection setting, and extend those findings to other immunodominant HCV-epitopes.

5.12.2. Limitations

Despite being the first study to dissect the general characteristics of protective immunity against HCV in a natural reinfection human setting, we are aware of some limitations that represent a challenge for studies with such cohorts in general. These include the limited number of patients and lack of specific time points for some patients (especially during the primary infection episode), the inability to specify the exact start-date of the infection/reinfection episode and the possibility of missing brief infection episodes. We tried to overcome these cohort-related limitations by narrowing the testing intervals for HCV-RNA in our patients, but this does not completely rule out the occurrence of brief episodes of HCV that are missed due to rapid clearance and/or low levels of viremia. Nevertheless, the testing intervals chosen represent the ideal compromise between the probability of not missing on any of the infection episodes and the cost/space, as well as other logistic limitations related to sample acquisition and preservation. These issues are recognized by researchers in the field and were previously addressed for such rare cohorts in general [357]. Besides, some technical caveats exist, where the number of epitopes that could be tested for a patient of a certain HLA-background is limited by the number of tetramers available for such an allele. We have tested the MHC class I tetramers available for the corresponding HLA alleles in each patient.

5.12.3. Probable causes of failure of protective immunity

These two studies provide an important insight for the first time into the general characteristics associated with protective immunity against HCV in a natural infection setting in humans. Nevertheless, more patients need to be examined to confirm the preliminary conclusions we drew from these studies and several important issues remain to be resolved to have a more complete picture about the causes of failure of the memory immune response in providing protection upon reinfection in the SR/CI group. Understanding these factors would be pivotal for successful vaccine design.

5.12.3.1. Defects in signaling pathways and transcription of HCV-specific T cells

Transcriptional control of memory CD8 T cell differentiation has been shown to include different transcription factors biasing the formation of effector T-cell populations

towards specific subsets (reviewed in [385]). Examining the transcriptome profile of HCV tetramer+ CD8 T cells at different time points during the reinfection episode would enable defining the molecular defects associated with failure in establishing protective immuity.

5.12.3.2. Suppression by Tregs

The role of other CD4 T-cell subsets in the establishment/failure of protective immunity needs to be defined. Tregs could be beneficial or detrimental during HCV infection, by either limiting immunopathology in the liver and preventing the apoptosis of highly activated CD8 T cells [363] or suppressing protective T-cell responses [386]. Acute resolving HCV is characterized by a strong Th1/Th17 response with specific expansion of IL-21 producing CD4 T cells that correlated with the frequency of HCV-specific CD8 T cells [269].

5.12.3.3. Absence of neutralizing Antibodies

The role of humoral immunity in spontaneous resolution of primary HCV infection and during reinfection episodes remains controversial [248, 249, 279, 338, 341, 354]. Its role during reinfection is even less defined. Examining the neutralizing capacity of longitudinal plasma samples using custom HCVpp with the variant E1/E2 sequences would enable the definition of the contribution of humoral responses to protective immunity.

5.12.3.4. Lack of particular T-cell clonotypes

It has been shown before within the context of HIV infection that certain epitope-specific clonotypes possess a higher avidity and flexibility in response to different variants of the epitope [89, 376]. Analyzing the TCR-repertoire from additional patients to verify whether specific clonotypes correlate with protection in response to the different variants of the epitope. This will also determine whether there is recurrent usage of specific TCR-clonotypes (public clonotypes) in protected patients.

5.13. Significance of the study

The importance of this study is that it provides a first insight into the general characteristics of the adaptive immune response associated with resolution of reinfection by a chronic virus in a human cohort. This signature of a protective immune response necessitates a deeper insight into the transcriptome and signalling correlates that ensure the generation of efficient memory populations capable of mounting a recall response that clears the reinfecting virus. In parallel, it is important to define the underlying causes of failure to mount such a response in patients who failed to clear HCV during the reinfection episode despite spontaneously resolving the primary infection. On another level, a more complete insight into the clonotypic requirements of epitope-specific CD8 T cells associated with protective immunity against different viral variants could be achieved by analyzing the TCR-repertoire in more patients and for various immunodominant epitopes. This would enable the identification of specific public clonotypes with optimum avidity and flexibility that confers such protection.

Despite the limited number of patients included in this study (which is a common limitation for such cohorts), we were able to draw some conclusions about the general signatures of the protective immune responses against HCV during reinfection episodes. Our results suggest that it would be very important for next-generation vaccine design to focus on epitopes that have the ability to generate responses inducing specific public clonotypes against immunodominant epitopes that possess superior functional avidity and polyfunctionality. Combining several immunodominant epitopes that would induce the generation high-avidity public clonotypes and that escape at a high fitness price would maximize the chances of a vaccine to eliminate the virus rapidly. This would inhibit the virus from establishing a foothold in the patient and acquiring the ability to manipulate the patient's immune response. This combination has to take into consideration the coverage of the most common HLA alleles in human populations to ensure a minimum response in patients with different genetic backgrounds. It is also essential to elucidate the kinetics associated with responses to different epitopes; both the kinetics of processing/presentation and the differential expression kinetics of the immunodominant protein regions that contains them. This could be a critical point for the choice of epitopes/proteins to be included in a vaccine and the precise design of

vaccination regimens that would ensure broad responses without having a specific response hamper the following responses or overshadowing the preceding responses to other epitopes/regions.

One major constraint of vaccine development for HIV and HCV is the heterologous nature of the sequences of these viruses where they circulate as quasispecies even within the same individual. This might be overcome by designing prime/boost vaccination regimens that would "stretch" the flexibility of the repertoire of epitope-specific T cells to the optimum, thus preparing the immune system for the expected challenge against the different possible variants of the same epitope. It is possible that rechallenge with a slightly different antigenic variant might favour expansion of T cells carrying a flexible T cell receptor that caould recognize these new variants. Thus, immunization and boosting with the different forms of the immunogen commonly observed in the population might afford a broader and more effective protection.

Accordingly, a successful prophylactic vaccine must have a dual capacity; the capacity to induce an effective immune response capable of surmounting the high mutational rate of HCV, and the capacity to generate high-quality memory immune cells capable of mounting a prompt recall response that outpaces HCV and prohibits it from producing enough viral proteins able to distort/antagonise the different arms of immune system.

The definition of the correlates of protective immunity would enable the tailored design of a vaccine containing the optimum HCV epitopes/regions and the adjuvants that would ensure induction of adaptive responses that establish memory T cell populations with the phenotype, functionality and repertoire that would confer protective immunity upon exposure to different HCV variants. Moreover, this definition would enable the testing of the right determinants that could predict whether the vaccine in question would be protective upon viral exposure. All present vaccine trials could be considered empirical, since they do not measure defined correlates for the efficacy of protection. With the recent advances in immunological and molecular biology techniques, it is inevitable to revise our strategies for vaccine design based on lessons learned from successful vaccines on how to trigger an

efficient memory CMI and based on the correlates of protective immunity in the setting of a natural human reinfection defined in this thesis and its future continuation studies.

CHAPTER 6:

PERSPECTIVES AND FUTURE DIRECTIONS

This study represents an initial effort to unravel the correlates of protective immunity and the characteristics that are associated with clearance of reinfection by a chronic virus. It enabled us to draw conclusions about the general determinants of protective immunity against HCV. Nevertheless, we still need to examine more patients to confirm the preliminary findings of this study. We also need to have a deeper insight into the underlying causes for failure of CD8 T cells recall responses in the non-protected group (transcriptome and individual-clonotype level), as well as a broader overview for the accompanying factors that could enhance/curtail such protection (roles for innate immunity, different CD4 T-cell subsets and nAbs). Another interesting group to be studied in parallel are patients who cleared a primary infection, but did not contract a second infection (exposed non-reinfected group) who could be privileged with a superior protection to that of the SR/SR group.

Several gaps in our knowledge about protective immunity against chronic viral infections remain to be elucidated. First, we need to define the genomic signatures and molecular mechanism(s) underlying the enhanced/failed recall response upon re-exposure, and verify whether the mechanisms identified in mouse-models are valid in the more complex setting of human chronic viral infections such as HCV. Second, we need to define the role of each of the different subsets of CD4 T-cells in sustaining a protective memory immune response. Third, we must verify whether neutralizing Abs play a role in protective immunity against HCV. Fourth, we have to study the impact of the heterogeneity of the entire HCV-genome sequence on the protective capacity of the memory immune response. Fifth, we need to monitor the specific contribution of individual TCR-clonotypes to protective immunity. Finally, we should verify whether there are differential elements affecting the migration of HCV-specific cells to the liver between the protected group and the un-protected group.

Further studies are needed to enable answering these important questions, which would be crucial for the rational design of next-generation vaccines with the ability to fine-tune the early adaptive immune response to ensure the generation of memory T cells with optimal qualities that would confer protection upon exposure to chronic viruses.

6.1. Transcriptome analysis to dissect underlying causes of failure of protective immunity

Transcriptional control of memory CD8 T cell differentiation has been shown to include an intricate interplay between different pairs of transcription factors. The balance between T-bet/EOMES and BLIMP1/Bcl-6 (among others) was shown to have a decisive role in the formation of short-lived terminally differentiated T cells versus long-lived memory T cells. Besides, the balance between different STATs (especially STAT3 and STAT4) is suggested to have a pivotal role in the differentiation of T cells and even biasing the formation of effector T-cell populations towards specific subsets (reviewed in [385]). A recent study also observed molecular and transcriptional profiles for exhausted CD4 T-cells that were distinct from those in their CD8 counterparts, and under the mixed control of both distinct and shared transcription factors [387].

Whether there are differential microenvironmental factors deriving the generation of high-quality versus low-quality memory through specific transcriptional and molecular mechanisms during the primary infection, thus favouring a specific fate in protected (SR/SR) versus non-protected (SR/CI) patients is important to be verified. The definition of these factors would be important to enable the adjustment of optimal conditions for generation of protective immunity using vaccination.

We will examine the transcriptome profile of sorted HCV tetramer+ CD8 T cells at the baseline, peak and post infection and reinfection episodes. Upregulated genes will be validated by real-time PCR. Gene set enrichment analysis (GSEA) will be used for identification of significantly modulated pathways [388]. GSEA has already been successfully used to define important immune pathways during HIV infection and Yellow Fever (YF) vaccination studies [112, 389]. The profiles could be compared between the SR/SR group and the SR/CI group, and a control group of SR patients could be included, as well.

6.2. Defining the role of different CD4 T-cell subsets in protective immunity against HCV

The help of CD4 T cells is essential to maintain efficient memory CD8 T cells [390, 391] and prevent their exhaustion [43, 392, 393]. We have already studied the role of helper CD4 T cells during the multiple episodes of HCV infection. Nevertheless, the role of other CD4 T-cell subsets in the human reinfection setting needs to be defined.

It has been previously shown that T-cell responses were differentially suppressed in patients infected with different strains of HCV, suggesting that T-cell responses are suppressed in a strain-specific fashion. Thus, even patients capable of clearing a primary infection, could develop persistent infection if reinfected by a heterologous strain of the virus, suggesting a role for virus-specific Tregs [394]. Tregs could be beneficial or detrimental during HCV infection, by either limiting immunopathology in the liver or suppressing protective T-cell responses [386]. Frequencies of CD25+ CD4+ T cells were higher in chronically infected patients, and exerted suppression on HCV-specific CD4 and CD8 T cells [267, 311]. Nevertheless, some studies suggested an immunomodulatory role preventing the apoptosis of highly activated CD8 T cells [363]. This is supported by a chimpanzee study showing that the frequency of Foxp3+ CD4+ CD25+ Tregs and the degree of suppression were similar in animals that spontaneously resolved and those that were persistently infected [395].

Our laboratory has already optimized the techniques for detection and measurement of the frequency and functions of different CD4 T-cell subsets. We were able to dissect the functions and ratio between two important CD4 T-cell subsets during acute primary HCV infection; Th17 and Tregs. We demonstrated that acute resolving HCV is characterized by a strong Th1/Th17 response with specific expansion of IL-21 producing CD4 T cells and increased IL-21 levels in plasma, which correlated with the frequency of HCV-specific CD8 T cells. In contrast, viral persistence was associated with lower frequencies of IL-21 producing CD4 T cells, reduced proliferation and increased exhaustion of HCV-specific CD8 T cells. Furthermore, progression to chronic infection was accompanied by expansion of a specific

subset of highly suppressive CD39-expressing Tregs and a reversal in the Treg/Th17 ratio. However, the dynamics of Th17 cells and IL-21 and the plasticity of different CD4 T-cell subsets during the memory phase and reinfection episodes and their role in protective immunity remain to be elucidated and is one of our perspectives (Figure 14).

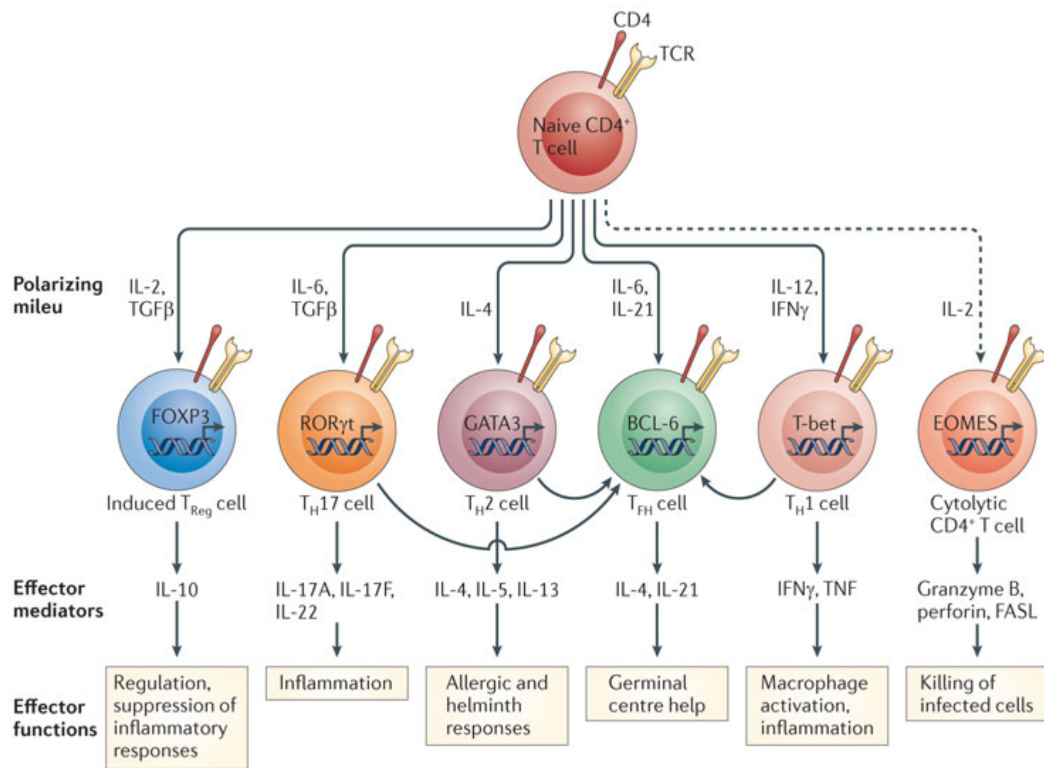

Figure 14: Plasticity of CD4 T-cell subsets in the context of viral infections.

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Legend: Following recognition of a specific antigen, naïve CD4 T cells become polarized into distinct effector T-cell subsets that differentially orchestrate the immune responses. The differentiation of polarized effector T cells is controlled by unique sets of transcription factors, the expression of which is determined by multiple signals during activation. Considerable plasticity exists within T-cell subsets *in vivo*, especially during responses to pathogens. Successful clearance of viruses in particular often depends on complex CD4 T-cell responses that encompass multiple T-cell subsets. Together, these subsets are capable of mediating direct antiviral functions, providing help for B cells and mediating cytotoxic killing of virus-infected cells. BCL-6, B cell lymphoma 6; EOMES, eomesodermin, FASL, FAS ligand; FOXP3, forkhead box P3; GATA3, GATA-binding protein 3; RORγt, retinoic acid receptor-related orphan receptor-γt; TCR, T cell receptor; TGFβ, transforming growth factor-β; TNF, tumour necrosis factor.

6.3. Defining the role of neutralizing antibodies in protective immunity against HCV

The role of humoral immunity in spontaneous resolution of primary HCV infection remains controversial [248, 249, 279]. Even the recently described broadly-neutralizing antibodies against HCV are generated months to years into the chronic infection [248]. The role of humoral immunity during reinfection episodes is far less defined. A rechallenge study in chimpanzees showed that HCV-envelope antibodies remained undetectable regardless of the outcome of the reinfection episode [341]. Another study also showed that chimpanzees that previously cleared a primary HCV infection were able to clear HCV upon rechallenge without any evidence of neutralizing antibodies [338]. On the other hand, Osbourn *et al.* demonstrated in a human cohort that spontaneous resolution upon HCV reinfection correlates with generation of nAbs that were cross-reactive when tested against heterologous HCVpp. Unfortunately, this was in a group of reinfected patients that all resolved the reinfection, not allowing comparison with a control group of non-resolvers [354].

We attempted to examine the neutralizing capacity of longitudinal plasma samples collected at different time points post reinfection (p.ri) against a panel of cell cultured HCV (HCVcc) representing the most common HCV genotypes in Canada (Genotypes 1a, 1b and 3a). However, no distinct difference could be detected between the SR/SR and SR/CI group which may be due to the low number of patients in each group (data not shown). Further investigation of the role of nAbs in a larger cohort using custom HCVpp with the autologous E1/E2 viral sequences isolated during the various episodes of infection will be conducted and are expected to be more reflective of the real infection setting.

6.4. Sequencing the HCV genome during the different episodes of infection

It has already been shown in an HCV-cohort undergoing IFN-therapy that diversity in the whole viral genome sequence correlated with the response to treatment, and these correlations were still valid even when all T-cell epitope regions were excluded from the

analysis. This suggests that these variations could not be solely attributed to differential immune selection [211]. It has also been demonstrated in chimpanzees that the ability to clear consecutive homologous rechallenges was associated with fixed polyprotein sequences. In contrast, persistent infection was associated with numerous changes in the sequences of reinfecting virus [339].

We have demonstrated that the changes in the sequences of the targeted epitopes in the unprotected group of patients (SR/CI) could be a probable cause of failure of the epitope-specific CD8 T-cell recall response. Nevertheless, it does not explain the diminished proliferative capacity and functionality in response to peptide pools. It also does not explain the failure in expansion in the general magnitude and breadth of the general immune response measured by ELISPOT. We will use deep sequencing techniques to sequence entire regions of the HCV genome during reinfection episodes, especially the immunodominant non-structural regions.

6.5. Dissecting the qualities of individual clonotypes associated with protection

It has been shown before within the context of HIV infection that certain epitope-specific clonotypes possess a higher avidity and flexibility in response to different variants of the epitope [89, 376]. The interplay between the virus and the immune system induces the mobilization of CD8 T-cell clonotypes with slightly lower avidity, but higher flexibility to cope with changes in the targeted epitope. Unfortunately, not all patients possess these clonotypes and are therefore unable to keep the mutated virus under check [375, 376].

We will generate cell-lines from individual epitope-specific clonotypes by sorting single tetramer+ CD8+ T cells and then expanding them [89]. We will test the avidity and polyfunctionality of the individual clonotypes against the different variants of the epitope, and verify the association between their avidity, functionality and flexibility.

This would enable us to identify the presence of specific clonotypes correlating with protection and clonotypes having higher flexibility in response to different variants of an epitope. It would also help us extend previous findings in cytochrome-c mouse model and

HIV about the link between the kinetics of the TCR-repertoire and the affinity of the pMHC-TCR binding [89, 372, 376].

6.6. Determining whether specific public clonotypes associate with protection

Analysis of the TCR repertoire of epitope-specific CD8 T cells in HIV controllers compared to progressors showed that their repertoires included public TCR sequences shared between different patients among the controllers' cohorts [371, 375].

We will perform clonotypic analysis of tetramer+ CD8+ T-cell populations targeting specific immunodominant epitopes from more patients to verify the recurrent usage of specific TCR-clonotypes in protected patients that could represent public protective clonotypes.

6.7. Concluding remarks

Successful control and eradication of smallpox, polio and yellow fever viruses causing infections with high rates of morbidity and mortality was achieved through the discovery of vaccines that provide protection upon exposure. Millions of humans are at risk of being infected with chronic viruses as HCV, HIV, HSV and CMV due to the lack of prophylactic vaccines against them. The main hindrance preventing the development of such vaccines is the lack of definition of the characteristics of a protective memory immune response against chronic viruses.

The work presented in this thesis provides a significant advancement in the understanding of the protective immune response as it provides a primary insight towards dissecting the correlates of the adaptive immune response associated with the protection. These results would have an important significance for the development of vaccines against HCV and other similar chronic viruses.

APPENDECIES

APPENDIX I: The candidate's contribution to the articles

Author's contribution to the first manuscript "Signatures of Protective Memory Immune Responses during HCV Reinfection"

Mohamed S. Abdel-Hakeem (MSA) participated in the design of the study. Experiments were planned under the supervision and guidance of the research supervisor Naglaa Shoukry (NS). MSA generated all the data represented in the figures, performed all the analysis and prepared all the figures/tables. MSA contributed with NS to the writing of the article. Nathalie Bédard shared in the optimization of experiments and preliminary tetramer screening for the patients. Donald Murphy performed HCV genotyping assays and viral quantification. Julie Bruneau recruited and provided clinical follow-up for study participants, as well as participated and gave opinion for the study design, data analysis and writing the manuscript. NS supervised the whole study including design, data analysis and writing the manuscript. The data and results presented in this article were all generated during the Ph.D. studies of the candidate (MSA). This article is in revisions at *Gastroeneterology*.

Author's contribution to the second manuscript "Dynamics of Virus-Specific CD8 T-cell Repertoire during HCV Reinfection"

Mohamed S. Abdel-Hakeem (MSA) participated in the design of the study. Experiments were planned under the supervision and guidance of the research supervisor Naglaa Shoukry (NS). MSA generated all the data represented in the figures, performed all the analysis and prepared all the figures/tables. MSA contributed with NS to the writing of the article. Julie Bruneau recruited and provided clinical follow-up for study participants, as well as participated and gave opinion for the study design, data analysis and writing the manuscript. Hugo Soudeyns provided support in the study design, data analysis and writing the manuscript. NS supervised the whole study including design, data analysis and writing the manuscript. The data and results presented in this article were all generated during the Ph.D. studies of the candidate (MSA). This article is to be submitted to the journal *Blood*.

APPENDIX II: Review article

Author's contribution to the review article "Protective Immunity against Hepatitis C; many Shades of Gray"

Mohamed S. Abdel-Hakeem (MSA) participated in the design of the general structure of the review article under the supervision of the research supervisor Naglaa Shoukry (NS). MSA contributed with NS to the writing of the article. MSA prepared all the figures.

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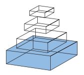

Protective immunity against hepatitis C: many shades of gray

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The majority of individuals who become acutely infected with hepatitis C virus (HCV) develop chronic infection and suffer from progressive liver damage while approximately 25% are able to eliminate the virus spontaneously. Despite the recent introduction of new direct-acting antivirals, there is still no vaccine for HCV. As a result, new infections and reinfections will remain a problem in developing countries and among high risk populations like injection drug users who have limited access to treatment and who continue to be exposed to the virus. The outcome of acute HCV is determined by the interplay between the host genetics, the virus, and the virus-specific immune response. Studies in humans and chimpanzees have demonstrated the essential role of HCV-specific CD4 and CD8 T cell responses in protection against viral persistence. Recent data suggest that antibody responses play a more important role than what was previously thought. Individuals who spontaneously resolve acute HCV infection develop long-lived memory T cells and are less likely to become persistently infected upon reexposure. New studies examining high risk cohorts are identifying correlates of protection during real life exposures and reinfections. In this review, we discuss correlates of protective immunity during acute HCV and upon reexposure. We draw parallels between HCV and the current knowledge about protective memory in other models of chronic viral infections. Finally, we discuss some of the yet unresolved questions about key correlates of protection and their relevance for vaccine development against HCV.

Keywords: hepatitis C, acute infection, HCV reinfection, innate immunity, adaptive immunity, NK cells, T cells, antibodies

INTRODUCTION

Hepatitis C virus (HCV), a member of the family *Flaviviridae*, is a non-cytopathic hepatotropic virus transmitted primarily through contaminated blood (1). The World Health Organization (WHO) estimates that there are 185 million individuals infected with HCV worldwide (2, 3). The prevalence is higher in developing countries, the highest being in Egypt where it is estimated that 15–20% are seropositive for HCV (4, 5). These numbers are probably an underestimate, since many HCV-infected individuals have not been tested and thus remain undiagnosed.

The acute phase of hepatitis C is empirically defined as the first 6 months following infection. Approximately, 20–30% of infected individuals are able to clear the virus spontaneously without any therapeutic intervention during this phase while 70–80% become persistently infected (1). As the virus continues to replicate in the liver of chronically infected individuals, they develop a variety of liver diseases over a period of 5–30 years including hepatic fibrosis, end-stage cirrhosis, and hepatocellular carcinoma (HCC) (6). Such patients represent 25% of the cirrhosis and HCC cases worldwide (7), making hepatitis C the most common indication for liver transplantation in North America (8, 9).

Despite the progress in understanding immunity against HCV infection and its pathogenesis and, the development of highly effective direct-acting antivirals (DAAs), a prophylactic anti-HCV vaccine is still lacking. In absence of such a vaccine, two million new HCV infections are estimated to occur every year (10). Such cases are highly prevalent among marginalized populations like injection drug users (IDUs), men who have sex with men (MSM), and individuals living in developing countries with limited access to screening and treatment (11, 12). Vaccine development against HCV is hampered by our limited knowledge of what constitutes an effective and protective immune response against HCV, as well as protection in real life exposure settings. Many shades of gray remain and constitute active areas of the current research on hepatitis C. In the following pages, we will attempt to review the correlates of protection against HCV and identify these outstanding questions and the ongoing efforts to address them.

THE VIRUS

The HCV genome consists of an uncapped positive single-stranded RNA (+ssRNA) of approximately 9.6 kb-pairs (13). The genome represents an uninterrupted open reading frame (ORF) encoding a polyprotein precursor of approximately 3000 amino acids (13). The viral genome includes 5′ and 3′ untranslated regions (UTRs) that contain secondary RNA structures essential for viral replication. The 5′ UTR harbors an internal ribosome

entry site (IRES) where protein translation is initiated. The HCV polyprotein is processed co- and post-translationally by a combination of cellular and viral proteases into three structural proteins (Core, E1, and E2) and seven non-structural (NS) proteins (P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (**Figure 1**). Core is the building unit of the viral nucleocapsid. The envelope glycoproteins E1 and E2 interact with the viral receptors on permissive cells and mediate viral entry. E2 also contains hypervariable regions (HVR) that are targeted by neutralizing antibodies (nAbs). P7 acts as a viroporin or ion channel. NS2 possesses an autoprotease activity necessary for the polyprotein cleavage between NS2 and NS3. NS3 acts as serine protease in combination with NS4A that acts as a cofactor to catalyze the processing of the HCV polyprotein. NS3 also harbors RNA helicase/NTPase activity that unwinds RNA–RNA substrates and is essential for viral replication [reviewed in Ref. (14, 15)]. The functions of NS4B and NS5A are poorly characterized. However, studies show that NS4B induces the formation of a membranous web compartment where viral replication takes place (16), and cell-culture adaptive mutations mapped to the NS5A enhance RNA replication suggesting its importance for viral replication (17, 18). NS5A was also shown to harbor a region that may determine response to alpha interferon (IFN-α) therapy known as interferon sensitivity determining region (ISDR) (19). NS5B is the viral RNA-dependent RNA-polymerase (RdRp) responsible for HCV–RNA replication (14). As with other RNA viruses, the HCV RdRp 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" (14).

Hepatitis C virus is classified into seven genotypes (1–7) and up to 67 different subtypes based on the nucleotide sequences of the Core/E1 and NS5B regions (20). The different genotypes show marked differences in geographic distribution, response to therapy, and pathogenesis (15).

VIRAL REPLICATION AND LIFE CYCLE

Hepatitis C virus replicates primarily in human hepatocytes. The first described receptors for HCV were CD81 (21) and scavenger receptor class B type I (SR-BI) (22). The LDL receptor and glycosaminoglycans (GAG) mediate initial binding to susceptible cells prior to interaction with CD81 and SR-BI (15). Tight junction proteins claudin-1 (CLDN1) and occludin (OCLN) act as receptors/co-receptors for HCV at cell junctions (23, 24). It was recently reported that the cholesterol absorption receptor Niemann–Pick C1-like 1 (NPC1L1) is an essential entry factor (25) and that the epidermal growth factor receptor (EGFR) and ephrin receptor type A2 are also required for HCV entry and possibly modulate the interaction between CD81 and CLDN1 (26). Human CD81 and OCLN are the minimally required receptors to render mouse hepatocytes susceptible to HCV entry (24). Binding of HCV to its receptors is followed by fusion of the viral envelope with the cellular membrane by clathrin-mediated endocytosis then fusion with the endosomal membrane and the viral genome is released into the cytosol (27). Cap-independent IRES-mediated translation of the HCV ORF generates a large polyprotein that is subsequently processed into mature structural and NS proteins (**Figure 1**). Junctions between structural proteins are processed by host signal peptidases from the endoplasmic reticulum. The viral NS proteins are processed by the NS2/3 autoprotease (14) and the NS3/4A serine protease (28). Replication takes place within ER derived structures known as the membranous web and is mediated by NS5B through a negative strand intermediate in a replication complex. Viral assembly occurs within lipid raft like structures. HCV nucleocapsid is built from units of the core protein with RNA, surrounded by a membrane derived from the human cell with embedded heterodimers of the envelope glycoproteins E1 and E2 [reviewed in Ref. (15)]. The virions associate with low-density and very-low-density lipoproteins (LDL and VLDL) forming lipoviroparticles (LVPs) that are pleomorphic (29).

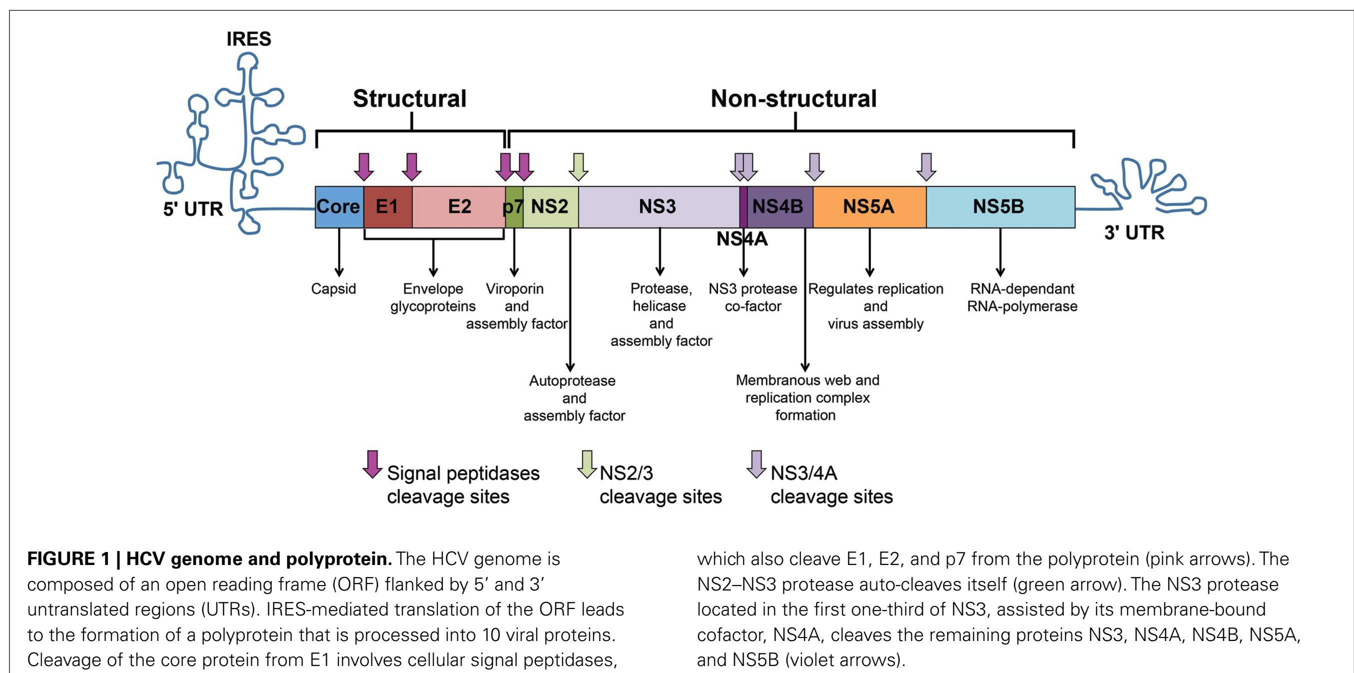

FIGURE 1 | HCV genome and polyprotein. The HCV genome is composed of an open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs). IRES-mediated translation of the ORF leads to the formation of a polyprotein that is processed into 10 viral proteins. Cleavage of the core protein from E1 involves cellular signal peptidases, which also cleave E1, E2, and p7 from the polyprotein (pink arrows). The NS2–NS3 protease auto-cleaves itself (green arrow). The NS3 protease located in the first one-third of NS3, assisted by its membrane-bound cofactor, NS4A, cleaves the remaining proteins NS3, NS4A, NS4B, NS5A, and NS5B (violet arrows).

MODELS FOR STUDYING HCV INFECTION AND IMMUNITY

IN VITRO MODELS

Hepatitis C virus replicates poorly in tissue culture. Earlier surrogate models to study HCV protein functions, virus–host interaction, and viral entry included vaccinia virus (VV) vectors expressing HCV proteins, direct transfection of HCV RNA, subgenomic, and full length replicons and viral pseudoparticles carrying HCV envelop glycoproteins on a capsid backbone of vesicular stomatitis virus or lentiviruses (HCVpp). It was not until 2005 that the first *in vitro* replicating strain was isolated from a Japanese patient with fulminant hepatitis termed JFH-1 virus, a genotype 2a isolate (30–32). Even with the development of this system, very few cell lines are permissive for its replication, often involving adaptive mutations within the viral genome and/or impairment in some of the cellular antiviral mechanisms [reviewed in Ref. (15, 33)]. These models have been instrumental in studying the innate antiviral response against HCV on a cellular level and identification of many of the underlying viral evasion mechanisms. The development of new cell lines or methods that allow HCV replication in primary human or mouse hepatocytes is an area of intense research.

IN VIVO MODELS

Humans and chimpanzees are the only two species that are susceptible to HCV infection. The chimpanzee model has been instrumental in the early studies of immunity against HCV where timing of the infection and infecting viral strains were known and it was possible to examine intrahepatic immune responses. Research on chimpanzees is now restricted (34) and the search for an alternate animal model is ongoing. Although considerable progress has occurred in developing humanized mice susceptible to HCV infection, these mice are generated on immune deficient backgrounds that preclude studying adaptive immune responses. Cotransplantation of human $CD34^{+}$ human hematopoietic stem cells and hepatocyte progenitors in mice with inducible liver damage demonstrated good engraftment of human leukocytes and hepatocytes. These mice became infected with HCV and demonstrated some HCV-specific immune responses and liver fibrosis (35). These data are preliminary and the model remains technically challenging. It will likely be a few more years before we have a suitable alternative to the chimpanzee model for studying HCV-specific immunity and preclinical testing of vaccine candidates [reviewed in Ref. (36)].

Due to the asymptomatic nature of HCV, a limited number of individuals present to the clinic with acute symptomatic infection. In that situation, it is usually difficult to determine the exact date of infection or exposure and the infecting viral strain(s). Most of our early knowledge about acute HCV came from studies of experimental infection of chimpanzees, or individuals infected following high risk exposures like needle stick injuries in health care workers, blood transfusions, as well as the few cases presenting with symptomatic acute HCV. Recent studies relied upon monitoring high risk individuals, in particular IDUs who currently represent the main population of novel HCV infection in developed countries. It is noteworthy that in these situations the definition of acute HCV can vary from one cohort to another and is dependent on the follow-up interval, where the date of infection is estimated at best. It is also ethically impossible to obtain liver biopsies during acute infection and our knowledge of acute intrahepatic responses is derived from the chimpanzee model.

CLINICAL COURSE OF HCV INFECTION

Hepatitis C virus RNA can be detected in the peripheral blood of infected individuals within one week following infection. Despite this high level of viral replication, HCV-specific immune responses remain undetectable in most infected individuals for several weeks suggesting that the virus outpaces the immune system and impairs its responses (37). Nevertheless, interferon stimulated genes (ISGs) are detected early in the liver and some mediators of innate immunity like natural killer (NK) cells are activated and can be detected in peripheral blood (discussed below). At approximately 4–8 weeks, HCV-specific CD4 and CD8 T cells become detectable in the liver and peripheral blood and are often but not always associated with an increase in liver transaminases in the blood (38, 39). Most individuals who are able to spontaneously clear HCV do so within the first 6 months and a smaller proportion may clear their infection within 12 months. The outcome of HCV infection is determined by a "ménage-à-trois" between host genetics, the virus and HCV-specific immunity. Other host factors that influence the outcome of infection include ethnicity, female sex, and accompanying co-morbidities (e.g., co-infection with human immunodeficiency virus (HIV), alcohol abuse, renal disease, obesity, or liver fibrosis) (40).

GENETIC FACTORS THAT INFLUENCE THE OUTCOME OF ACUTE PRIMARY HCV

Several genetic factors, mostly in genes related to innate and adaptive immune functions, influence the outcome of acute HCV infection. Individuals homozygous for the killer inhibitory receptor (KIR) gene KIR2DL3 and its ligand HLA-C1 alleles are more likely to clear HCV infection than individuals with other KIR2DL:HLA-C combinations (41). KIRs interact with their cognate MHC class I ligands and regulate the activation and function of NK cells. KIR2DL3 has a lower affinity for HLA-C1 than other KIR2DL alleles, so HLA-C1-mediated inhibition of NK cells is thought to be weaker in individuals homozygous for KIR2DL3 and HLA-C1 thus enhancing their capacity to inhibit HCV replication or kill virally infected hepatocytes (42). The same KIR/HLA-C combination was consistently more frequent in IDUs with high risk exposure to HCV who remain seronegative/aviremic as compared to those with chronic infection (43).

Genome-wide association studies (GWAS) demonstrated a correlation between several single-nucleotide polymorphisms (SNPs) near the IL28B (IFNλ3) gene locus and the spontaneous resolution of infection, as well as response to IFN therapy (44–47). Although several SNPs were identified, the rs12979860 SNP has become the most relevant (48). The favorable allele was identified as CC and the non-favorable allele CT or TT for heterozygous or homozygous individuals, respectively. These alleles were found to be unequally distributed among individuals of different ethnicities (Asians, Caucasians, and African Americans) and correlated with their differential response to IFN therapy (44, 47). Recent data have reported a novel transiently induced region that carries a dinucleotide variant ss469415590 (TT or

ΔG), which is in high linkage disequilibrium with rs12979860. This resulted in a frameshift variant that created a novel gene, designated IFNL4, encoding the interferon-λ4 protein (49). This mutation was also associated with higher levels of induction of IL28B and interferon gamma-induced protein 10 (IP-10) thus providing a potential mechanism for its enhanced role in viral clearance (50).

It is not yet clear how polymorphism in the IL28B and/or IFNL4 gene influences immune responses and the capacity to spontaneously eliminate HCV. Given that the IL28B and IL29 (IFNλ1) genes are in opposite orientation, this SNP is up stream of the two genes and may influence expression of both. The responder genotype was linked to higher expression of the IL28A/B as measured by qRT-PCR in PBMCs or protein levels in plasma and lower HCV viral load, but results were not consistent among different studies (45, 51, 52). IL-29 was upregulated during acute and chronic HCV and its expression correlated with induction of ISGs but not acute infection outcome (52, 53). The non-favorable IL28B genotype was associated with higher expression of ISGs in the liver (54–56), considered a predictor of non-response to IFN therapy. Interestingly, ISGs were differentially expressed in liver versus macrophages, where the non-responder genotype was associated with lower ISG expression in macrophages (56).

It was also suggested that IL28B polymorphism may influence NK cell function or the cross-talk between innate and adaptive immunity. Dring et al. have demonstrated that KIR2DS3 synergizes with IL28B to predict chronic evolution of HCV (57). Suppiah et al. have demonstrated that polymorphism in IL28B, HLA-C, and KIRs additively and interactively predict response to therapy in chronic HCV infection (58). However, functional data supporting this possibility are still lacking.

A more recent GWAS study demonstrated that in addition to IL28B, another SNP near the gene for the HLA class II molecule DQB1*03:01, was independently associated with spontaneous resolution of HCV infection (59). Indeed, several HLA HLA class I and class II alleles were previously associated with the outcome of HCV [reviewed in Ref. (60)]. The HLA class I alleles HLA-A*03, HLA-B*27, and HLA*B57 were associated with spontaneous clearance [reviewed in Ref. (60)]. Interestingly, the HLA-B*27 and -B*57 alleles are also associated with better viral control in HIV infected individuals (61). The HLA class II alleles HLA-DRβ1*0101, -DRB1*0401, -DRB1*1101, and DQB1*0301 were strongly associated with HCV clearance, whereas HLA-DRβ1*0701 was associated with HCV persistence (62–64). It is suggested that such HLA class I and II alleles favor the generation of immune responses targeting conserved epitopes or epitopes that are less likely to mutate because of the high fitness cost associated with such mutations and viral replication (65).

Other genetic factors that were associated with spontaneous clearance of HCV include: ISGs, toll-like receptors (TLRs), chemokines, cytokines, and their receptors [reviewed in Ref. (60)], as well as tapasin associated with peptide loading on MHC class I (66). Finally, HCV was also shown to exploit "holes" in the T cell repertoire to facilitate its escape from detection by virus-specific CD8 T cells (67).

INNATE IMMUNE RESPONSES DURING ACUTE PRIMARY HCV

VIRAL SENSING AND INTRACELLULAR INNATE IMMUNITY

Foreign RNA molecules are recognized by pattern recognition receptors (PRRs) including TLRs and/or intracellular nucleic acid-binding proteins (68, 69). In hepatocytes, TLR3, protein-kinase R (PKR), and retinoic-acid-inducible gene I (RIG-I) are the main PRRs recognizing HCV upon entry and replication. PKR and RIG-I activation signals are relayed via the mitochondrial antiviral signaling protein (MAVS), while TLR3 signals are relayed via the TIR domain-containing adapter inducing IFN-β (TRIF) [reviewed in Ref. (70)]. Downstream of these adaptor proteins signaling cascades are activated culminating in the secretion of type I IFNs (70). Binding of auto- and paracrine type I IFNs to their receptors activates signaling along the JAK–STAT signaling pathway. This induces the expression of hundreds of ISGs in the infected cells and neighboring cells (71) and creates a general antiviral state in the liver that limits HCV RNA replication and cell-to-cell spread (72). ISGs expressed in response to HCV infection include several proteins with known antiviral effects, as well as some others that were shown to promote HCV replication *in vitro*, as ISG15 and USP18 (73, 74).

Upregulation of ISGs is detected in the liver early after HCV infection irrespective of the outcome, suggesting that most HCV isolates are resistant to the antiviral effects of this early innate response (75–78). Nevertheless, the pro- and anti-viral roles of the different ISGs would suggest differential induction of specific ISGs in patients with different outcomes of HCV infection. Recent work on primary hepatocytes infected with HCV demonstrated that type III IFNs (IFNλs) could be the earliest responders and that they lead to induction of ISGs shared with type I IFNs, as well as other distinct ISGs (79, 80). Preliminary studies suggest that although similar ISGs are induced by type I and type III IFNs, they may signal through two distinct pathways (81), have different kinetics (82) maybe cell type specific (56) and/or IFNλ allele dependent (83).

Infected hepatocytes are not the only source of type I and type III IFNs but liver resident Kupffer cells (84), BDCA3^{+} myeloid dendritic cells (mDCs) (85), and plasmacytoid DCs (pDCs) (86, 87) can detect HCV RNA and produce type I and type III IFNs, thus contributing to the intrahepatic antiviral state and inducing inflammatory cytokines and chemokines like IP-10 that recruit virus-specific T cells to the liver.

Despite the rapid viral sensing and induction of antiviral responses, HCV is able to persist as it has devised several mechanisms to evade recognition and inhibit IFN-signaling pathways. NS3/4A was shown to use its serine protease activity to act as the viral "Swiss army knife" to disrupt viral sensing pathways at early steps by splicing MAVS and TRIF downstream of the major sensors of HCV–RNA [reviewed in Ref. (70)]. Other HCV proteins as core, E2, and NS5A interfere with various early and late steps along the JAK–STAT signaling pathway that is induced by type I and III IFNs further contributing to inhibiting the IFN response (88–90). However, ISGs remain highly expressed in individuals who develop chronic infection and their level of expression correlates inversely with the response to IFN based therapies (91). This suggests that

sustained viral replication and induction of ISGs induce a state of tolerance within hepatocytes that become refractory to external IFN stimulation.

NATURAL KILLER CELLS

Natural killer cells are one of the earliest lines of innate immune defense. They exert their function by killing virally infected cells via secretion of cytotoxic molecules like granzymes and perforin or through tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated killing. They also secrete cytokines that can regulate innate and adaptive immunity like IFNγ, TNFα, IL-10, and IL-21. NK activity is governed by the balance between activating and inhibitory signals. One of the major determinants of such activity is the strength of the interaction between the inhibitory KIRs expressed on NK cells and their MHC class I ligands expressed on target cells (92). NK cells are highly enriched in the liver and are expected to play a major role in controlling hepatotropic infections (93, 94). NK cells were activated early in healthcare workers following accidental percutaneous exposure to HCV and may have contributed to protection from infection in 11/12 individuals in this group who remained aviremic (95). NK cells were also activated in high risk IDUs and expression of the NK cells activating receptor NKp30 correlated with protection from HCV infection (96). Hyperactivation of NK cells was observed during acute HCV infection irrespective of the outcome (97–99). Increased IFNγ production was enriched in KIR2DL3 expressing NK cells as suggested by genetic studies demonstrating that this allele was associated with spontaneous resolution of HCV potentially due to less NK cell inhibition (98). Reduced expression of NKp30 could predict the outcome of acute HCV (97). Notably, NK cells from HCV acutely infected individuals and during chronic infection were biased toward cytotoxicity rather than cytokine production (99, 100) and NK cell degranulation, a surrogate marker of cytotoxicity, correlated with the magnitude of HCV-specific T cells (99). This suggests that NK cell cytotoxic activity may kill infected hepatocytes and facilitate the transfer of HCV antigens to the draining lymph nodes as they are picked up and presented by DCs.

During chronic HCV infection, intrahepatic NK cells are generally highly activated and this activation correlates with the degree of liver inflammation (101, 102). Peripheral NK cells express variable levels of the NK activation markers NKp30, NKp44, NKp46, NKG2C, and NKG2D and increased expression of the inhibitory receptor NKG2A as compared to healthy individuals [reviewed in Ref. (103–105)]. NK cell numbers and functions, especially cytotoxic activity, were shown to be lower in chronic hepatitis C patients as compared to healthy donors but results varied from one study to another probably reflecting various experimental designs and patient cohorts (106–109). Recombinant HCV E2 protein was reported to directly bind CD81 on the surface of NK cells and inhibit its functions (110, 111). HCV-infected hepatocyte cell lines but not purified virions reproduced a similar phenomenon (112, 113).

Recent data from the lymphocytic choriomeningitis virus (LCMV) model has demonstrated that NK cells may act as rheostat to control CD4-mediated help for CD8 T cell functions during chronic infection (114). Similarly, in the hepatitis B virus (HBV) model, it was shown that NK cells can selectively eliminate activated T cells expressing the TNF-related apoptosis-inducing ligand death receptor 2 (TRAIL-R2) and thus, negatively regulate antiviral immunity (115). NK cells also interact with DCs and this cognate interaction regulates both innate and adaptive immunity. NK cells produce IFNγ and TNFα that induce maturation of DCs and enhance their capacity to prime virus-specific T cells. In return, DCs produce IL-12 and IL-15 that enhance activation of NK cells (116). It would be interesting to examine if such cross talk exists during acute HCV infection and cross-regulates adaptive immunity.

DENDRITIC CELLS

Dendritic cells are one of the major antigen-presenting cells (APCs) in the body. They bridge innate and adaptive immunity and may impact priming of HCV-specific immune responses. DCs rapidly differentiate into mature DCs in response to various "danger" signals like activation through pathogen associated molecular patterns (PAMPs) in particular TLR ligands, interaction with innate lymphocytes (NK and NKT cells), cytokines, and inflammatory mediators (117). There are two main subsets of DCs, mDCs representing the majority of DCs and mostly associated with antigen processing and presentation and pDCs that can sense viral infections and are the main producers of type I and type III IFNs. pDCs can detect HCV RNA in a TLR-7 specific manner when presented as part of an infected cell (86). This activation can be mediated by transfer of exosomes containing HCV–RNA from infected cells to pDCs (87). As such, DCs are considered a main orchestrator of the HCV innate and adaptive immune response.

The role and function of DCs during acute and chronic HCV infection remain highly controversial. The frequencies of mDCs and pDCs were shown to correlate with the outcome of infection, where reduced frequencies were associated with chronic infection (118–121). Our group has recently demonstrated that sustained hyperresponsiveness of DCs was associated with resolution of HCV infection suggesting better priming of HCV-specific T cells (122). Various groups have reported that DCs are defective in chronic HCV, in particular, in response to TLR ligands (123–126), may be infected (123–126), and may induce proliferation of Tregs (127) while others have demonstrated that they are functional (118, 120, 128, 129). How do DCs influence priming of HCV-specific immune responses? Is it through a direct effect on antigen presentation or through better NK–DC interaction? Are DCs presenting HCV antigens more tolerogenic or targets for elimination by NK cells? These remain as some of the gray areas in the HCV field.

ADAPTIVE IMMUNE RESPONSES DURING ACUTE PRIMARY HCV

The importance of cell-mediated immunity in HCV clearance is demonstrated by the correlation between specific HLA class I and class II alleles and spontaneous resolution and underscored by depletion studies in the chimpanzee model showing that both CD4 and CD8 T cells are required for viral clearance and that they have complementary rather than exclusive roles. The role of nAbs is slowly being unraveled. In the following sections, we will summarize the current state of knowledge on this topic.

ONSET OF ADAPTIVE IMMUNE RESPONSES

HCV-specific CD8 and CD4 T cell responses appear late around 6–8 weeks following primary HCV infection despite high levels of viral replication. This delay in the onset of virus-specific T cells could be attributed to the tolerogenic nature of the liver environment and the time required for viral antigens to reach the draining lymph nodes to be presented by professional APCs (38, 94). It might also be indicative of the capacity of the virus to evade the innate immune system, interfere with maturation of DCs or impair their capacity for antigen processing and presentation. Onset of HCV-specific CD8 T cells in blood and/or liver and the detection of IFNγ, CD3, CD4, and CD8 transcripts in the liver are kinetically linked to decline in viral loads (76, 77).

CD8 T CELL RESPONSES DURING ACUTE PRIMARY HCV

The breadth of the CD8 T cell response is a key determinant of spontaneous resolution. Up to nine different epitopes were recognized simultaneously in chimpanzees and humans with acute resolving HCV while much fewer epitopes were recognized in individuals developing chronic infection (130, 131). Responses targeting the NS proteins were immunodominant and correlated with spontaneous clearance (132), but the individual epitopes targeted were different, even among individuals with shared HLA alleles (133, 134).

Escape mutations within targeted CD8 T cell epitopes are associated with viral persistence and represent a major immune evasion mechanism used by the virus. They typically occur early and become fixed in the viral quasispecies (135–137). Mutated epitopes are mostly associated with loss of binding to the restricting MHC and are thus not recognized or poorly induce a new T cell response (135, 136). Escape mutations are also dependent on the interplay between the virus and host genetics. The host HLA alleles enforce selective pressure on their cognate epitopes. This is evident as these mutations revert to their wild type sequences when transmitted to an individual not carrying the same HLA allele and where the epitope is no longer under selection pressure (138). Host HLA may also induce selection pressure on a population level resulting in viral adaptation within a specific genetically related population (139). Finally, viral fitness limits the variability within some epitopes (140, 141). Certain HLA-alleles like HLA*B27 are considered protective as they prime responses to highly constrained epitopes that are less likely to mutate because of the high fitness cost (65, 142, 143).

The epitope-specific CD8 T cell receptor (TCR) repertoire may also limit escape mutations. One study in the chimpanzee model suggested that generation and maintenance of a repertoire with higher diversity was associated with resolution of HCV infection and limited the emergence of escape mutations in the targeted epitopes (144). Another study correlated a specific mutation in an MHC class I restricted epitope ($NS3_{1406}$) to the scarcity of specific TCRs that could recognize the mutant, thus exploiting this "hole" in the T cell repertoire (67). Several studies in HIV cohorts demonstrated an association between the ability to control HIV replication and progressing to AIDS with the presence of specific CD8 T cell clonotypes that possessed superior functional avidity, as well as cross-reactivity to the different variants of the targeted epitope (145). Additional studies examining the dynamics of the TCR repertoire, functionality, and escape mutations during acute and chronic HCV in relation to changes in viral quasispecies would be of interest.

The magnitude of responding CD8 T cells also correlates with spontaneous resolution. Studies using MHC class I tetramers demonstrated that T cells specific for one single epitope can reach up to 8% of CD8 T cells in spontaneous resolvers (131, 146). The frequency of HCV-tetramer positive population was several folds higher in the liver than in peripheral blood of chimpanzees during acute resolving HCV with elevated expression of CD69, a marker of T cell activation (147).

The use of MHC class I tetramers allowed direct *ex vivo* phenotypic characterization of the HCV-specific CD8 T cells. Early expression of the IL-7 receptor alpha (CD127) on virus-specific CD8 T cells emerged as a major predictor of spontaneous resolution while its loss was associated with viral persistence (148, 149). CD8 memory T cell populations generated following clearance of primary HCV infection were $CD127^{hi}$ and $Bcl\text{-}2^{hi}$ (146). This is consistent with data from the LCMV model showing that CD127 is a marker of cells destined to become long-lived memory T cells (150). On the other hand, HCV-specific CD8 T cells express variable levels of PD-1 during acute infection suggesting that PD-1 acts more as an activation rather than an exhaustion marker (151–153). Other exhaustion markers like the T cell immunoglobulin and mucin domain 3 (Tim-3), cytotoxic T lymphocyte associated antigen-4 (CTLA-4), CD160, KLRG-1, and 2B4 are differentially expressed during acute and chronic HCV suggesting a spectrum of exhaustion that correlated with function and persistent infection (154, 155). The Tim-3 ligand galectin-9 (Gal-9) was upregulated in the plasma during acute infections progressing to chronicity (155, 156) and in the liver of chronic individuals (156), thus contributing to T cell exhaustion. Blockade of PD-1, Tim-3, and CTLA-4 separately or in combination rescued HCV-specific CD8 T cells from exhaustion suggesting that they act synergistically (157–159). *In vitro* supplementation of IL-21, a mediator of CD4 T cell help, also rescued HCV-specific T cells from Tim-3/Gal-9-mediated apoptosis (155).

HCV-specific CD8 T cells become difficult to detect in the peripheral blood of individual who develop chronic infection but are readily detectable in the liver and continue to express a highly activated and exhausted phenotype (160, 161). HCV-specific intrahepatic CD8 T cells from chronic HCV patients expressed TIM-3, PD-1, and 2B4, while CD8 T cells from patients who had cleared the virus following IFN therapy (i.e., in absence of active viral replication) and T cells specific for cytomegalovirus lacked TIM-3 and expressed higher levels of LAG-3; these cells also exhibited different memory phenotypes and proliferative responses (162).

HCV-specific T cells in the peripheral blood might be slightly impaired in their proliferative and cytokine producing capacity when they first appear in blood suggesting a "stunned" phenotype (163). Analysis of multiple effector functions simultaneously identified the presence of a polyfunctional population of virus-specific CD8 T cells that correlated with spontaneous clearance of HCV (146), similar to what was observed during control of HIV infection (164) and in the LCMV model (165). The functions analyzed included secretion of the effector cytokines IFNγ, the T cell growth

factor IL-2 and the degranulation marker CD107a, a surrogate marker of cytotoxicity (146). Assays on sorted cells demonstrated that polyfunctionality was localized within the $CD127^+$ HCV-tetramer reactive CD8 T cells providing further evidence to the importance of this T cell subset in mediating viral clearance (146).

Differences in CD8 T cell functions become increasingly apparent as the infection progresses and are associated with loss of helper CD4 response, as well (discussed below). In patients who become chronically infected T cells show progressive loss of function, reduced polyfunctionality and diminished proliferative capacity (146, 166). CD8 T cells detected in chronic HCV patients were arrested in an early maturation stage, impaired in cytokine production, cytotoxicity, and/or proliferative capacity (167–169). The loss in function also correlates with the degree of exhaustion as described above and were reversible *in vitro* upon blockade of the PD-1, CTLA-4, and/or Tim-3 pathways (170). However, *in vivo* blockade of the PD-1 pathway in chronically infected humans and chimpanzees had limited efficacy suggesting that a threshold of functional HCV-specific T cells is required for such immunotherapeutic strategies to work (171, 172). Functional exhaustion could be attributed to the persistence of the antigen, where it was shown in the LCMV model that prolonged exposure to viral antigens is the main cause for reduced frequency and impaired effector functions of virus-specific CD8 T cells (173, 174). Confirming this hypothesis are data demonstrating that HCV-specific CD8 T cells detected in chronic patients where the respective cognate epitope was mutated (i.e., no longer stimulated by the cognate epitope) were functional and expressed lower levels of PD-1 and increased CD127, a marker of long-lived memory cells and a predictor of spontaneous resolution (175–177). Similarly, early therapeutic intervention and sustained virologic response to IFN therapy rescued $CD127^+$ long-lived memory T cells (146, 176).

In summary, spontaneous resolution of acute HCV correlates with early emergence of $CD127^+$ HCV-specific CD8 T cells. The response is also broad, of high frequency and polyfunctional in nature. Several factors contribute to failure of this response in individuals who become persistently infected. First, escape mutations in targeted epitopes facilitate viral evasion of the immune system. Second, loss of CD4 help or a switch to an immunoregulatory profile further compromises the antiviral capacity of virus-specific CD8 T cells (discussed below). Third, continued viral replication contributes to exhaustion of HCV-specific T cells through persistent antigenic stimulation leading to progressive loss of function and diminished survival of virus-specific CD8 T cells. Fourth, exhaustion is exacerbated by increased expression of the ligands to the inhibitory receptors like Gal-9.

CD4 T CELL RESPONSES DURING ACUTE PRIMARY HCV

The importance of CD4 T cells in clearance of primary acute HCV was first demonstrated by the correlation between CD4 proliferative responses targeting the HCV NS proteins and viral clearance (178, 179), whereas the loss of such responses was associated with viral recurrence (37, 131, 180). The breadth and vigor of the CD4 response also correlated with spontaneous resolution (179, 180). CD4 T cell responses targeting up to 14 different epitopes were detected in individuals with acute resolving HCV (181) and some of these epitopes were identified as promiscuous epitopes that can be presented by multiple HLA-DR alleles (182, 183). Unlike CD8 epitopes, escape mutations were uncommon in MHC class II restricted CD4 T cell epitopes suggesting that this is an unlikely mechanism of CD4 T cell failure (184, 185).

Despite the development of MHC class II tetramers and multimers, direct *ex vivo* detection and characterization of HCV-specific CD4 T cells has been limited due to the low frequency of tetramer reactive T cells, very often requiring enrichment. In addition, the restricted number of epitopes and tetramers that are available limited such analysis. Direct *ex vivo* studies have demonstrated that HCV-specific CD4 T cells are detectable by tetramers mostly during the acute phase. The frequency declines and they remain detectable in humans who resolve the infection during the memory phase but require enrichment where they were shown to express markers of central memory T cells ($CCR7^+CD45RA^-CD27^+$) (186, 187). Nevertheless, HCV-specific CD4 T cells were detectable directly *ex vivo* by MHC class II tetramers in one chimpanzee at 7 years post resolution of acute primary HCV (152). The use of tetramers also revealed that broad virus-specific CD4 T cells were present early in most individuals acutely infected with HCV irrespective of the outcome, but rapidly lost the proliferative capacity and cytokine production, specifically IL2, and disappeared from the periphery with the establishment of chronic infection (188, 189). This challenged the prevailing hypothesis that HCV persistence is the result of a failure to prime virus-specific CD4 T cells and suggested that it is rather T cell exhaustion and the failure to sustain such CD4 T cell responses that leads to chronicity. Indeed, *in vitro* blockade of the PD-1, IL-10, and TGF-β pathways rescued the proliferative and cytokine producing capacity of CD4 T cells from individuals with chronic HCV (190).

The importance of CD4 T cell help in maintaining a functional CD8 T cell response during HCV infection was clearly demonstrated by CD4 depletion studies in chimpanzees, where the frequency of virus-specific CD8 T cells and their cytokine producing capacity gradually declined resulting in accumulation of escape mutations in the targeted CD8 epitopes (191). So how do HCV-specific T cells provide help for CD8 T cells? Our group recently demonstrated that expansion of $CD161^{hi}CCR6^+CD26^+CD4^+$ Th17 T cells correlated with spontaneous resolution of HCV. These cells produced large amounts of IL-17A and IL-21 and this correlated with the increased plasma concentration of these two cytokines and spontaneous resolution of HCV (155). Specifically, increased plasma levels of IL-21 during the late acute phase correlated with the frequency of virus-specific CD8 T cells and rescued them from Tim-3/Gal-9-mediated apoptosis (155). This underscored the role of IL-21 as a major helper cytokine similar to previous observations on the LCMV model and HIV (192). Other sources of IL-21 like NK cells and follicular helper T cells (Tfh) remain unstudied and may play an active role in mediating CD4 help.

Virus-specific Th17 and regulatory T cells (Tregs) were detected during acute and chronic HCV (193–195) and Th17-like CD4 T cells were enriched in the livers of individuals with chronic HCV (196). The preferential expansion of Tregs with viral persistence may represent another level of inhibition of HCV-specific CD4 and CD8 T cells. $CD39^+CTLA4^+$ Tregs were expanded during acute HCV infections progressing to chronicity with a shift in the

Th17/Treg balance (155). Such Tregs can be a source of Gal-9 further contributing to the inhibition/exhaustion of CD4 and CD8 T cells expressing Tim-3 (155). Tregs are also expanded in chronic HCV but it is not yet clear if expansion of Tregs is a cause or an effect of HCV persistence as a feedback mechanism to limit virus induced immunopathology and inflammation. A recent report suggested that subinfectious exposures to HCV in the chimpanzee model may predispose to the development of Tregs that can later suppress HCV-specific responses upon subsequent infection (197) suggesting that elevated Tregs can be a cause of persistence. Finally, the flexibility and reversible nature among the different T-helper subsets is a domain of intense research (198) and whether HCV-specific CD4 T cells are endowed with such plasticity and the pathways involved remain to be seen.

In summary, broad HCV-specific CD4 T cell responses are primed during most acute HCV infections. However, contraction of this CD4 T cell population and its failure in sustaining a robust CD8 T cell effector response is a hallmark of HCV persistence. Early induction of IL-21 producing Th17 cells is critical to mediate help and limit exhaustion of virus-specific CD8 T cells. However, CD4 T cell exhaustion, leads to sequential loss of IL-2 production, proliferative capacity, and IFNγ production in addition to an imbalance in the Th17/Treg ratio. Tregs may contribute to exhaustion and inhibition of CD4 and CD8 T cells through production of the Tim-3 ligand Gal-9 and the regulatory cytokines IL-10 and TGF-β.

HUMORAL RESPONSES DURING ACUTE PRIMARY HCV

Although, HCV RNA reaches high serum titers by week 2 post infection, anti-HCV antibody response (seroconversion) is usually not detected before week 8 (199, 200). Early studies showed that antibodies (Abs) targeting the HVR-1 region of the E2 glycoprotein of HCV are neutralizing *in vitro* and *in vivo* (201, 202), but select for mutations in the envelope region (203). Chimpanzee studies showed that generation of Ab responses was not necessarily associated with viral clearance (204, 205). In humans, Ab responses were delayed, were of low titers and declined rapidly in individuals who cleared HCV spontaneously (206–208). One study showed that nAbs appeared in patients after HCV has already established chronic infection and were unable to clear the virus and selected for escape mutants (209). However, another study showed that early induction of nAbs was associated with spontaneous resolution of HCV primary infection (210). Broadly nAbs that could protect against heterologous HCV infection were recently reported (211, 212). Similar broadly nAbs confer protection against most pathogens, yet, they did not necessarily correlate with the control of infection or protection against superinfection in some chronic viral infections like HIV (213).

One of the major limitations for understanding humoral immunity against HCV is the lack of proper tools to measure precisely the levels of nAbs. The current method measures neutralization of HCVpp or infectious virions carrying HCV E1–E2 envelope glycoproteins corresponding to a limited number of HCV genotypes reference sequences, and thus do not necessarily represent the autologous E1/E2 sequences circulating in the patient (214, 215). An HCVpp library comprised of 19 genetically-distinct sequences that represent the natural variability of genotype 1 E1/E2 was recently used to demonstrate the evolution of the HVR-1 sequences in response to nAbs (216). A recent study using this HCVpp library demonstrated that resolution of HCV infection was associated with a broad nAb response generated early during the infection (217).

Another antiviral role for anti-HCV Abs could be through induction of antibody-dependent cellular cytotoxicity (ADCC). ADCC is a mechanism whereby the variable region of Abs binds to infected cells while the constant region (Fc) is recognized by Fc receptors expressed by various innate immune cells including NK cells. Binding to the FcγR3a (CD16) triggers cytokine production and degranulation of NK cells, resulting in lysis of the targeted cell. Several studies suggest that ADCC plays a role in controlling viral replication during HIV infection (218, 219) and may have contributed to the success of the HIV RV144 vaccine trial performed in Thailand (220). So far, only one study has demonstrated the presence of ADCC-inducing antibodies specific for the E2 envelop protein in several HCV patients during acute and chronic HCV infection, as well as after spontaneous clearance (221). Further studies will be required to confirm these results and determine if ADCC-inducing antibodies play a role in the spontaneous clearance of HCV infection and in the protection against HCV infection and reinfection.

Some major challenges exist for generating protective humoral immunity against HCV. First, the envelope proteins are not highly immunogenic, causing the Ab response to be slow and weak during primary infection (216). Second, Abs mostly target the HVR of E2, a region with high mutation rates, which facilitates the selection of viral sequences that are highly resistant to Ab neutralization (222). This selection was shown to take place as the cellular immunity collapsed and the infection progressed to chronicity (223). Third, the epitopes targeted by nAbs are shielded by heavy glycosylation and complexation with host lipoproteins, which limits their efficacy *in vivo* (224).

In summary, recent data suggest that nAbs are induced earlier than what was previously thought and may play an active role in spontaneous resolution but these results remain inconclusive and further investigation using autologous E1/E2 sequences is required to understand this role. The recent elucidation of the crystal structures of E2 (225) and E2 bound to the broadly nAb AR3C (226), as well as structural mutational studies should provide better insights into how E1/E2 interact with their receptors and nAbs. In addition, the role of CD4 T cells, in particular Tfh cells, in providing help for antibody production by B cells remains undefined.

SUMMARY OF ACUTE PHASE IMMUNE RESPONSES AND MECHANISMS OF IMMUNE EVASION

Hepatitis C virus infection of hepatocytes activates innate immune sensing mechanisms like TLRs, RIG-I, and PKR that activate signaling cascades resulting in production of type I and type III IFNs and induction of several antiviral ISGs in infected hepatocytes. HCV can also be transferred to DCs or Kupffer cells via exosomes leading to activation of innate pathways and additional secretion of IFNs. Intrahepatic NK cells are activated by IFNs and recognize and kill HCV-infected hepatocytes generating apoptotic bodies containing HCV antigens. These apoptotic bodies are taken up by APCs like Kupffer cells and DCs that process and present HCV

antigens as peptides bound to MHC class I and II and prime HCV-specific CD8 and CD4 responses, respectively. Infected hepatocytes also present processed HCV antigens in the context of MHC class I and prime virus-specific CD8 T cells that contribute to killing and generation of additional apoptotic bodies containing viral antigens. The transfer of HCV antigens from the liver to draining lymph nodes via migratory DCs is thought to be critical for priming of efficient immune responses. Several populations of HCV-specific CD4 helper T cells are primed including Th1 cells that provide help for CD8-mediated killing of infected hepatocytes via production of IFNγ and TNFα, Th17 cells that produce IL-21 that limits exhaustion of HCV-specific CD8 T cells and rescues them from apoptosis and Th2 cells that provide help for antibody producing B cells and generation of nAbs via IL-4 and IL-6. The role of Tfh cells and their support for development of nAbs remain undefined during acute HCV, as well as the potential contribution of ADCC to viral control. Similarly, cross-talk between NK cells and DCs and how this interaction influences priming of virus-specific T cells and regulates their function are unknown. HCV can evade innate immunity by interfering with the IFN-signaling pathway, inhibiting NK cells' functions (even transiently), escape mutations of targeted CD8 T cell epitopes, or exhaustion of virus-specific CD4 and CD8 T cells through upregulation of exhaustion molecules like PD-1, Tim-3, CTLA-4, and others. Tregs are also induced and they can dampen HCV-specific responses directly through secretion of the immunoregulatory cytokines IL-10 and TGF-β or production of Gal-9 that can induce apoptosis of Tim-3$^+$ virus-specific Th1, Th17, and CD8 T cells (**Figure 2**).

PROTECTIVE IMMUNITY AGAINST HCV

HCV-specific memory CD4 and CD8 T cells were detectable in the peripheral blood of humans who have spontaneously resolved primary acute HCV for up to 20 years following viral clearance while antibody responses waned (227). Similarly, memory CD4 and CD8 T cells were detectable in the peripheral blood and liver of HCV-resolved chimpanzees at 7 years following resolution of primary infection (147). Virus-specific CD4 and CD8 T cells were also detectable in individuals with potential occupational, household contact, or high risk behavior like injection drug use, in absence of detectable antibody responses or history of infection suggesting subclinical exposure, and priming of HCV-specific memory T cells (228–231).

The frequency of HCV-specific memory T cells was stable for several years after the resolution of primary infection in chimpanzees (147). Rechallenge studies demonstrated no sterilizing immunity but were characterized by a shorter duration of viremia and lower viral loads and were associated with protection from virus persistence in most (147, 232–235), but not all studies (236). Expansion of HCV-specific memory CD4 and CD8 T cells in peripheral blood and the liver was associated with rapid control of HCV infection upon rechallenge. IFNγ producing CD4 and CD8 T cells were detected at a higher frequency and much earlier during secondary infection as compared to primary infection in the same chimpanzees (147, 191, 232–235). The hierarchy of CD4 helper T cell epitopes was preserved during secondary infection years later (182). Control of viral replication in animals challenged with HCV following experimental vaccination was associated with priming of CD127$^+$ PD-1^{lo} CD8$^+$ T cells that persisted at high levels for prolonged periods and were bifunctional, producing both IFNγ and TNFα (237). Another study demonstrated that clearance of HCV reinfection upon heterologous rechallenge involved the activation of both innate and adaptive immune responses. Higher intrahepatic gene expression of CD56, CD8, type I and II IFN, and ISGs were observed in one out of two chimpanzees who were able to clear the rechallenge virus (238). This response correlated with expansion of HCV-specific CD4 and CD8 T cells in the peripheral blood in absence of nAbs (238).

The protective role of memory CD4 and CD8 T cells was confirmed using antibody-mediated depletion. CD4 T cell depletion was associated with low level viremia. Although memory CD8 T cells initially provided some control for viral replication, they rapidly waned in absence of CD4 T cell help leading to accumulation of escape mutations in targeted epitopes and viral persistence after more than 1 year of follow-up (191). In contrast, memory CD8 T cell depletion led to a significant delay in control of viremia, and this control coincided with the recovery of HCV-specific T cells in blood and liver (147). Altogether, these data demonstrate a protective role for memory T cells upon reexposure to HCV but the role of nAbs was not sufficiently analyzed due to lack of the proper tools and the generally weak anti-HCV antibody response induced in chimpanzees.

As described above, HCV-specific memory CD4 and CD8 T were detectable 20 years post-clearance of the virus, whereas antibodies were barely detectable (227). Phenotypic characterization of HCV-specific CD4 T cells in spontaneously resolved cases showed they were CCR7$^+$, CD45RA$^-$, and CD27$^+$, characteristic of central memory T cells (186). Spontaneous resolution from a primary HCV infection was associated with the generation of polyfunctional HCV-specific memory population expressing CD127^{hi} Bcl-2^{hi} profile (146, 148, 149) consistent with a profile of long-lived memory CD8 T cells (150). It is not clear how HCV-specific memory CD4 and CD8 T cells are maintained for prolonged periods. Apart from their intrinsic long-lived properties and transcriptional profile, memory T cells can be maintained through homeostatic proliferation in response to the γ-chain cytokines IL-7, IL-15, and IL-21 (239, 240). They may also be maintained through periodic restimulation with cross-reactive viral or self-antigens. Finally, it is possible that minor quantities of HCV RNA are maintained for a prolonged period following viral clearance and contribute to periodic restimulation of such memory T cells (241, 242).

The protective role of HCV-specific immunological memory is difficult to address in humans. Nevertheless, epidemiological studies demonstrated that high risk IDUs who have spontaneously resolved one HCV infection were less likely to be reinfected than HCV-naïve individuals despite repeated high risk exposures (243–246), but the reported rate of incidence of reinfections was variable because of the different HCV RNA testing interval where reinfections of shorter duration or accelerated viral clearance may have been missed (247). Other factors include differences in the characteristics of individual subjects, as age, ethnicity, and risk behaviors (247). Nevertheless, the rate of spontaneous clearance of reinfection was higher than that for primary infection (243, 245, 248). This provided preliminary evidence of protective immunity against HCV. Osburn et al. monitored a group of high risk IDUs at

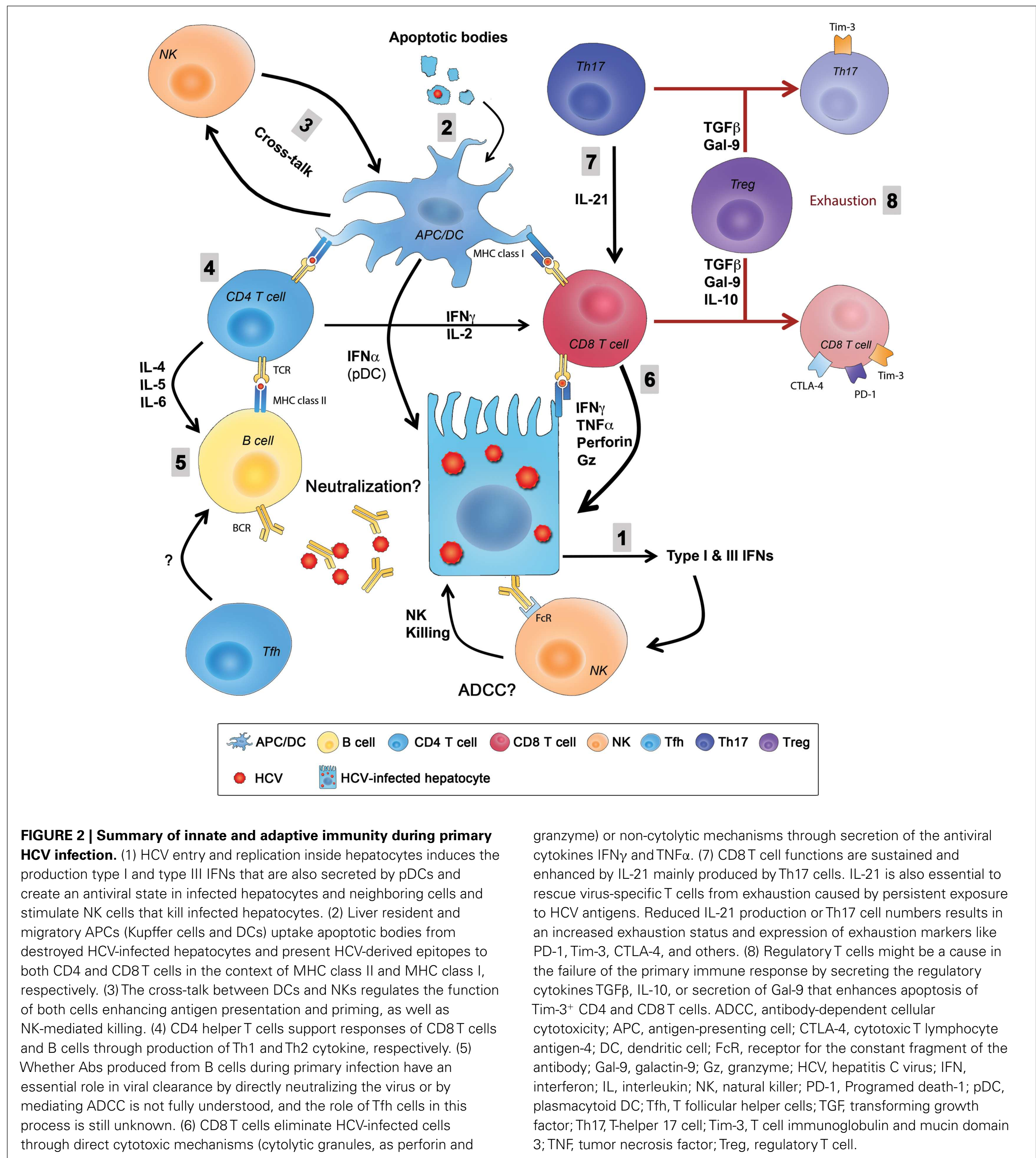

FIGURE 2 | Summary of innate and adaptive immunity during primary HCV infection. (1) HCV entry and replication inside hepatocytes induces the production type I and type III IFNs that are also secreted by pDCs and create an antiviral state in infected hepatocytes and neighboring cells and stimulate NK cells that kill infected hepatocytes. (2) Liver resident and migratory APCs (Kupffer cells and DCs) uptake apoptotic bodies from destroyed HCV-infected hepatocytes and present HCV-derived epitopes to both CD4 and CD8 T cells in the context of MHC class II and MHC class I, respectively. (3) The cross-talk between DCs and NKs regulates the function of both cells enhancing antigen presentation and priming, as well as NK-mediated killing. (4) CD4 helper T cells support responses of CD8 T cells and B cells through production of Th1 and Th2 cytokine, respectively. (5) Whether Abs produced from B cells during primary infection have an essential role in viral clearance by directly neutralizing the virus or by mediating ADCC is not fully understood, and the role of Tfh cells in this process is still unknown. (6) CD8 T cells eliminate HCV-infected cells through direct cytotoxic mechanisms (cytolytic granules, as perforin and granzyme) or non-cytolytic mechanisms through secretion of the antiviral cytokines IFNγ and TNFα. (7) CD8 T cell functions are sustained and enhanced by IL-21 mainly produced by Th17 cells. IL-21 is also essential to rescue virus-specific T cells from exhaustion caused by persistent exposure to HCV antigens. Reduced IL-21 production or Th17 cell numbers results in an increased exhaustion status and expression of exhaustion markers like PD-1, Tim-3, CTLA-4, and others. (8) Regulatory T cells might be a cause in the failure of the primary immune response by secreting the regulatory cytokines TGFβ, IL-10, or secretion of Gal-9 that enhances apoptosis of Tim-3^{+} CD4 and CD8 T cells. ADCC, antibody-dependent cellular cytotoxicity; APC, antigen-presenting cell; CTLA-4, cytotoxic T lymphocyte antigen-4; DC, dendritic cell; FcR, receptor for the constant fragment of the antibody; Gal-9, galactin-9; Gz, granzyme; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; NK, natural killer; PD-1, Programed death-1; pDC, plasmacytoid DC; Tfh, T follicular helper cells; TGF, transforming growth factor; Th17, T-helper 17 cell; Tim-3, T cell immunoglobulin and mucin domain 3; TNF, tumor necrosis factor; Treg, regulatory T cell.

monthly intervals and observed a rate of reinfection at almost 50% (11/22). Spontaneous clearance of reinfection was associated with broadened T cell responses and generation of cross-reactive Ab responses (248). However, phenotypic and polyfunctional characterization of HCV-specific memory CD4 and CD8 T cells was not performed. In this study, 10/11 subjects examined resolved the second infection, thus a control group of unprotected patients that would enable the definition of the exact correlates of protection was lacking. Preliminary work from our group suggests that protection from HCV persistence upon reinfection is variable and

associated with expansion of HCV-specific memory CD8 T cells that are polyfunctional in nature with expansion of a $CD127^{lo}$ population consistent with effector memory T cells (249).

Early treatment of HCV could rescue long-lived HCV-specific memory CD4 and CD8 T cells (146, 176, 189) but the protective role of natural T cell memory generated after viral clearance versus treatment induced memory generated after therapeutic-mediated clearance remains to be evaluated. An equally protective memory immune response would argue in favor of early treatment of high risk individuals as this will provide them with long-term protection if they are reexposed. Comparison of the capacity of the new DAAs and/or IFN free regimens to rescue such protective memory responses should also be evaluated.

In summary, long-lived T cell memory responses are generated following spontaneous HCV clearance and data from chimpanzees and limited human studies suggest that they can provide some protection. However, the signatures of a protective immune response upon reexposure are far less defined, especially in terms of the phenotype and functionality of HCV-specific protective memory immune responses. We propose a model inferred from our current knowledge of protective immunity during acute primary HCV infection (**Figure 3**). We hypothesize that protection upon reexposure is associated with the maintenance of higher frequency, breadth, and polyfunctionality of HCV-specific memory CD4 and CD8 T cells. Such long-lived memory T cells are likely maintained through homeostatic proliferation in response to γ-chain cytokines. Upon reexposure, these memory T cells expand rapidly and eliminate the infecting virus. Similar to murine models of viral infections, repeated exposure will select for the virus-specific memory T cells with the highest proliferative capacity and functional avidity (250). It is likely that such long-lived memory T cells will acquire a genomic signature that will facilitate their capacity to expand rapidly and effectively upon reexposure. Individuals who cannot maintain such long-lived, broad, and polyfunctional memory T cells because of reduced homeostatic cytokine or proliferation or predisposition to generation of Tregs and increased T cell exhaustion are less likely to be protected upon reexposure. Host genetics, nAbs and homology between the infecting viral strains during primary and secondary infection can tip the balance toward viral clearance or persistence of the second infection.

Protective immunity in the context of real life exposure remains the most understudied area of HCV due to the limited number of organized high risk cohorts suitable for such studies and the need for follow-up at close intervals to detect multiple infection episodes (247). Concerted efforts to study such response on a large scale in matched cohorts with different ethnic backgrounds are underway and should yield important knowledge in the future (251, 252). In addition, the organization and characterization of such cohorts provides the perfect setup to evaluate vaccine candidates. Use of novel genomic and proteomic tools should yield better insights about the pathways involved in protective immunity. Comparative analysis of virus-specific cellular and humoral responses in relation to variations in the infecting viral strain is essential to understand the interplay between the virus and immune system and cross-protective immunity.

IMPLICATIONS FOR VACCINE DEVELOPMENT

The need of a prophylactic HCV vaccine to prevent viral transmission remains an urgent need to reduce the future disease burden worldwide (11). The plethora of data suggesting that cellular immunity is the major arm mediating spontaneous clearance during primary HCV infection led to the assumption that it should be the target for vaccine development. Nevertheless, the recent data on the role of nAbs in resolution of primary infection suggest a possible contribution of humoral immunity in both primary HCV clearance and prophylaxis.

There are two challenges facing the development of HCV vaccines. First, the genetic diversity of HCV sequences and its high rate of mutability. Second, the ability of HCV proteins to disrupt and evade the different arms of the immune system. This necessitates the design of an effective vaccine that generates memory immune cells capable of mounting a prompt recall response upon reexposure to diverse HCV strains in a fashion that outpaces the virus and prohibits it from crippling the immune system.

Three major approaches have been adopted for vaccine design against HCV. The traditional approach uses recombinant envelope proteins to induce nAbs. It has gained more potential recently with the discovery of broadly nAbs. Vaccination with recombinant E1/E2 proteins was shown to induce cross-reactive nAbs that might contribute to protective immunity upon exposure to HCV (253–255). The second approach uses virus-like particles (VLPs) that express HCV structural proteins to induce both humoral and cellular immunity, but it failed to induce humoral responses when tested in chimpanzees. Furthermore, upon homologous rechallenge 2/4 vaccinated chimpanzees demonstrated sporadic very low levels of viremia during 1 year of follow-up (256). The third and most promising approach is designing an HCV vaccine that would induce a potent T cell immune response. Replication-defective recombinant viral vectors [e.g., adenovirus (Ad), vaccinia virus (VV), modified vaccinia Ankara (MVA)] were used to deliver HCV antigens to prime T cell responses. Another promising vector that was successful in the simian immunodeficiency virus (SIV) model, but has not yet been tested in HCV is cytomegalovirus (257). HCV proteins could also be delivered using DNA vaccines, where recombinant plasmids expressing various proteins could be injected into the host [reviewed in Ref. (11)].

A combination of viral vector prime and DNA or recombinant protein boost is preferred to prevent neutralization of the subsequent boosts by vector-specific antibodies that could have been generated during priming. Two candidate vaccines using this strategy showed promising preliminary results and are at an advanced stage of development. The first vaccine uses heterologous prime/boost regimens with chimpanzee adenovirus Ad3Ch3 and a rare strain of human adenovirus (Ad6) expressing the entire NS region of genotype 1b BK strain (NS3-5B). This vaccine was tested in a phase I clinical trial in humans (ClinicalTrials.gov NCT01436357). Both vectors primed broad CD4 and CD8 T cell responses that were capable of responding to heterologous strains of HCV genotypes 1a and 3a. Polyfunctional HCV-specific T cells ($IL\text{-}2^{+}$ $IFN\gamma^{+}$ $TNF\alpha^{+}$) could be sustained for at least a year after boosting with the heterologous vector (258). This vaccine

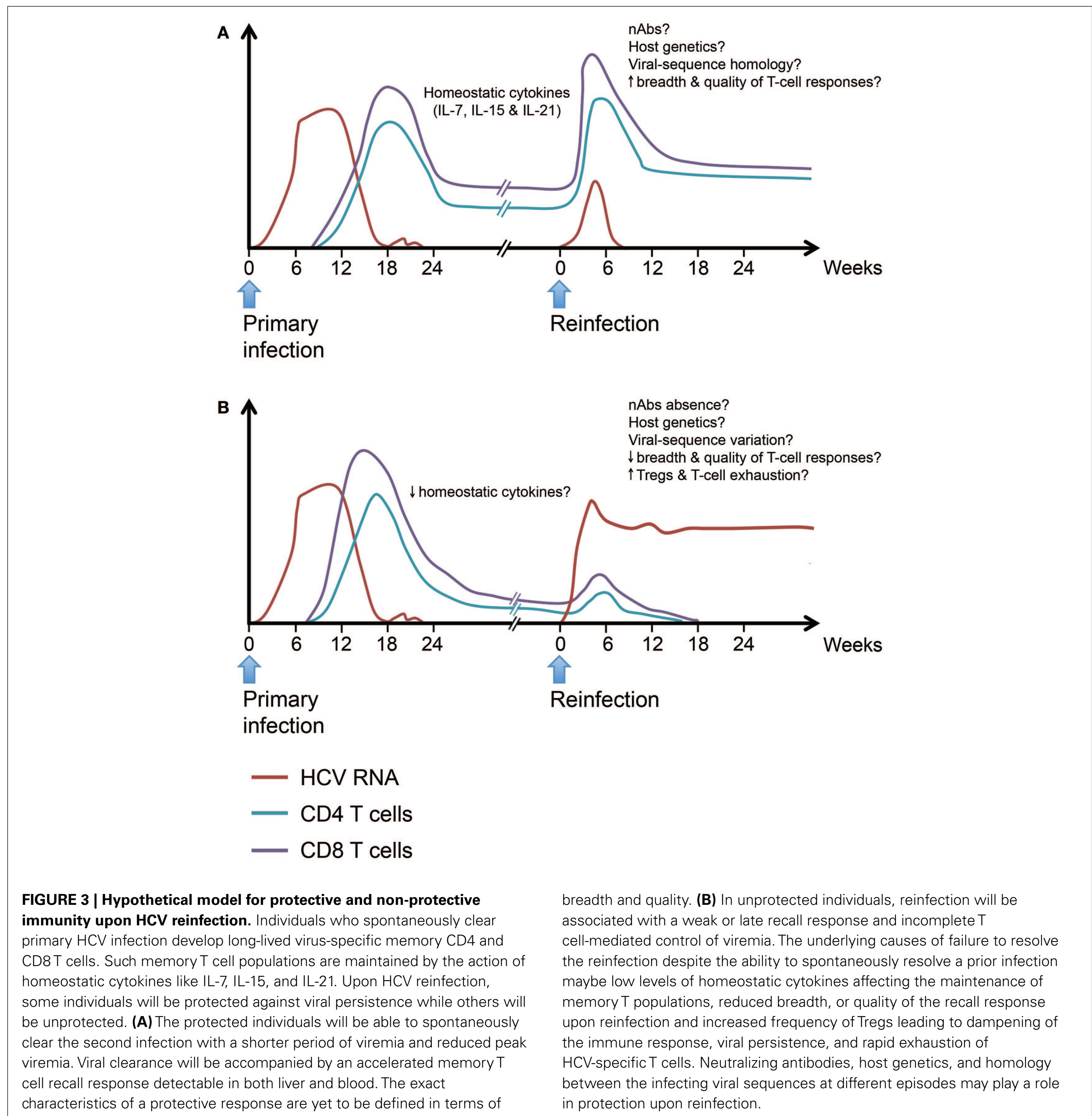

FIGURE 3 | Hypothetical model for protective and non-protective immunity upon HCV reinfection. Individuals who spontaneously clear primary HCV infection develop long-lived virus-specific memory CD4 and CD8 T cells. Such memory T cell populations are maintained by the action of homeostatic cytokines like IL-7, IL-15, and IL-21. Upon HCV reinfection, some individuals will be protected against viral persistence while others will be unprotected. **(A)** The protected individuals will be able to spontaneously clear the second infection with a shorter period of viremia and reduced peak viremia. Viral clearance will be accompanied by an accelerated memory T cell recall response detectable in both liver and blood. The exact characteristics of a protective response are yet to be defined in terms of breadth and quality. **(B)** In unprotected individuals, reinfection will be associated with a weak or late recall response and incomplete T cell-mediated control of viremia. The underlying causes of failure to resolve the reinfection despite the ability to spontaneously resolve a prior infection maybe low levels of homeostatic cytokines affecting the maintenance of memory T populations, reduced breadth, or quality of the recall response upon reinfection and increased frequency of Tregs leading to dampening of the immune response, viral persistence, and rapid exhaustion of HCV-specific T cells. Neutralizing antibodies, host genetics, and homology between the infecting viral sequences at different episodes may play a role in protection upon reinfection.

is currently in a phase II trial in high risk IDUs. The second vaccine uses a regimen of priming with Ad6 encoding NS3-5B of genotype 1b BK strain and boosting with NS3-5B-encoding plasmid DNA. A study in chimpanzees that received this vaccine then challenged with HCV demonstrated that control upon HCV challenge following vaccination was associated with $CD127^{+}$ $PD\text{-}1^{lo}$ CD8 T cells that persisted at high levels for prolonged period and were bifunctional ($IFN\gamma^{+}$ $TNF\alpha^{+}$). Nevertheless, three out of five chimpanzees were not protected suggesting that a more efficient immune response may be required (237, 259).

Correlates of protective immunity against HCV need to be clearly defined so that they could be monitored following vaccination to predict the degree of protection that may be conferred. This should involve characterization of the genomic and proteomic pathways involved and identification of biomarkers that can be used as benchmarks to monitor vaccine success in a non-invasive way. Strategies targeting both cellular and humoral immunity are likely to be more successful (260). The use of immune modulators like PD-1 blockers, TLR agonists, or modulators of biological pathways like transcription factors involved

in memory T cell generation may be useful as vaccine adjuvants to enhance immunogenicity and favor the development of the desirable immune response (250).

FUTURE RESEARCH DIRECTIONS

There is a general misconception that this is the end of HCV and that the new treatments will completely eliminate HCV within the next few years. Unfortunately, this is highly unlikely since the new DAAs remain expensive and are associated with a fair number of side effects. Moreover, their use in special populations like individuals with advanced liver disease or HIV coinfection remains experimental and limited at this point. Most importantly, many of the individuals infected with HCV have not been tested and continue to infect others. Future research should focus on the many shades of gray that still cloud our understanding of what constitutes a long-term protective immune response and the interaction between the virus and the host. This requires the use of organized and well characterized cohorts and standardized methods to evaluate immunity on a large scale with sufficient statistical power. Areas of interest involve better understanding of the role of genetic predictors of HCV outcome like IL28B and IFNL4 in viral clearance and long-term protection; understanding the nature of CD4 T cell help for both CD8 T cells and antibody producing B cells; elucidation of the role of neutralizing and non-nAbs during primary and secondary HCV infections; development of better small animal models for preclinical testing of vaccines and the use of novel genomics and proteomics tools to identify implicated pathways and non-invasive markers of efficient immune response. Collaboration with other fields, including research on vaccines for other viruses like yellow fever virus and HIV, as well as work with more physiologically relevant *in vitro* models like primary hepatocytes should provide a clearer image of the protective immunity against HCV and better success for ongoing and future vaccine development strategies.

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394. Sugimoto, K., et al., *Strain-specific T-cell suppression and protective immunity in patients with chronic hepatitis C virus infection.* J Virol, 2005. **79**(11): p. 6976-83.
395. Manigold, T., et al., *Foxp3+CD4+CD25+ T cells control virus-specific memory T cells in chimpanzees that recovered from hepatitis C.* Blood, 2006. **107**(11): p. 4424-32.
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Curriculum Vitae

EDUCATION:

May 2009 – Aug 2014	**Ph.D. in Microbiology and Immunology** Faculté de médecine, Université de Montréal
May 2007 – April 2009	**M.Sc. in Microbiology and Immunology** Faculté de médecine, Université de Montréal
Sept 1994 – May 1999	**Bachelor degree in Pharmaceutical Sciences (B. Pharm.)** Faculty of Pharmacy, Cairo University

DISTINCTIONS, AWARDS AND HONOURS:

FELLOWSHIPS:

2011-2014	Fredrick Banting & Charles Best Doctoral Fellowship Percent rank: top 4.68%	Canadian Institutes for Health Research (CIHR)
2011-2014	FRSQ Doctoral Fellowship Rank: 1st in committee	Fonds de Recherche en Santé du Québec (FRSQ) (Declined for holding 2 fellowships)
Sept 2012	Bourse de Perfectionnement	Centre de Recherche de Centre Hôspitalier de l'Université de Montréal (CRCHUM)
Dec 2011	Bourse d'Excellence	CRCHUM
Jan 2011	Bourse de Perfectionnement	CRCHUM
2009–2013	Exemption from tuition fees	Faculté des études superieur et postdoctorales (FESP), Université de Montréal
2009–2013	Doctoral fellowship	National Canadian Research Training Program for Hepatitis C (NCRTP-HepC)
2008–2009	Graduate fellowship	Université de Montréal
2007–2008	Graduate fellowship	Université de Montréal
2007–2009	Exemption from international student fees	Ministère de l'éducation, loisir et sport du Québec
1994–1999	Undergraduate Student's Excellence Fellowship	Cairo University (Renewed every year)
1991–1994	Exemption from tuition fees	Orouba Language School, Egypt

AWARDS AND PRIZES:

July 2014	Travel Award	21st International Symposium on HCV & Related Viruses. Banff, Canada. Sept, 2014
Mar 2014	Oral Presentation Prize	Journée Dépt. Microbiologie et Immunologie, Université de Montréal
Mar 2013	Basic Research Award	Canadian Association for the Study of the Liver (CASL)
Dec 2012	Poster Presentation Prize	Journée de Recherche, CRCHUM
Nov 2012	Oral Presentation Prize	Journée du Réseau SIDA/MI du FRSQ
Aug 2012	Bill & Melinda Gates Foundation Global Health Travel Award	Keystone Symposium for Immunological Mechanisms of Vaccination. Ottawa, Canada. Dec, 2012.
Nov 2011	Oral Presentation Prize	Journée du Réseau SIDA/MI du FRSQ
Oct 2011	Poster Presentation Prize	Journée Dépt. Microbiologie et Immunologie, Université de Montréal
Mar 2010	Basic Research Award	CASL
Jan 2010	Excellence Award	Egyptian Cultural and Educational Bureau, Montréal, Canada
Aug 2009	Young Investigator Award	16th International Symposium on HCV & Related Viruses. Nice, France. Oct, 2009.
Feb 2009	Research Award	Egyptian Student Association of North America (ESANA)
Nov 2008	Oral Presentation Prize	Journée du Réseau SIDA/MI du FRSQ
April 2000	Excellence Award for graduating at the top 2%	The Arab Co. for Pharmaceutical and Chemical Industries

HONOURS AND CREDENTIALS:

Jun 2010	Dean's Honours list (M.Sc. graduation)	Faculté des études superieur et postdoctorales, Université de Montréal
Jun 2010	Dean's Honours list (M.Sc. graduation)	Faculté de médecine, Université de Montréal
April 2009	M.Sc. thesis chosen as one of the top 10% in the discipline	Jury of the M.Sc. thesis
July 1999	Licence of practicing pharmacist	Egyptian Pharmacists Syndicate
May 1999	Dean's Honours list (B.Pharm. graduation)	Faculty of Pharmacy, Cairo University

REFEREED PUBLICATIONS:

1. **Abdel–Hakeem MS**, Bruneau J, Soudeyns H and Shoukry NH. Dynamics of the Virus-Specific CD8 T-cell Repertoire during HCV Reinfection. Submitted to Blood journal.
2. **Abdel–Hakeem MS**, Bédard N, Murphy D, Bruneau J and Shoukry NH. Signatures of Protective Memory Immune Responses during HCV reinfection. Gastroenterology, 2014.
3. **Abdel–Hakeem MS** and Shoukry NH**.** Protective Immunity against Hepatitis C: Many Shades of Gray. Invited Review for Frontiers in Immunology, Vol.5, 2014.
4. **Abdel–Hakeem MS**, Bédard N, Badr G, Ostrowski M, Bruneau J, Sékaly RP, Willems B, Heathcote JE and Shoukry NH. Comparison of Immune Restoration in Early Versus Late Interferon Alpha Therapy against Hepatitis C Virus. Journal of Virology, Vol.84, 2010.
5. Badr G, Bédard N, **Abdel–Hakeem MS**, Trautmann L, Villeneuve JP, Willems B, Haddad EK, Sékaly RP, Bruneau J and Shoukry NH (2008). Early Interferon Therapy Rescues Poly-functional Long-lived Virus-specific CD8+ Memory T cells. Journal of Virology, Vol.82, 2008.

ORAL PRESENTATIONS:

1. **Abdel–Hakeem MS**, Bruneau J, Soudeyns H and Shoukry NH. Dynamics of the Virus-Specific CD8 T-cell Repertoire during HCV Reinfection. 21st International Symposium on Hepatitis C Virus and Related Viruses. Banff, AB, CANADA. Sept 7–11, 2014.
2. **Abdel–Hakeem MS**, Bruneau J, Soudeyns H and Shoukry NH. Dynamics of the Virus-Specific CD8 T-cell Repertoire during HCV Reinfection. International Union of Microbiological Societies (IUMS) – XVIth International Congress of Virology. Montréal, QC, CANADA. July 28–August 2, 2014.
3. **Abdel–Hakeem MS**, Bruneau J, Soudeyns H and Shoukry NH. Dynamiques de répertoire de cellules T pendant la réinfection par le VHC. Journée du département de microbiologie et immunologie, Université de Montréal. Montréal, QC. March 3, 2014.
4. **Abdel–Hakeem MS**, Bédard N, Murphy D, Bruneau J and Shoukry NH. Les signatures des réponses immunitaires protectrices pendant la reinfection par le VHC. Journée du Réseau SIDA/MI du FRSQ. Montréal, QC. November 1, 2013.
5. **Abdel–Hakeem MS** *et al*. Signatures of a protective immune response against HCV infection. Li Ka Shing Institute of Virology, Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB. April 10, 2013.
6. **Abdel–Hakeem MS** *et al*. Analysis of a protective immune response during multiple episodes of HCV infection (Abstract ABS005). 2nd Canadian Symposium on HCV, Victoria, BC. March 4, 2013.
7. **Abdel–Hakeem MS** *et al*. Analysis of a protective immune response during multiple episodes of HCV infection (Abstract A43). Oral presentation, CASL Basic paper session (session 4), Annual Canadian Association for Studying the Liver (CASL) meeting. Victoria, BC. March 2-5, 2013.

8. **Abdel–Hakeem MS** *et al*. Le rôle protecteur du système immunitaire lors de la réinfection par le VHC. Journée des étudiants, Réseau SIDA/MI du FRSQ. Montréal, QC. November 3, 2012.
9. **Abdel–Hakeem MS** *et al*. Analysis of a protective immune response during multiple episodes of HCV infection (Abstract O-25). Oral presentation No. 25, 19th International Symposium on Hepatitis C Virus and Related Viruses. Venice, Italy. 3-7 October, 2012.
10. **Abdel–Hakeem MS**, Bédard N, Badr G, Ostrowski M, Bruneau J, Sékaly RP, Willems B, Heathcote JE and Shoukry NH. Viral Determinants of the Outcome of Hepatitis C Viral Infection and Response to Antiviral Therapy. Journée des étudiants, Réseau SIDA/MI du FRSQ. Montréal, QC. November 18, 2011.
11. **Abdel–Hakeem MS**. Comparison of immune restoration in early versus late interferon alpha therapy against hepatitis C. Department of Microbiology & Immunology, Faculty of Pharmacy Cairo University. May, 7th 2011.
12. **Abdel–Hakeem MS**. Viral determinants of the outcome of interferon therapy against hepatitis C virus. Ph.D. seminar II, Université de Montréal. January, 2011.
13. **Abdel–Hakeem MS**, Bédard N, Badr G, Ostrowski M, Bruneau J, Sékaly RP, Willems B, Heathcote JE and Shoukry NH. Comparison of immune restoration in early versus late interferon alpha therapy against hepatitis C. (Abstract A002). Oral presentation, CASL Basic paper session, Annual Canadian Association for Studying the Liver (CASL) meeting. Toronto, ON. February 27 – March 2, 2010.
14. **Abdel–Hakeem MS**. Viral Determinants of the Outcome of Hepatitis C Viral Infection and Response to Antiviral Therapy. Ph.D. seminar I, Université de Montréal. November, 2009.
15. **Abdel–Hakeem MS**, Bédard N, Badr G, Ostrowski M, Bruneau J, Sékaly RP, Willems B, Heathcote JE and Shoukry NH. Restauration de la réponse immunitaire contre le virus de l'hépatite C en comparant le traitement à l'interféron alpha précoce et tardif. Journée des étudiants, Département de microbiologie et immunologie, Université de Montréal. Montréal, QC. October 23, 2009.
16. **Abdel–Hakeem MS** *et al*. Comparison of immune restoration in early versus late interferon alpha therapy against hepatitis C. (Abstract O-42). Oral presentation No. 42, General session 5, 16th International Symposium on Hepatitis C Virus and Related Viruses. Nice, France. 3-7 October, 2009.
17. **Abdel–Hakeem MS** *et al*. Restauration de la réponse immunitaire contre le virus de l'hépatite C en comparant le traitement à l'interféron alpha précoce et tardif. Journée des étudiants, Réseau SIDA/MI du FRSQ. Montréal, QC. November 14, 2008.
18. **Abdel–Hakeem MS** *et al*. Early but Not Late Interferon Alpha Therapy Against Hepatitis C Rescues Polyfunctional CD4+ and CD8+ Memory T Cells. (Abstract # 760). Oral presentation No. LB-265, Late Breaking General Session, 7th Joint Conference of the ISICR (International Society for Interferon and Cytokine Research) and the ICS (International Cytokine Society). Montréal, QC, CANADA. October 12-16, 2008.
19. **Abdel–Hakeem MS**. Virus–Host Interaction during Therapy against Hepatitis C Virus. M.Sc. seminar, Université de Montréal. January 2008.

POSTER PRESENTATIONS:

1. **Abdel–Hakeem MS**, Bruneau J, Soudeyns H and Shoukry NH. Dynamics of the virus-specific CD8 T-cell repertoire during HCV reinfection. 3rd Canadian Symposium on HCV, Toronto, ON. February 7, 2014.
2. **Abdel–Hakeem MS**, Bédard N, Murphy D, Bruneau J and Shoukry NH. Signatures of Protective Memory Immune Responses during HCV reinfection. Poster 001. Immunology Montréal Symposium 2013, December 6, 2013.
3. **Abdel–Hakeem MS** *et al*. Les signatures des réponses immunitaires protectrices pendant la reinfection par le VHC. Poster 34. 16e Congrés annuel scientifique des étudiants, stagiaires et résidents de CRCHUM. CRCHUM, Montréal, QC.
4. **Abdel–Hakeem MS**, Bédard N, Murphy D, Bruneau J and Shoukry NH. Signatures of Protective Memory Immune Responses during HCV reinfection. Keystone Symposium on Advancing Vaccines in the Genomics Era (T1), Rio de Janeiro, Brazil. November 1-4, 2013.
5. **Abdel–Hakeem MS** *et al*. Analysis of a protective immune response during multiple episodes of HCV infection. Poster presentation, NCRTP PAC meeting, Victoria, BC. March 2-3, 2013.
6. **Abdel–Hakeem MS** *et al*. Analysis of a protective immune response during multiple episodes of HCV infection. Poster presentation No. 77, 15e Congrés annuel scientifique des étudiants, stagiaires et résidents de CR-CHUM. Hôpital Notre-Dame du CHUM, Montréal, QC. December 18, 2012.
7. **Abdel–Hakeem MS** *et al*. Analysis of a protective immune response during multiple episodes of HCV infection (P1001), Keystone Symposium on Immunological Mechanisms of Vaccination (S3), Ottawa, ON, CANADA. December 13-18, 2012.
8. **Abdel–Hakeem MS**, Fabre T, Gélinas J-F, Elfar M, Murphy D, Breneau J, Willems B and Shoukry NH. Viral determinants of the outcome of interferon therapy against hepatitis C virus. Poster presentation (P102), Keystone Symposium on Antiviral Immunity (X6), Keystone, CO, USA. March 21-26, 2012.
9. **Abdel–Hakeem MS** *et al*. Viral determinants of the outcome of interferon therapy against hepatitis C virus. Poster presentation, Annual Canadian Association for Studying the Liver (CASL) meeting, Montréal, QC. February 26, 2012.
10. **Abdel–Hakeem MS** *et al*. Viral determinants of the outcome of interferon therapy against hepatitis C virus. Poster presentation, NCRTP PAC meeting, Montréal, QC. February 24-25, 2012.
11. **Abdel–Hakeem MS** *et al*. Viral determinants of the outcome of interferon therapy against hepatitis C virus. Poster presentation, 1st Canadian Symposium on HCV, Montréal, QC. February 23, 2012.
12. **Abdel–Hakeem MS** *et al*. Viral determinants of the outcome of interferon therapy against hepatitis C virus. Poster presentation No. 41, 14e Congrés annuel scientifique des étudiants, stagiaires et résidents de CR-CHUM. Hôpital Notre-Dame du CHUM, Montréal, QC. December 13, 2011.
13. **Abdel–Hakeem MS** *et al*. Viral determinants of the outcome of interferon therapy against hepatitis C virus. Journée des étudiants, Département de microbiologie et immunologie, Université de Montréal. Montréal, QC. October 23, 2011.

14. **Abdel–Hakeem MS** *et al*. Viral determinants of the outcome of interferon therapy against hepatitis C virus. Poster presentation (P10.23), 18th International Symposium on HCV and related viruses, Seattle, WA, USA. September 8-12, 2011.
15. **Abdel–Hakeem MS** *et al*. Viral determinants of the outcome of interferon therapy against hepatitis C virus. Poster presentation, NCRTP-HepC 2011 PAC, Vancouver, BC. February 25-27, 2011.
16. **Abdel–Hakeem MS**, Bédard N, Badr G, Ostrowski M, Bruneau J, Sékaly RP, Willems B, Heathcote JE and Shoukry NH. Restauration de la réponse immunitaire contre le virus de l'hépatite C en comparant le traitement à l'interféron alpha précoce et tardif. Journée des étudiants, Réseau SIDA/MI du FRSQ. Montréal, QC. November 18, 2010.
17. **Abdel–Hakeem MS** *et al*. Restauration de la réponse immunitaire contre le virus de l'hépatite C en comparant le traitement à l'interféron alpha précoce et tardif. Journée de VIH CR-CHUM. Montréal, QC. May 18, 2010.
18. **Abdel–Hakeem MS** *et al*. Comparison of immune restoration in early versus late interferon alpha therapy against hepatitis C. Poster presentation, NCRTP-HepC 2010 PAC, Toronto, ON. Feb 25-27, 2010.
19. **Abdel–Hakeem MS** *et al*. Comparison of immune restoration in early versus late interferon alpha therapy against hepatitis C. Poster presentation, 2e Journée d'immunologie du CR-CHUM. Hôpital Notre-Dame du CHUM, Montréal, QC. April 24, 2009.
20. **Abdel–Hakeem MS** *et al*. Early but Not Late Interferon Alpha Therapy Against Hepatitis C Rescues Polyfunctional CD4+ and CD8+ Memory T Cells. Poster presentation No. 23, 11e Congrés annuel scientifique des étudiants, stagiaires et résidents de CR-CHUM. Hôpital Notre-Dame du CHUM, Montréal, QC. March 19, 2009.
21. **Abdel–Hakeem MS** *et al*. Early but Not Late Interferon Alpha Therapy Against Hepatitis C Rescues Polyfunctional CD4+ and CD8+ Memory T Cells. (Abstract # 760). Poster presentation No. PP9-265, 7th Joint Conference of the ISICR (International Society for Interferon and Cytokine Research) and the ICS (International Cytokine Society). Montréal, QC, CANADA. October 12-16, 2008.
22. **Abdel–Hakeem MS** *et al*. Virus–Host Interaction During Antiviral Therapy Against Hepatitis C Virus in the Chronic Phase. (Abstract # 117). Poster presentation, 21st Annual Spring Meeting of the Canadian Society for Immunology (CSI). Mont Tremblant, QC. April 11-14, 2008.
23. Badr G, Bédard N, **Abdel–Hakeem MS**, Trautmann L, Villeneuve JP, Willems B, Haddad EK, Sékaly RP, Shoukry NH. Early Interferon Therapy Rescues HCV-specific CD8+ T cells and Selects for CD127hi Long-lived Memory T Cells. (Poster # P–112). Poster presentation, 14th International Symposium on Hepatitis C Virus and Related Viruses. Glasgow, Scotland, UK. 9-13 September, 2007.
24. Yehia SA, El-Gazayerly O, Baselios E, **Salah El-Din M**. Preparation and Evaluation of Fluconazole Mucoadhesive Buccal Films. (Abstract T3172). The AAPS Journal, Vol. 8, No. S2, 2006.

CONTRIBUTION TO SCIENTIFIC NOTES:

1. Participating in the preparation of the scientific notes for the practical studies in the field of Basic Microbiology, Immunology and Medical Microbiology. Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, 2006.

REVIEWING ACTIVITY:

1. Member of the reviewing committee for the 2nd Canadian HCV Symposium, 2013.

TECHNICAL EXPERTISE AND TRAINING:

- **Cell biology techniques:** Tissue culture, working with human patients' samples, working with infectious material under BSL2 and BSL3 conditions.
- **Immunological flow cytometry techniques:** Intracellular staining (ICS), proliferation assays (CFSE dilution), tetramer staining and cell-sorting techniques using BD LSR II multiparameter flow cytometer, and analysis using FlowJo® and SPICE® software.
  Other Immunological techniques: Enzyme linked immunospot (ELISpot) assays and Immune Flourescence (IF).
- **Virological techniques:** viral RNA extraction, culturing infectious virion particles *in vitro* and design of chimeric plasmids.
- **Molecular biology techniques:** PCR, Real-time PCR, DNA sequencing, cloning, protein separation, immunoblotting, using Lasergene® software for the analysis of mutations, design of cloning strategies and primers.
- **Basic bacteriological techniques:** bacterial culture, bacterial counting, bacterial identification, biochemical reactions and assessment of antimicrobial activity.

- Training on viral neutralization techniques at the laboratory of Dr. Michael Houghton, Li Ka Shing Institute of Virology, University of Alberta. April, 2013.
- Training on viral culture techniques at the laboratory of Dr. Arash Grakoui, Emory Vaccine Centre, Emory University, Atlanta, GA, USA. February, 2011.