University of Alberta

Development of an ex vivo assay of hepatitis C specific T-cell responses using QuantiFERON®

by

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If we would have new knowledge, we must get a whole world of new questions.

Susanne Langer, 1895 - 1985

Abstract

Cellular immune responses to Hepatitis C (HCV) epitopes are crucial for successful host response to HCV infection. We investigated a platform to assess specific and global immune responses in HCV infection. We identified 57 HCV peptides from literature (24 of CD4+, 33 of CD8+ specificity) and tested them in two peptide pools to assess specific response in non-transplanted and post-liver transplant (LT) patients. Robust interferon-gamma (IFN) response to CD4+ peptide and mitogen stimulation was seen in sustained virological clearance. IFN response to the CD4+ peptide pool could differentiate between SVR and NR with 82% accuracy.

In patients with recurrent HCV post-LT, HCV-specific responses were attenuated, but global immune responses were preserved. Significantly lower specific (CD4+) and global immune responses (mitogen response) were observed in patients with advanced allograft disease (fibrosis score>2). Quantiferon-HCV may identify patients likely to respond to anti-HCV treatment, as well as post-LT patients with aggressive HCV recurrence.

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Abbreviations

Hepatitis C virus	HCV
Orthotopic liver transplantation	LT
Interferon- gamma	IFN- γ
Cytotoxic lymphocytes	CTL
Cytomegalovirus	CMV
Peripheral blood mononuclear cells	PBMC
Centres for disease control	CDC
Enzyme-linked immunospot	ELISPOT
Spot-forming Unit	SFU
Ribonucleic acid	RNA
Sustained virological response	SVR
Alanine aminotransferase	ALT
Hepatocellular carcinoma	HCC
Envelope protein 1	E1
Envelope protein 2	E2
Non-structural protein 2	NS2
Non-structural protein 3	NS3
Non-structural protein 4A	NS4A
Non-structural protein 4B	NS4B
Non-structural protein 5A	NS5A
Non-structural protein 5B	NS5B
Alternate frame reading protein	AFRP
Pathogen-associated molecular patterns	PAMPs
Toll-like receptors	TLRs
Interleukin	IL
Antigen presenting cell	APC
Pegylated interferon alpha	PEG-IFN
Ribavirin	RBV
T-Cell Receptor	TCR
Dendritic cell	DC
Major histocompatibility complex	MHC
Interferon gamma release assay	IGRA
Allograft rejection	AR

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Chapter 1

Introduction

Hepatitis C virus (HCV) infection affects 170 million people, or 3% of the world's population (1). Currently, the most effective treatment for chronic HCV is the combination of peg-IFN-and ribavirin, but <50–60% of treated people have sustained benefit from antiviral therapy (2). In Western countries, genotype 1, which infects 70–80% patients, is associated with a poor response to IFN-therapy (2). End-stage liver disease due to HCV infection is the most common indication for orthotopic liver transplantation (LT) in the Western world (3). Unfortunately HCV recurrence is almost universal post-LT, and 20-30% of such patients develop cirrhosis within 5 years of transplant.

The importance of virus-specific CD4+ and CD8+ T cell response for the outcome of HCV infection has been shown in patients with acute self-limiting hepatitis C who frequently display a vigorous virus-specific CD8 + T cell response, and in chronically infected patients with a sustained response to antiviral therapy (4). In contrast, chronic course of HCV infection has been associated with a weak or transient HCV-specific CD8+ and CD4 + T cell response (5).

There are several technical limitations concerning the analysis of HCV-specific T-cell response. Direct ex-vivo tests avoid the need for prolonged in vitro expansion, but are unhelpful if only a very limited number of cells are available. Production of interferon- γ (IFN- γ) is predictive of CD8+

response to hepatitis C infection, and IFN-γ production can be a functional surrogate for the identification of HCV-specific cytotoxic lymphocytes (CTLs). Functional tests assessing IFN- γ production include include the ELISPOT test, and flow cytometry assessment of intracellular IFN- γ production.

Quantiferon assays have been used to quantify IFN- γ production in CMV disease, as well as in the detection of latent tuberculosis. It provides a useful marker of disease-specific cell-mediated-immunity. Stimulation of peripheral blood with cytomegalovirus (CMV) T-cell peptide epitopes resulted in high levels of IFN-γ production, which were readily detected by the assay. The sensitivity of the Quantiferon assay for HCMV epitopes was at least equivalent and in some cases more sensitive than the ELISPOT. Most notably the Quantiferon assay does not require separation of peripheral blood lymphocytes (PBMCs) and the assay can be carried out on whole blood (6).

This study will develop and validate an HCV immunity assays using the basic QuantiFERON® - assay platform as a simple, reproducible, and reliable test for the detection of IFN-γ in response to HCV -specific T-cell epitopes.

1.1 Incidence and global burden of Hepatitis C

Hepatitis C virus (HCV) is a globally important cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Approximately 170 million people are estimated to have the infection worldwide (1). Chronic HCV infection has almost doubled in the last decade; it is now the leading indication for liver transplantation in developed nations and will continue to pose an important health and economic burden during the next 10–20 years (3,7). Although the incidence of HCV infection has decreased, the number of HCV-related deaths is likely to rise over the next decade owing to the long latency of the disease.

The prevalence of chronic HCV in Canada is relatively low, believed to be between 0.8% and 1.0%, but is likely to increase because of an increasing number of people with chronic disease and because of migration from highly endemic areas such as Africa and Asia. Around 250,000 Canadians are thought to have HCV disease, and only around 65% of all cases have been identified (7).

The hepatitis C virus (HCV) is transmitted by blood-to-blood contact. In developed countries, it is estimated that 90% of persons with chronic HCV infection were infected through transfusion of unscreened blood or blood products or via injection drug use or sexual exposure. In developing countries, the primary sources of HCV infection are unsterilized injection

equipment and infusion of inadequately screened blood and blood products. Injection drug use is the most common route of HCV infection in the developed world (1).

1.2 Progression of disease

The Centres for disease control (CDC) estimate that of every 100 persons infected with HCV, approximately 75–85 will go on to develop chronic infection, 60–70 will go on to develop chronic liver disease, 5–20 will go on to develop cirrhosis over a period of 20–30 years and 1–5 will die from the consequences of chronic infection (liver cancer or cirrhosis). Prior infection with HCV does not protect against later infection with the same or different genotypes of the virus.

End-stage liver disease secondary to hepatitis C infection (HCV) accounts for about 40–45% of all transplants in the Western world (7).
Unfortunately, HCV recurrence following transplantation is a universal phenomenon. HCV RNA can be detected in virtually all patients post-transplant (8, 9) with at least 50% of recipients demonstrating histological evidence of HCV-induced hepatitis at 1 year (10).

1.3 Treatment of Chronic HCV infection

Therapy with pegylated interferon in combination with ribavirin has been shown to have the best results. Combination therapy increases the

proportion of patients who have a sustained viral response (SVR), reaching 40%-50%, compared with response rates of 15%-25% with interferon alone (2). Genotype determinations influence treatment decisions. Currently the best indicator of effective treatment is a sustained viral response, defined by the absence of detectable HCV RNA in the serum, as shown by HCV RNA assay with lower limit of detection of 50 IU/mL or less at 24 weeks after the end of treatment.

About 50% of patients respond to interferon-based therapy by normalizing ALT at the end of therapy, but half of these relapse within the 6 months of follow-up after IFN withdrawal. The long-term biochemical response falls then to 20-25%. Only a minority of these have a persistent disappearance of HCV RNA from serum. The duration of therapy depends on the genotype and level of viremia. In patients with genotype 2 or 3, the duration is 24 weeks, while patients with genotype 1 need 48 weeks of treatment. Infections with genotype 1 strains of HCV are less responsive to interferon than infections with other genotypes of HCV.

Liver transplantation is indicated in patients with life-threatening cirrhosis or hepatocellular carcinoma (HCC). Patients with cirrhosis, who have a life expectancy of 1-2 years without transplantation because of recurrent or refractory ascites, Child-Pugh C cirrhosis, uncontrolled gastrointestinal bleeding, severe encephalopathy, or bacterial peritonitis, are considered for liver transplantation (8).

1.4 Clinical course of Hepatitis C after liver transplantation

Liver grafts are rapidly re-infected with HCV, and HCV viremia increases as early as 3 days post-transplant. HCV RNA levels increase progressively, and at 1 month, the HCV RNA level may be dramatically increased compared to pre-transplant values, often peaking within 7 days after transplantation (8-10). Post-transplant hepatitis C viral load at 1 year is typically about 10–20-fold greater than the pre-transplant levels (11). The progression to liver allograft cirrhosis following HCV recurrence is significantly enhanced with a median time to cirrhosis of 10 years compared to 30–40 years in HCV-infected immunocompetent patients (12, 13). Liver allograft failure and mortality rates are higher in patients with post-transplant HCV recurrence than non-HCV-infected recipients.

There are several factors that promote progression to allograft failure following HCV recurrence in liver allograft recipients (14). These can be classified as: (A) Recipient factors—increasing age, female sex, severity of disease pre-transplant and race; (B) Donor factors—increasing age, warm ischemia time and allograft steatosis; (C) Virological—high pre-transplant HCV load and (D) Other—immunosuppression, high-steroid use, concomitant CMV infection and early onset HCV recurrence.

1.5 Viral Characteristics

HCV is an enveloped RNA virus with a diameter of about 50 nm, classified as a separate genus (Hepacivirus) within the Flaviviridae family. HCV was identified as the infectious agent responsible for the majority of transfusion-associated non-A, non-B hepatitis, with the isolation of the first HCV genomic clones in 1989.The positive stranded RNA genome is about 9.6 kb in length. The precursor polyprotein encoded by the large open reading frame that is processed into at least 10 proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) by cellular and viral proteases (15).

HCV is highly heterogeneous. Eleven HCV genotypes with several distinct subtypes have been identified throughout the world. Genotypes 1-3 have a worldwide distribution (1). Types 1a and 1b is the most common, accounting for about 60% of global infections. They predominate in Northern Europe and North America, and in Southern and Eastern Europe and Japan, respectively. Type 2 is less frequently represented than type 1. Type 3 is endemic in South-east Asia and is variably distributed in different countries. Genotype 4 is principally found in the Middle East, Egypt, and central Africa. Type 5 is almost exclusively found in South Africa, and genotypes 6-11 are distributed in Asia.

These diversities have distinct consequences, in that different genotypes vary in their responsiveness to interferon/ribavirin combination therapy. Moreover, such heterogeneity hinders the development of vaccines.

1.6 Viral life cycle

Translation of the single, long open reading frame yields a polyprotein of approximately 3000 amino acids. These proteins include the highly conserved core (C) protein, the glycosylated envelope proteins (E1 and E2), and a short peptide p7, which mediates membrane permeability and secretion. Several nonstructural (NS) proteins are involved in proteolysis (NS2, NS3/NS4A), formation of replication complexes (NS4B), RNA binding (NS5A), and function as RNA-dependent RNA polymerase (NS5B). The polyprotein is processed during and after translation by host and viral proteases to release the ten individual proteins making up the viral particle and the viral replication machinery. HCV replication and assembly take place in association with endoplasmic reticulum–derived membranous structures in the cytosol of infected cells. The structural proteins core, E1, and E2 are at the amino terminus of the polyprotein. The viral RNA is believed to assemble with core and the E1 and E2 glycoprotein (GPs) in a particle with a lipid envelope (16). The infectious form of the virus in vivo is still incompletely understood, but the virus may associate with lipid particles. Non-enveloped particles also have been observed, although the significance of these particles is unclear. The

nonstructural proteins, p7–NS5B, are present in infected cells but probably not in the viral particles. Another protein, known as F (frame shift) or ARFP (alternative reading frame protein), may be expressed from the core region of the genome as a result of translational frame shift. The function of this protein is poorly understood.

1.7 Mechanisms of evasion of immune response [Figure-1]

Up to 50–80% of patients infected with HCV fail to eliminate the virus, leading to long-term viral persistence and an increased risk of liver-related morbidity and mortality. The virus has evolved several strategies to escape host immune response. These strategies can broadly be grouped as:

a) High viral replication and mutation rates

b) Action on the innate immune response

c) Altered Natural Killer cell (NK) activity

d) Impaired CD4+ and CD8+ antigen presentation

e) Primary T-cell failure and exhaustion

f) Action on the adaptive immune response

1.7.1 Replication and mutation:

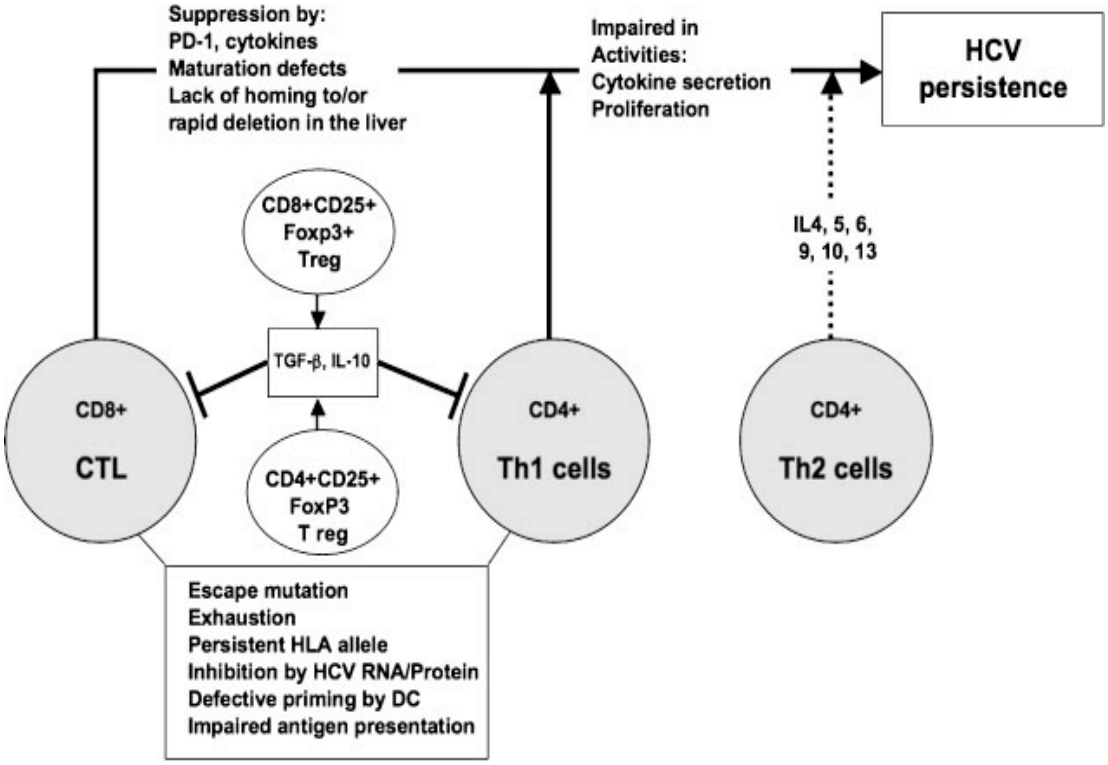
The high HCV replication rate (10^{12} virions/per day) and lack of proof-reading activity of the HCV RNA-dependent RNA polymerase leads to a high mutation rate – approximately 1.5–2.0 x10^{-3}/per site per genome per year, generating significant genetic diversity (17). HCV commonly exists as a mixture of variants (quasispecies) under the selective pressure of host immune responses and antiviral drugs, even in a single patient. Mutations in the epitopes of MHC restricted HCV-specific CD4+ and CD8+ T cells may be one of the most important mechanisms for failure T cell response. Mutations in CD8+ + T cell epitope Regions can occur within 1 year of acute infection in HCV high-risk populations. Mechanisms for evasion of T cell immunity via escape mutations include

(1) Lower binding affinity of epitope peptide to MHC class I molecule;

(2) Decreased TCR recognition of mutant peptides;

(3) Impaired antigen processing by proteasomes. (16,17).

Figure-1.1 Mechanisms of immune escape by Hepatitis C virus (16)



1.7.2 Action on the innate immune response

HCV is recognized by innate virus-sensing mechanisms and induces a rapid interferon (IFN) response. The virus also uses multiple strategies to target and disable host mechanisms responsible for IFN production and IFN responses. It is estimated that no more than 10% of hepatocytes support HCV replication (18). This supports the hypothesis that an antiviral state is induced rapidly in most cells of the liver, perhaps mediated by IFNs produced early in infection. Type I IFNs and IFN response pathways are induced in the liver early in infection regardless of the outcome of infection. Infected hepatocytes may produce the first IFNs, and then this early signal is possibly amplified by surrounding immune cells. Recently, it has been shown that genetic polymorphisms near the human IL28B gene, encoding interferon lambda 3, are associated with significant differences in response to the treatment (19).

An antiviral state is mainly dependent on the induction of type 1 interferon (IFN-a/b). The activation of this system is mediated by pathogen-associated molecular patterns (PAMPs), and toll-like receptors (TLRs). TLRs are a type of pattern recognition receptor and recognize molecules that are broadly shared by pathogens referred to as PAMP (15,16). There are two pathways leading to an IFN response. One is mediated by RIG-I/MDA535–39 while MyD88 (myeloid differentiation primary response gene 88) activates the other. RIG-1 senses triphosphorylated single stranded HCV RNA and MDA5 recognizes dsRNA. Both act on Cardif,

which transmits the activation signal to IKKe and TANK-binding kinase-1 (TBK-1). These two kinases in turn phosphorylate the interferon regulator factor-3 (IRF-3) that activates the IFN-b promoter (15). Double-stranded HCV RNA is also recognized by TLR-3, which activates IKKe/TBK-1, via TRIF (TIR-domain-containing adapter-inducing interferon-b) joining the RIG-I/MDA5 pathway. In the other pathway, TLR7 senses single-strand HCV RNA and via the MyD88 adaptor protein activates IRAK4/IRAK1. These kinases stimulate IFN-a synthesis via the transcription factor of interferon response factor 7 (16).

HCV infection blocks viral activation of interferon regulatory factor 3 (IRF-3), a transcription factor that plays a pivotal role by targeting the RIG-I and TLR3 adaptor proteins, MAVS and TRIF, for specific proteolysis by the viral NS3/4A protease. The HCV core protein interferes with the JAK/STAT pathway, by activating the JAK–STAT signaling inhibitor SOCS-3. HCV NS5A inhibits the antiviral actions of IFN by secretion of interleukin-8 (IL-8) (19).

1.7.3 Altered Natural Killer cell (NK cell) activity

NK cells contribute significantly to the innate immune response, and a deficient NK cell activity is associated with persistent HCV infection. Activating receptors in human NK cells include natural cytotoxicity receptors (NCRs), TLRs, and CD161, the Fc receptor for immunoglobulin G, which mediates antibody-dependent cellular cytotoxicity. (20).

HCV E2 binding to CD8+ directly inhibits NK cell activity. HCV core protein Stabilizes HLA-E expression and inhibits cytolysis of NK cells.

1.7.4 Impaired Antigen Presentation for CD4+ and CD8+ T cells

DCs initiate the activation of CD8+ and CD4+ T-cells by presenting MHC I and II restricted HCV antigens to the corresponding T cell receptors. Defective monocyte-derived DCs, in number, phenotype, or function, have been reported in HCV infection. Direct infection of DCs is one possible mechanism of DC dysfunction in persistent HCV infection. HCV interacts with DC-SIGN, a receptor on dendritic cells that has the capacity to regulate their maturation and promote Th2-type microenvironment (21-23). HCV associated NK cell dysfunction and abnormal expression of cytokines, such as IL-12 and IL-15, disrupts NK: DC cross talk, resulting in impaired DC priming, and Th1 CD4+ -cell induction and CD8+ -CTL development (19-21).

1.7.5 Primary T Cell Failure and T Cell Exhaustion

Acutely HCV-infected patients lose their initial CD4+ T cell response (called T cell exhaustion) within 5 to 10 months followed by HCV recurrence, and weak, narrow epitope(s)-specific CD4 + or CD8+ cell responses are frequently observed in chronic HCV infection. T cell failure or exhaustion may be due to ineffective antigen presentation by DCs and macrophages or the depletion of virus-specific T cells (20, 24).

1.7.6 Action on the adaptive immune response

Neutralizing antibodies can be detected in sera of HCV-infected chimpanzees and humans, but HCV-specific humoral response(s) are not sufficient, because it does not prevent re-infection. The high variability of the HCV genome especially in the E2 region is a major target for antibody responses. The development of neutralizing antibodies is significantly delayed in acute infection, and the antibody response lags behind viral evolution. Antibodies do not mediate sterilizing immunity, due to continued viral mutation and antibody-dependent selection.

Other mechanisms of persistent HCV infection include failure of T cell maturation, immunosuppression by HCV RNA and Proteins and suppression of regulatory T-cell activity (20,21,25).

1.8 The role of the T-cell response

1.8.1 CD4+ and CD8+ specific T cell responses

CD4+ T lymphocytes represent the "helper" T cell population, while CD8+ T lymphocytes represent the antigen specific cytotoxic T lymphocytes (CTL), which respond to and kill cells which are infected with intracellular pathogens such as viruses, some intracellular bacteria (e.g. Listeria), some intracellular protozoa (e.g. malaria parasites) and virus infections.

In contrast to immunoglobulin on B cells or soluble antibody molecules, T cells do not recognise free soluble or surface bound antigen, but require the antigen to be processed and presented by an antigen-presenting cell. T lymphocytes (via their TCR) recognise antigen in the form of short peptides presented in association with "self" class I or class II major histocompatibility complex (MHC) molecules at the surface of an antigen-presenting cell (APC).

Human MHC genes are termed HLA -A, B and C for MHC class I, and HLA - DR, DP and DQ for MHC class II. These genes are highly polymorphic, and each individual inherits 2 copies of each gene. The degree of polymorphism is such that most individuals will express genes encoding 6 different HLA class I molecules and 6 different HLA class II alleles. Different HLA molecules bind a different set of peptides, so the polymorphism within MHC genes maximises the number of different (antigenic) peptides, which can be bound and presented to an individual's T cells.

T cell recognition of antigen involves direct cell-cell contact between the antigen-specific TcR on the T lymphocyte and an MHC/peptide complex at the surface of a MHC compatible antigen-presenting cell. In general, class I MHC molecules present antigen to CD8+ T cells, and class II MHC molecules present antigen to CD4+ T cells. Class I MHC molecules are expressed constitutively on almost all nucleated cells of the body, while

constitutive expression of class II molecules is restricted to certain cells of the immune system - B cells, macrophages and dendritic cells - although expression of class II MHC may be induced on other cell types at sites of inflammation. There are distinct pathways of processing of antigen for presentation in association with class I and II MHC molecules. Antigens bound for presentation in association with MHC class I molecules are derived from the cell's cytosol, and are usually endogenously synthesised within the cell (for instance, viral antigens); in contrast, the majority of antigens which are presented by MHC class II molecules are derived from exogenous antigens, such as soluble proteins or extracellular organisms.

1.8.2 CD4+ cell responses

CD4+ T cells recognize antigen, which has been processed and is presented in association with a self-class II MHC molecule. This interaction takes place in the lymphoid tissue, and involves T cell interaction with "professional antigen presenting cells" -B cells, macrophages and dendritic cells - which take up, process and present the relevant antigen. Once a CD4+ T cell has been activated in this way, it is capable of recognizing the antigen presented by any cell, which expresses the appropriate class II MHC molecule. Antigen-presenting cells primarily express MHC class II molecules, but they can be unregulated in most cells as an inflammatory response.

CD4+ lymphocytes perform the following helper functions:

(1) Help B cells develop into antibody-producing plasma cells;

(2) Help CD8+ T cells to become activated cytotoxic T cells;

(3) Help macrophages effect delayed hypersensitivity (e.g., limit infection by M. tuberculosis).[Adapted from Levinson W, Review of Medical Microbiology and Immunology, 10e].

There are two primary subpopulations of CD4+ cells: Th-1 cells help activate cytotoxic T cells by producing IL-2 and help initiate the delayed hypersensitivity response by producing primarily IL-2 and gamma interferon, whereas Th-2 cells perform the B-cell helper function by producing primarily IL-4 and IL-5. Either Th-1 or Th-2 cells, play a dominant role in the response to bacterial or viral disease.

One important regulator of the balance between Th-1 cells and Th-2 cells is interleukin-12 (IL-12), which is produced by macrophages. IL-12 increases the number of Th-1 cells, thereby enhancing host defenses against organisms that are controlled by a delayed hypersensitivity response. Another important regulator is gamma interferon, which inhibits the production of Th-2 cells. CD4+ cells make up about 65% of peripheral T cells and predominate in the thymic medulla, tonsils, and blood. In chronic HCV infection, a strong Th-1 phenotype, evidenced by a robust interferon-gamma response is associated with viral clearance.

1.8.3 Antigen recognition

The activation of helper T cells requires that their TCR recognize a complex on the surface of antigen-presenting cells (APCs), e.g., macrophages and dendritic cells, consisting of both the antigen and a class II MHC protein.

After ingestion of the foreign protein (or microbe) into the APC, it is cleaved into small peptides that associate with the class II MHC proteins. The complex is transported to the surface of the macrophage, where the antigen, in association with a class II MHC protein, is presented to the receptor on the CD4+ positive helper cell (Figure-1.2).

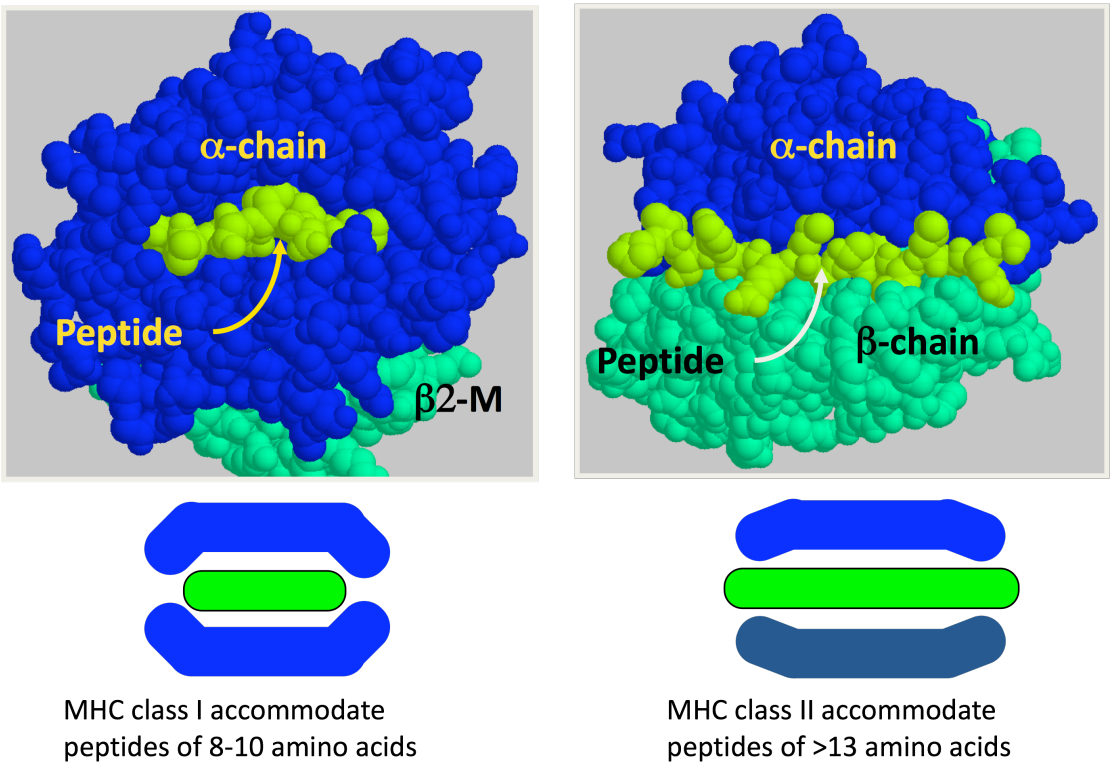
CD4+ T cells bind an epitope consisting of an antigen fragment lying in the groove of a class II histocompatibility molecule. CD4+ T cells are essential for both the cell-mediated and antibody-mediated branches of the immune system.

CD4+ cells tend to recognise larger peptide fragments (12-15 mers), and have a more promiscuous response to foreign antigen in contrast to CD8+ cells, which bind short peptide sequences (8-10mers) but are highly specific, and often result in cytolysis or the death of the antigen-presenting cell.

1.8.4 CD8+ responses (26,27)

Virus-specific CD8+ cytotoxic T lymphocytes (CTL) are the primary effector cells of the immune system. These cells recognize viral antigens which have been synthesized within cell's nucleus or cytosol, and which have been degraded. They are presented at the cell's surface as short peptides associated with self-class I MHC molecules. The recognition of antigen by CD8+ T cells is, therefore, distinct from that of CD4+T cells in several respects. It requires synthesis of the target antigen within the cell (and is therefore restricted largely to virally infected or tumor cells); it is restricted by class I MHC molecules (as opposed to MHC class II restriction for CD4+ T cells). MHC class I molecules are expressed on almost all cells, so virtually any cell, on infection with virus, can act as a target cell for antigen specific CTL (contrasts with the limited tissue distribution of class II MHC); recognition of an antigen presenting cell (APC) by an antigen-specific CTL usually results in the destruction of the APC.

Figure-1.2 Differences in peptide binding between CD4+ (MHC Class-II) & CD8+ (MHC-Class I) cells



[Adapted from Gumperz et al Immunology and Cell Biology (2004) 82, 285–294.]

1.9 Specific responses to HCV infection

1.9.1 CD4+ responses to HCV infection (28-32)

Cellular immune responses are often first evident in circulating T cells 4–8 weeks after the onset of infection (28-30). The onset of cellular immunity corresponds to a spike in serum transaminases, suggesting a transient immune-associated liver injury (28). Spontaneous resolution of HCV is associated with a robust and sustained T cell response targeting multiple HCV epitopes at once and with intrahepatic production of IFN-γ (28-30). CD8+ T cells in the blood and liver display IFN-γ production and cytotoxic T lymphocyte (CTL) activity in response to HCV peptides (31,32). T cell proliferative responses must be differentiated from antigen-stimulated IFN-γ production; only the latter is associated with control of viremia.

Strong, broad, and sustained CD4+ and CD8 + T cell responses have been observed in 79–100% of individuals who were able to clear HCV. The frequency of CD4+ or the Th1 cytokine secreting CD4+ T cells is much higher, (0.01–0.1%) in the peripheral blood mononuclear cells (PBMC) from patients with spontaneous or treatment-induced viral clearance; while it is only 0.001% or undetectable in persistently infected patients. In resolved HCV infection, antiviral mechanisms of CD4+ T cells may include :

(1) Secretion of Th1 cytokines (such as interleukin-2, IL-12), IFN-γ, and TNF-a

(2) Activation of HCV-specific B cells (Th2 profile)

(3) Activation of CD8+ T cell responses (Th1 profile) to produce IFN-γ

(4) Increasing the expression of HLA-molecules on infected cells to enhance the immune recognition by CD8+ T cells and NK cells (31-33). The lack of an optimal CD4+ T helper 1 response (which is characterized by the secretion of interferon gamma) is associated with fibrosis/cirrhosis development in patients with chronic hepatitis C infection. Notably, loss of HCV-specific CD4+ T cell accompanied by recurrent HCV viremia and hepatitis has been observed in some patients with initial HCV clearance during acute HCV infection (33).

1.9.2 CD8+ responses to HCV infection (34,35)

HCV-specific CD8+ T cells are more frequent than the CD4+ T cells in the peripheral blood. Similar to the CD4+ T cell response, a strong, multi-specific CD8+ T cell response during the first 6 months after infection is associated with viral clearance; moreover, stronger polyclonal CTL responses in PBMC and in the liver were linked to lower viremia.

Mechanisms of CD8+ specific T cell response include direct killing of infected cells and noncytotoxic mechanisms (secretion of antiviral cytokines, IFN-γ, or TNF-a). IFN-γ, produced by virus-specific CD8+ T

cells can significantly inhibit HCV RNA replication. Conversely, weak and narrow epitope specific Th cell and cytotoxic T lymphocyte (CTL) responses are associated with viral mutation and persistent infection. In established chronic infection, CD8+ T cells specific for HCV epitopes can be abundant, making up as much as 1–2% of total CD8+ T-cells in the peripheral blood or liver. However, many of these cells are functionally deficient in that they have limited ability to produce IFN-γ, to proliferate, or to kill cells presenting their cognate antigen.

HCV can persist despite the presence of CD8+ cytotoxic T cells (36-38). Therefore, it has been suggested that CD8+ T cell response is less effective in clearing HCV compared to other viruses like influenza and Hepatitis B. CD4+ T cells, on the other hand, have a dominant role both in the initiation and propagation of antiviral immunity (37). The balance between Th1/Th2 CD4+ T cells may also influence the expansion of CD8+ T cells (38,39). While Th1 predominance has been associated with HCV resolution, Th2 response is frequently observed in non-transplant patients with chronic infection and cirrhosis.

1.10 Measurement of Hepatitis C Specific T-cell responses (40)

The analysis of HCV-specific T-cell responses includes the assessment of

(i) T-cell proliferation in response to HCV antigens,

(ii) The frequency of cytokine-secreting HCV-specific T cells (Figure-1.3)

(iii) Changes in T-cell specificity and viral sequence during the infection.

Proliferation assays are methods for measuring clonal proliferation of lymphocytes in peripheral blood mononuclear cells (PBMCs) in response to HCV antigens. Thymidine incorporation assay has been the most frequently used assay, because of extensive published data available for comparison, the small number of cells required, and its high throughput requirement. The amount of proliferation is detected after culture with antigens by measurement of incorporation of thymidine into the DNA of proliferating lymphocytes. However, PBMC proliferation cannot be equated with CD4+ T-cell proliferation because B-cells and CD8+ T-cells have also been shown to proliferate in response to recombinant viral proteins and/or their breakdown products.

Table –1.1 Methods of assessing T-cell function

Function assessment	Methods
Proliferation capacity	3H-thymidine uptake
	flow cytometry using CFSE
Cytokines/chemokines	
secretion	
– IFN-g and IL-2	ELISPOT
– MIP-1b and TNF-a	ICS
	ICS
Cytotoxicity	
– target cell lysis	(Chromium -51) Cr^{51} release
– perforin/granzyme B/release	Flow cytometry
– CD107a/b mobilization/ degranulation	ELISPOT
– target cell caspase cleavage	Flow cytometry
activity	Flow cytometry

CFSE, 5, 6-carboxy-fluorescein diacetate succinimidyl ester; ICS, intracellular
cytokine staining; TNF-a, tumor necrosis factor-a, MIP-1b, macrophage inflammatory protein-1b

1.10.1 Cytotoxicity assays

Cytotoxicity is an essential CD8+ T-cell function and implicated in the killing of virus-infected hepatocytes, that display viral peptides within cell surface MHC class I molecules. In vitro assays are therefore either a direct measure of cytotoxicity, i.e. based on target cell lysis (Cr^{51} release assay, flow cytometry CTL (FCC) or caspase assay), or an indirect measure, based on the detection of specific molecules that CD8+ T-cells use to lyse target cells (flow cytometric CD107a/b degranulation assay, perforin/granzyme ELISPOT assay).

The Cr^{51}-release assay is the historical assay in which cytotoxic T-cell (CTLs) lines are co-cultured for 4–6 h with HLA-matched Cr^{51} labeled target cells, which do or do not express the cognate antigen. Cytotoxicity is indicated by the amount of Cr^{51} released into the supernatant from lysed target cells. The advantages of this assay are its high sensitivity (especially when performed with T-cell lines and/or clones) and the direct detection of target cell lysis (41).

Indirect measures of cytotoxicity monitor activation of cytotoxic T-cells rather than lysis of target cells. These include the detection of CD107a/b on the surface of CD8+ T cells by flow cytometry (42) and the release of granzyme (43) or perforin (44) by ELISPOT analysis. However, granule

content depends on the maturation status of the CD8+ T cell (45) and granules of central memory cells may not contain perforin or granzymes.

1.10.2 Analysis of cytokine secretion:

Hepatitis C virus-specific T-cell gamma interferon and proliferative responses may be more common in perihepatic lymph nodes than in peripheral blood or liver, but recent studies indicate that the kinetics of hepatitis C virus-specific CD8+ T cell responses in the blood mirror those in the liver, at least in acute hepatitis C virus infection.

1.10.2.1 Intracellular cytokine staining (41):

Detection of intracellular cytokines using intracellular IFN-gamma staining by flow cytometry has been used extensively for several viral infections, such as CMV, but a consensus conference concluded that it is not sensitive enough to detect low frequency HCV-specific T-cells ex vivo in HCV infection, except in some cases with acute hepatitis C infection.

1.10.2.2 The ELISPOT assay

The Enzyme-linked immunospot (ELISPOT) assay is a method for identification and enumeration of cytokine-secreting T cells. ELISPOT assays were first established to quantitate antibody-secreting B-cells (47) and were then modified to detect cytokine-secreting T-cells (48). ELISPOT

assays are the method of choice to monitor HBV- and HCV-specific immune responses, followed by proliferation assays, tetramer staining and intracellular cytokine staining.

ELISPOT assays can be performed in a semi-automated manner, allowing rapid screening of many individual, antigen-specific responses in complete PBMC. Using overlapping peptides, the breadth of CD4+ and CD8+ T cell responses in the context of all autologous HLA alleles can be analyzed and multiple cytokines can be tracked simultaneously (49). The ELISPOT assay is considered superior to the proliferation assay because it yields good responses even with cryopreserved PBMC (50). It is considered superior to the intracellular cytokine staining/flow cytometry assay because of its 10- to 100-fold higher sensitivity.

1.10.2.3 The role of ELISPOT in Hepatitis C infection

Several studies applying a comprehensive approach to analyse HCV-specific T cell responses using overlapping peptides have been published in recent years. Mizukoshi et al [2008] studied HCV-specific immune responses in long-term IDUs of duration, >10 years, by proliferation, enzyme-linked immunospot (ELISPOT), interferon (IFN)-gamma secretion, and cytotoxicity assays as well as enzyme immunoassays for HCV-specific antibodies (51). They found that the reduced risk of HCV persistence in IDUs previously recovered from HCV infection correlated

with T cell responses, and prolonged antigenic stimulation was required to maintain humoral response. Overall, HCV-specific T cell responses were stronger and broader after resolved infections than in chronically infected patients. Responses tend to be heterogeneous between different individuals with no particular dominant epitopes consistently recognized in chronically infected and recovered patients.

ELISPOT assays vary in patients with HCV infection with or without cirrhosis. Anthony et al [2001] used an ELISPOT assay to analyze a cross-section of HCV-infected patients with or without cirrhosis, for recall responses to HCV Core and NS3 proteins (52). Peripheral blood lymphocytes (PBL's) from HCV-infected patients without cirrhosis responded to NS3 and Core proteins, producing predominantly IFN-gamma, with little IL-4 or IL-5. In contrast, PBMCs from HCV-infected patients with cirrhosis responded to NS3, but not to the Core protein, suggesting a selectively altered immune state during cirrhosis.

Knowledge of HCV-specific T cell responses in individuals who cleared HCV either spontaneously or after interferon-based treatment could provide some ideas on the potential risk for re-infection after successful therapy which may also have consequences for the management of high-risk individuals who have mild liver disease but are potentially infectious. Wertheimer et al (2003) reported that pretreatment HCV-specific immunity is associated with response to combination antiviral therapy. 43% of

patients in their study who had more than 168 SFUs/$10^{(6)}$ peripheral blood mononuclear cells (above background) experienced SVR compared to 28% of those who did not (P= 0.007). They concluded that the CD4+ specific ELISPOT response was independently associated with SVR by multivariate analysis (53).

There is a lot of conflicting evidence on the evolution of peripheral T cell responses during interferon-alpha therapy. Burton el al, reporting for the Virahep study group (2008) reported that total HCV-specific IFN-γ CD4+ T cell ELISPOT responses did not increase with therapy, but rather decreased by 8 weeks and remained below baseline 24 weeks after cessation of therapy (54). They did not find statistically significant differences with respect to viral kinetics, race and virologic outcome. In contrast, viral relapse after treatment was associated with a three-fold increase in HCV-specific responses. Other studies have shown a partial restoration of HCV-specific T cell immunity by interferon-treatment of chronic hepatitis C. Cramp et al studied CD4+ T lymphocyte proliferation together with interferon (IFN)-γ and interleukin (IL)-10 production from peripheral blood mononuclear cells in response to 4 HCV antigens (core, NS3, NS4, and NS5) in 25 patients with chronic hepatitis C undergoing antiviral therapy with IFN alone or in combination with ribavirin, prospectively, before, during, and after treatment. They concluded that treatment-induced control of hepatitis C viremia is associated with the development of HCV-specific T-cell responses with enhanced IFN-γ and

low IL-10 production, and that the greater efficacy of combination therapy with IFN-α plus ribavirin may be related to its ability to suppress HCV-specific IL-10 production (55).

The matrix approach is an epitope screening method that employs peptide pools in a matrix array in ELISPOT assays (56). The identity of individual, recognized peptides can be deduced from the pattern of recognized peptide pools. Klade et al (2009) studied a matrix approach using 393 15mer peptides from conserved HCV regions overlapping by 13 amino acids in 52 HCV-recovered individuals. While only 33% of HCV-recovered individuals recognized recombinant HCV proteins, 81% of individuals tested positive in the matrix approach ($p < 0.001$) (56). The strength, frequency and breadth of HCV-specific T cell responses were similar in spontaneously recovered patients than in interferon-recovered patients. This approach underscores the utility of using peptide combinations expressing multiple antigenic epitopes, to increase the sensitivity of the assay.

1.10.2.4 Limitations of the ELISPOT assay

The main limitation of the ELISPOT assay is the requirement of purification of PBMCs, which adds to the complexity of the operation. Additionally ELISPOT analysis cannot be performed on fresh whole blood. The variability of ELISPOT has been a major challenge for assay performance, standardization, optimization, and reproducibility (57). The

recent use of serum-free media and co-stimulation, particularly the use of interleukin-7, has been shown to increase the sensitivity of spot detection. Martinuzzi et al [2008] demonstrated that two parameters were found to greatly enhance detection sensitivity (i.e., to specifically increase epitope-driven signal while keeping background noise to a minimum): use of human serum-free vs. serum-supplemented culture medium (2.4-fold median increase) and addition of low dose IL-7 (1.5-fold increase). Incorporating both of these parameters into the ELISPOT procedure proved capable of greatly amplifying (35.1-fold increase) the low-grade CD8+ T cell responses. Other studies using co-stimulation with anti- CD28 and IL-15 have also reported amplification of response (58-60).

Often, IFN-γ ELISPOT is performed with cryopreserved peripheral blood mononuclear cells (PBMC). However, it has not been well defined yet to what extent diminished cell viability of PBMC following cryopreservation affects IFN-γ responses in ELISPOT. Lenders et al [2010] assessed the influence of apoptotic cells on the number of spot-forming cells (SFC) in IFN-γ ELISPOT using a gradient of UV-irradiated apoptotic PBMC and viral antigens derived from Varicella-Zoster virus (VZV) and cytomegalovirus (CMV). They found that the presence of apoptotic cells among viable T cells hampered the detection of SFC following stimulation (61).

1.11 QuantiFERON® assay for IFN- γ production

Quantiferon assays for interferon-gamma release have been developed to study interferon-gamma release in an in-tube fashion. Quantiferon assays have been used to quantify IFN- γ production in tuberculosis disease, as well as in the detection of CMV infection post-transplant. These assays have significant advantages over existing methods- it provides a T cell assay comparable in cost and labour to a simple immunoassay. There is no requirement for lymphocyte isolation. The logistics for large-scale use are more favourable, as it uses inexpensive and readily available laboratory equipment. Thus, direct comparison of results from multicenter studies is more readily possible. The incubation of whole blood preserves the in vivo cellular and biochemical environment for lymphocyte stimulation, maximising cellular response.

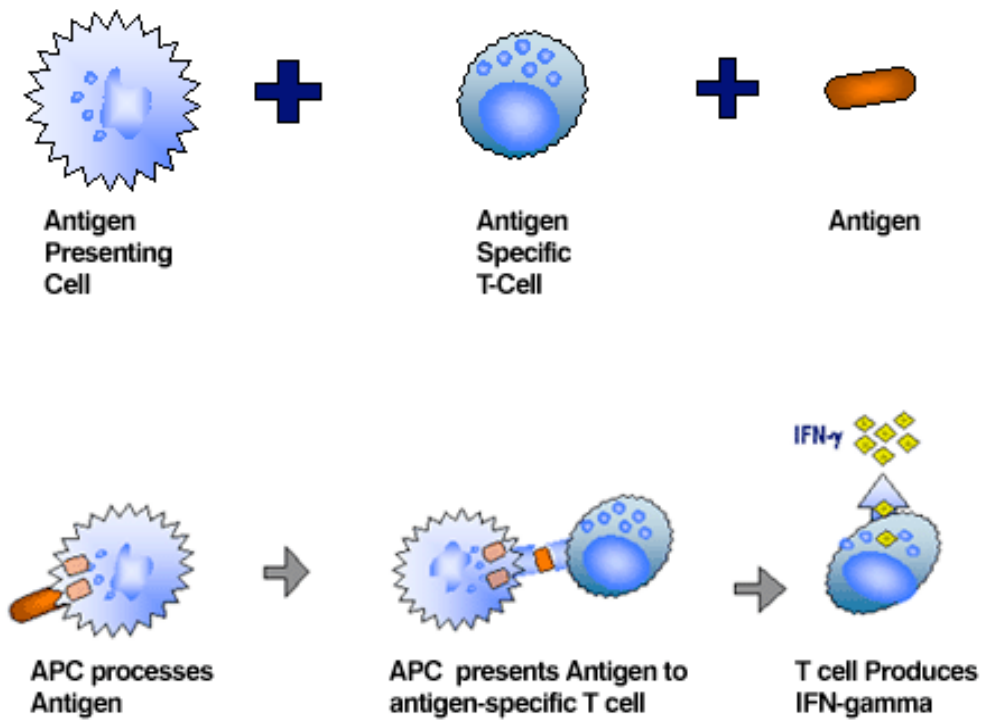


Figure-1.3 Principle of interferon-gamma release assays [Source: cellestis.com]

1.11.1 QuantiFERON® and Tuberculosis (TB) infection:

Quantiferon testing for tuberculosis was approved by the FDA 5 years ago, and seems set to become the gold standard for detection of active TB, TB in high-risk healthcare workers and latent TB detection.

Quantiferon-TB and TB-gold are interferon-gamma release assays (IGRAs) based on proteins derived from the M. tuberculosis RD1 genomic segment , which encodes proteins, such as early secretory antigenic target (ESAT)-6, that are absent from M. bovis bacille Calmette-Guérin (BCG) and most environmental mycobacteria. Mori et al presented a meta-analysis of 21 reports using Quantiferon for the diagnosis of TB infection. The range of observed sensitivity was 62% to 95%; with a pooled value was 80% (62).

A United Kingdom UK Health technology assessment (HTA) conducted a systematic review on the effectiveness of available rapid diagnostic tests to identify tuberculosis (TB) infection. They found that IGRA's correlate better with intensity of exposure, and therefore are more likely than TST/purified protein derivative (PPD)-based assays to detect latent TB infection (LTBI) accurately. They were also found to be more cost-effective in screening high-risk populations (63).

1.11.2 Comparison of Quantiferon to ELISPOT

A commercially available ELISPOT kit for TB (T-spot) provides an opportunity to compare the results of Quantiferon assays to ELISPOT. Pai et al (64) presented a meta-analysis on interferon –gamma detection assays in TB diagnosis. The pooled sensitivity was 78% (95% CI, 73% to 82%) for Quantiferon -TB Gold, 70% (CI, 63% to 78%) for Quantiferon -TB Gold In-Tube, and 90% (CI, 86% to 93%) for T-SPOT.TB. The pooled specificity for both Quantiferon tests was 99% among non-BCG-vaccinated participants (CI, 98% to 100%) and 96% (CI, 94% to 98%) among BCG-vaccinated participants. They concluded that IGRAs have excellent specificity that is unaffected by BCG vaccination, and that T-SPOT.TB appeared to be more sensitive than both Quantiferon tests and TST (Mantoux).

1.11.3 Quantiferon and Cytomegalovirus (CMV) infection

Quantiferon -CMV measures interferon-γ responses to a variety of HLA class I restricted CMV T-cell epitopes. The sensitivity of the Quantiferon assay for HCMV epitopes was at least equivalent and in some cases more sensitive than the ELISPOT. Kumar et al [2009] studied the utility of cell-mediated immunity (CMI) measured by Quantiferon in predicting Cytomegalovirus disease in high-risk solid organ transplant recipients. CMV disease occurred 5.3% patients with a detectable interferon-γ

response versus 22.9% patients with a negative response ($p = 0.038$). They concluded that monitoring of CMI might be useful for predicting late-onset CMV disease (65).

No data exists on the use of this test platform for the assessment of T-cell response in Hepatitis C. Development of an HCV-specific T-cell assay may help in predicting outcome of HCV infection after treatment.

Research questions

- Which combination of peptides will be most useful to assess CD8+ and CD4+ IFN-γ release?
- Can a Quantiferon based HCV-specific assay (Quantiferon-HCV) be used as a diagnostic test for the detection of HCV infection, and for assessing response to treatment?
- Will Quantiferon-HCV be a useful clinical tool for predicting timing and severity of HCV recurrence in HCV-infected patients who have undergone liver transplantation?

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Chapter 2

Which combination of peptides will be most useful to assess CD8+ and CD4+ IFN-γ release?

Introduction:

T lymphocytes recognize MHC molecules that have bound peptide epitopes derived from the intracellular processing of antigens. Thus, the immunogenicity of a given epitope is dependent upon: the generation of the appropriate fragment, the presence of an MHC molecule that binds this fragment and the presence of T cells capable of recognizing the complex.

Recognition of an antigenic peptide-MHC complex by an αβ T cell receptor (TCR) initiates an intracellular signaling cascade leading to a T cell response. T cells recognize only specific antigen presented by one of the host MHC molecules in which they developed (also termed MHC restriction). Although antigen specificity is considered a hallmark of cellular immunity, the TCR is cross-reactive, binding and responding to multiple peptide-MHC ligands. Molecular mimicry, in which cross-reactive ligands share key structural and chemical features is a mechanism commonly used to explain TCR cross-reactivity.

This aspect of the study assessed the optimal concentrations and combinations of peptide pools to elicit a specific gamma-interferon response from HCV infected individuals.

Methods:

Assessment of appropriate peptides, and combinations was carried out by ELIPSOT assay. In the first instance, individual peptides were tested in duplicate among patients known to have a Sustained viral response (SVR) or with evidence of spontaneous clearance (SC), to determine which peptides produced an interferon-gamma response.

After this initial assessment, two peptide pools each were constituted for the CD4 specific peptides, which were then assessed for efficacy in 11 subjects. Two pools of 12 peptides each were constituted, and matched for hydrophobic amino acid residues, representation of all parts of the HCV proteome and the amount of DMSO in each pool. These were compared against a composite pool of all 24 peptides to assess if there was an incremental response to peptide pooling (1-19).

CD8 specific peptides were used in a single pool to maximize the coverage across HLA specificities. For the purposes of this study, a pool of peptides representing 35 previously identified HLA A1, A11, A2, A24, A26, A3, B7, B8, B27, B35, B44, B51 B57 and B60 restricted HCV cytotoxic T lymphocyte epitopes were used to study CD8+ specific cell responses (20-29).

Peptide selection and reconstitution

Candidate peptides were identified from the immune epitope database (hcv.lanl.gov, and immuneepitope.org) (30). Peptides were selected on the

basis of known ability in the literature to elicit interferon-gamma response from T cells on HCV infected individuals, either by ELISPOT or by flow cytometry. CD8 peptides were selected from the database to ensure a combination of HLA restrictions, which would provide population coverage of more than 90% for all major North American ethnic groups. The peptides utilized are listed in Tables 2.1 and 2.2.

Peptides of 95-99% purity were obtained from a single source (CanPeptide Inc., Montreal, QC). Peptides were initially suspended in 10-15 µL of DMSO to give a concentrated solution stock with a concentration of 60-80 mg/mL. Peptides were then mixed in pre-assigned pools (CD4 and CD8 specificities, as defined by literature). The volume used for each experiment was limited to 10µL DMSO, to limit the dilution effect of excess vehicle, and to restrict the final DMSO concentration per reaction to 0.1%(v/v), as DMSO can attenuate IFN- gamma release at levels greater then 0.5% (v/v). The peptide mix was then separated into multiple aliquots and stored at -20 degrees before use.

Potential subjects were contacted by telephone or directly on a clinic visit by the author, or by the lead clinician or practice nurse in charge of the patients care. The study was discussed in brief, and patients who indicated interest were invited to a special clinic, where the study was discussed in detail, consent sought and blood collected by venupuncture.

Healthy controls of known HCV negativity (immunocompetent and HCV antibody negative) were selected from within hospital and laboratory staff. A total of 20 millilitres of blood was collected by venupuncture – 8-10 ml in ACD (Acid citrate dextrose- yellow cap) tube, 5 ml in a lithium-heparin (green top) tube and 5-7 ml in a serum collection tube. While blood was alliquoted within 2 hours of collection into the Quantiferon tubes, and blood from the ACD tube was used for PBMC extraction. This study was conducted on freshly extracted PBMCs alone.

Density gradient separation of Peripheral blood mononuclear cells (PBMC)

PBMC extraction was carried out using a Lympholyte density gradient. Lympholyte-Mammal is a density separation medium specifically designed for the isolation of viable lymphocytes and monocytes from the peripheral blood -it consists of Sodium Diatrizoate combined with Dextran. Standardization experiments performed in the lab (data not shown) found the PBMC isolation yield of Lympholyte to be comparable to the Ficoll-density gradient PBMC isolation.

Briefly, heparinized blood was mixed 1:1 volume with Phosphate-buffered saline (PBS), and 4 ml of the solution was layered slowly over 3 ml of Lympholyte. The tube was then centrifuged at 800 g for 20 minutes. The

well-defined lymphocyte layer was removed, and diluted with complete RPMI before centrifugation at 800 g for 10 minutes. The cell pellet was re-suspended in a fixed volume of media, and a cell count performed. The suspension was re-centrifuged and the pellet re-suspended in a volume of media to give a final concentration of $2x10^{6}$ PBMCs/ml of media.

ELISPOT

ELISPOT was performed from freshly extracted lymphocytes using the ELISPOT Human interferon-gamma ready-set-go kit (Ebiosciences, Catalog number 88-7386). The Human IFN-γ ELISPOT Ready-SET-Go! Reagent set contains the necessary reagents for performing enzyme linked immunosorbent spot (ELISPOT) assays for high-resolution frequency analysis of IFN-γ-secreting cells, or spot-forming units (SFUs). The kit components are as follows:

- **Capture Antibody (**Pre-titrated, Functional Grade (low endotoxin) purified antibody)
- **Detection Antibody (**Pre-titrated, biotin-conjugated antibody), ELISA/ELISPOT Coating Buffer (Phosphate buffered saline), and
- **Detection enzyme** (pre-titrated Avidin-HRP) and ELISPOT wash buffer (PBS –Tween)

Procedure

96-well polyvinylidene difluoride (PVDF) backed plates (MAIP S 45; Millipore, Bedford, MA) were coated with 15 µg/ml of anti–IFN-γ monoclonal overnight at 4°C. Plates were then washed 6 times with ELISPOT wash buffer (PBS with 0.5% Tween) and blocked with RPMI-1640 supplemented with streptomycin, penicillin, and 10% heat-inactivated fetal bovine serum (FBS) for 1 h. PBMCs were separated from heparinized whole blood on Lympholyte, washed 3 times, and re-suspended in complete RPMI to a concentration of 2 x 10^{6} PBMCs/ml. These PBMCs were then added in 100 µl RPMI/well to the pre-coated plates, in triplicate wells. The cells were then stimulated with 100µl peptide mix divided into four groups- a negative control group, a group stimulated with a peptide pool known to elicit a CD4+ response, a third group with a peptide pool that elicited a CD8+ specific response and a mitogen group stimulated with phytohemagglutinin (PHA). An additional group, studying the effect of interleukin-7 co stimulation (CD4 +IL-7) was also studied, with a concentration of IL-7 of 5ng/mL. The plate was then incubated at 37°C, 5% CO2 humidified incubator for 24 hours.

The medium and cells were then decanted and the plate washed 3 times with ELISPOT Wash Buffer. The biotinylated detection antibody was diluted in assay diluent according to instructions on the Certificate of Analysis, provided with the reagent set- 100 µl/well to plate microwells and

incubated at room temperature for 2 hr. The antibody solution was decanted, and the plate washed 4 times with ELISPOT wash buffer, with the wells being allowed to soak for 1 minute for each wash. Avidin-HRP reagent was diluted in assay diluent according to instructions on the Certificate of Analysis, and 100 µl/well of Avidin-HRP was added and incubated at room temperature for 45 minutes. The plate was then washed 3 times with ELISPOT Wash Buffer, and then 2 times with 1X PBS (no Tween-20). Fresh AEC substrate (100 µl/well) was added, and spots allowed to develop at room temperature for 10-60 minutes. The substrate reaction was stopped by washing wells 3 times with 200 µl/well of distilled water.

The plate was air-dried overnight, and the spots counted using an automated ELISPOT reader (AID, Strassberg, Germany). The T-cell precursor frequency for each peptide pool was based on the total number of PBMC in the well and the number of peptide-specific spots per well, over an average of three wells. The number of peptide-specific spots was also calculated by subtracting the negative control values, which consisted of PBMC with media, without peptide (an average of three wells), from the test wells.

Results

A successful T-cell response to HCV involved specific T-cell responses to different parts of the HCV proteome, especially non-structural proteins.

Analysis of T cell responses by ELISPOT to different peptides was carried out by plating $2x10^5$ PBMCs in duplicate with each peptide in the pool individually for patients with SVR and spontaneous clearance of the disease. Only CD4 specific peptides were included in this assay, as CD8 specific peptides are HLA Class-I restricted in action.

Individual peptide assays were carried out in five patients, 4 with SVR and one with SC. There were robust responses observed to peptide sequences from all HCV proteins. The highest responses in both SVR and the SC group were for peptide sequences from the NS3 protein, (Figure-2.1). Responses were similarly distributed in the SVR and the SC groups.

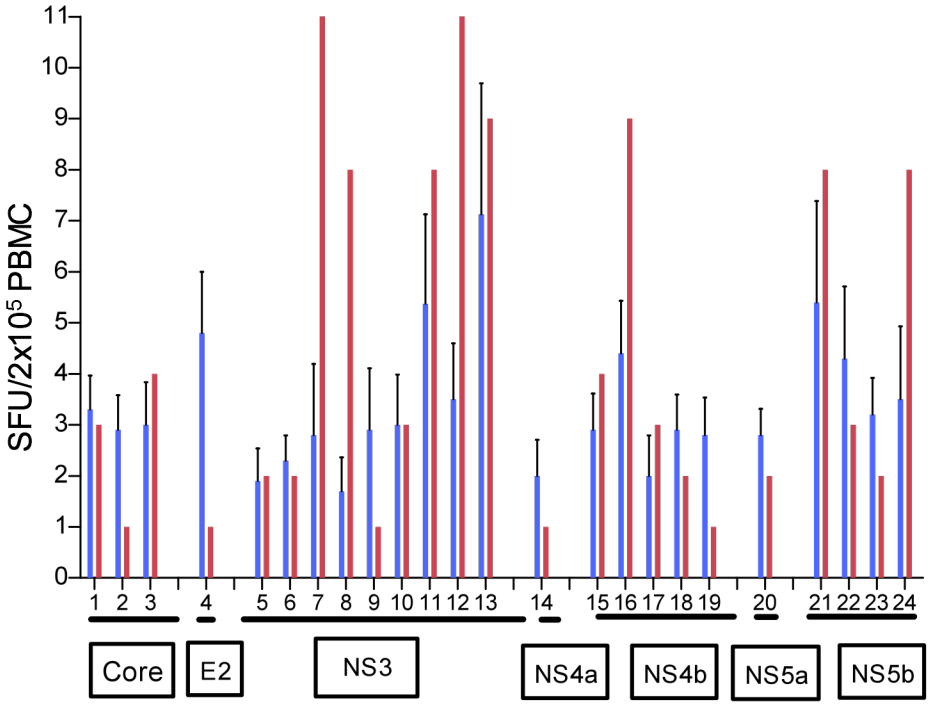

Figure-2.1 Figure showing distribution of elicited T-cell responses by ELISPOT to individual peptides from the CD4 pool. Bars in blue represent patients with SVR (Mean ± SEM) to disease (n=4), while red bars represent patients with SC (n=1). Numbers 1 to 24 represent peptide sequences labeled, as per Table-3.

CD4+ specific responses:

Peptides of known CD4+ specificity (n= 24 peptides) representing all parts of the proteome were combined in two different pools of 12 peptides each, at 10 micrograms/ml. These combinations were tested separately on 11 subjects (6 patients with HCV, and 5 controls), to assess the degree of response.

There was an incremental response to using all 24 peptides as compared to groups of 12 peptides (Median SFU for HCV+ patients in pool A: 47 (IQR 13-61), Pool B:13 (10-22), Pool C [Combined] 64 (24-83), as compared to the controls ; Pool A:8 (5-11), Pool B:12 (3-23) , and Pool C [Combined] 10 (7-23). [Figure 2.2]

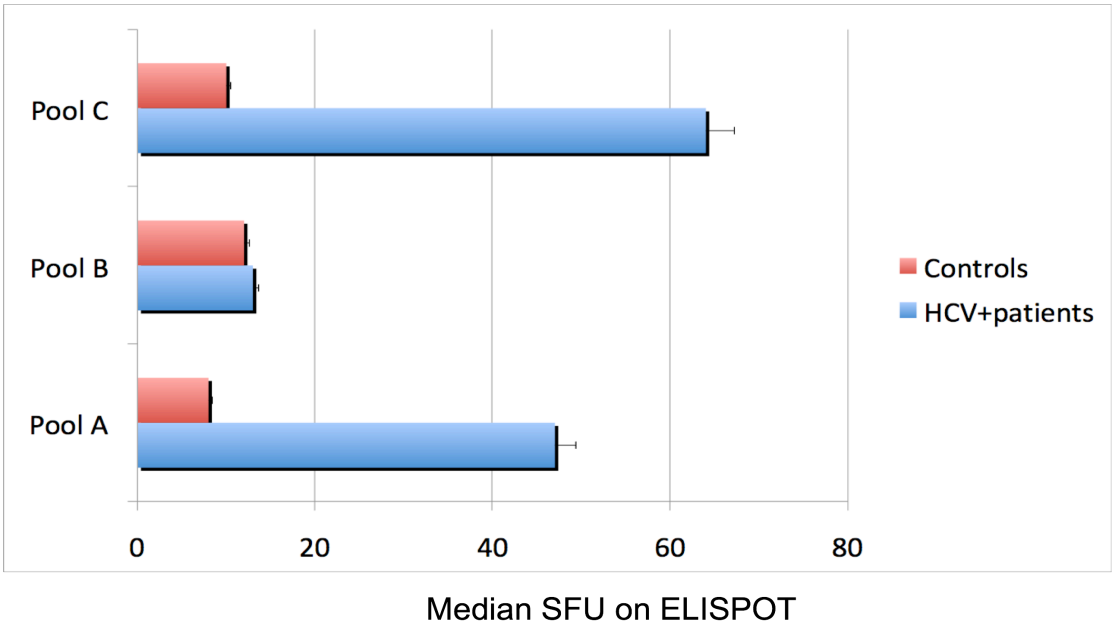

Figure-2.2 Figure showing additive response of a composite peptide pool on elicited IFN response

CD8+ specific peptides

The CD8 peptide pool was also assessed in these 11 patients to assess whether a difference between HCV+ patients and healthy controls could be elicited. The response elicited was lower than that among the CD4 peptide pool, but higher in HCV subjects than in controls (Healthy controls 4 SFU (2-11) , cases 9 SFU (5-21) (p value=0.06).

Discussion

In this initial part of the study, we identified a group of peptides known to elicit interferon-gamma production from either CD4 or CD8 cells. We also demonstrated that this response covers peptide sequences from different parts of the proteome, although a successful response demonstrates high interferon-gamma production with non-structural proteins.

MHC Class II specific antigen recognition is associated with DR DP and DQ molecules. CD4+ cells tend to recognise larger peptide fragments (12-15 mers), and have a more promiscuous response to foreign antigen. The conformation of the receptor allows it to recognize peptide fragments as part of larger protein and peptide chains.

Broad, sustained CD4+ responses are considered essential for clearance of HCV infection- some authors suggest that the CD4 response may be

more important than the CD8 responses (30), since lack of help from CD4+ cells has been correlated with impaired CD8 T cell function and viral persistence. Studies have also shown that the anti-viral CD4+ response during acute HCV infection is a critical determinant of disease resolution.

HLA class I restricted cytotoxic CD8+ T lymphocytes (CTL) recognize a complex composed of the MHC-encoded class I heavy chain, the β_2-microglobulin light chain and a short antigenic peptide, 8-11 mer in length, derived from the degradation of intracellular proteins. Since the recognition of cognate antigen is highly specific to the tertiary receptor conformation, it was initially believed that the immune system might be capable of generating a unique TCR for almost every antigenic peptide. Recognition of these peptides is HLA restricted, which poses a considerable challenge for devising a peptide pool offering coverage to an ethnically diverse population.

HLA class I alleles have been grouped by some investigators into supertypes, which reflect similarities in MHC sequence and peptide-binding patterns, eg, HLA A2, A3, B7, and B44 supertypes . The peptide pools were designed to provide HLA coverage exceeding 94% of the population for three major ethnic groups.

The weights used were 71.9% Caucasian, 12.1% African American, 3.7% Asian, 11.6% Latino, 0.7% Native American (calculated from Mori and co-workers report on the serological typing of over 1.35 million volunteers from the United States) (31,32). An algorithm for increasing population coverage as expressed by Longmate et al was used to identify peptides of appropriate HLA specificity. A combination of peptides of the following HLA specificities – HLA A2, HLA-A24, HLA-A24, HLA-A1, HLA-A3, HLA-A11, HLA-A23, HLA-A30, HLA-A32, HLA-A*0206, HLA-B*0702, HLA-A74, HLA-A26, HLA-A31, HLA-A*0202, HLA-A*0207, HLA-A68,- would represent 93.2% of the Asian population, 90.9% of the black population, and 94.4% of the Caucasian population, thus providing coverage for an aggregate 94.1% of the North American population (31).

However, frequent recognition of HLA class I-restricted T cell epitopes on several alternative alleles across HLA class I supertypes and encoded on different class I loci has been reported. Frahm et al studied the extent of promiscuity of HLA class I peptides by eliciting responses to 242 well-defined viral epitopes tested in 100 subjects regardless of the individuals' HLA type. Half of all detected responses were seen in the absence of the originally reported restricting HLA class I allele, and only 3% of epitopes were recognized exclusively in the presence of their original allele.

Theories to explain T-cell cross reactivity include induced fit of peptides, differential TCR docking, structural degeneracy, molecular mimicry and antigen-dependent tuning of peptide-MHC flexibility (36).

Two pools of peptides, one of known CD4 and the other on known CD8 specificity representing all major HCV proteins were identified and constituted to limit the amount of DMSO which could attenuate the response.

There was a marked difference in the ELISPOT response between the peptide pools analyzed for CD4 response. Both pools were matched to ensure representation of all major HCV proteins and controlled for the amount of DMSO. However, pool B had a larger number of hydrophobic amino acid residues, particularly valine- the significance of this is unclear. Previous studies looking at the effects of individual peptides have not compared the effects between peptides, and it is possible that the residues in Pool B simple represented a less immunogenic peptide pool of HCV peptides. A composite pool of all 24 peptides showed an additive effect on overall response.

In summary, this part of the study defined and tested a pool of HCV – specific peptides to elicit a specific IFN-γ response.

Table 2.1 – Pool ‘A’ of peptides (CD4 specificity)

Amino acid position	HCV protein	Amino acid sequence
aa 21-40	Core	DVKFPGGGQIVGGVYLLPRR
aa 141-155	Core	GAPLGGAARA LAHGV
aa 393-410	E2	GFATQRLTSLFALGPSQK
aa 1241-1260	NS3	PAAYAAQGYKVLVLNPSVAA
aa 1248-1267	NS3	GYKVLVLNPSVAATLGFGAY
aa 1384-1401	NS3	VIKGGRHLIFCHSKKKCD
aa 1539-1554	NS3	LRAYMNTPGLPVCQDH
aa 1746-1765	NS4b	IAPAVQTNWQKLETFWAKHM
aa 1771-1790	NS4b	GIQYLAGLSTLPGNPAIASL
aa 1907-1926	NS4b	GPGEGAVOWMNRLIAFASRG
aa 2268-2282	NS5a	VSVPAEILRK SRRFA
aa 2941-2960	NS5b	CGKYLFNWAVRTKLKLTPIA

Table 2.2– Pool ‘B’ of peptides (CD4 specificity)

Amino acid position	HCV protein	Amino acid sequence
aa 31-45	Core	VGGVYLLPRR GPRLG
aa 1248-1261	NS3	GYKVLVLNPSVAAT
aa 1251-1259	NS3	VLVLNPSVA
aa 1411-1426	NS3	GINAVAYYRGLDVSVI
aa 1531-1550	NS3	TPAETTVRLRAYMNTPGLPV
aa 1581-1600	NS3	ENLPYLVAYQATVCARAQAP
aa 1686-1705	NS4a	VVLSGKPAIIPDREVLYREF
aa 1767-1786	NS4b	NFISGIQYLAGLSTLPGNPA
aa 1806-1818	NS4b	TLLFNILGGWVAA
aa 2571-2590	NS5b	KGGRKPARLIVFPDLGVRVC
aa 2841-2860	NS5b	ARMILMTHFFSVLIARDQLE
aa 2941-2955	NS5b	CGKYLFNWAV RTKLK

Table -2.3 CD4 + restricted epitopes [Pool C]

	Amino acid position	HCV protein	Amino acid sequence	Epitope ID	HLA-restriction
1	aa 21-40	Core	DVKFPGGGQIVGGVYLLPRR	10639	DRB1*1101,DQB1*0301
2	aa 31-45	Core	VGGVYLLPRR GPRLG	68673	DRB1*1101
3	aa 141-155	Core	GAPLGGAARA LAHGV	18725	DRB1*1101
4	aa 393-410	E2	GFATQRLTSLFALGPSQK	19501	DRB1*1101
5	aa 1241-1260	NS3	PAAYAAQGYKVLVLNPSVAA	46755	DRB1*15, DRB1*0301 +
6	aa 1248-1261	NS3	GYKVLVLNPSVAAT	23390	DR4, DRB1*1101
7	aa 1248-1267	NS3	GYKVLVLNPSVAATLGFGAY	23393	DQB1*0301
8	aa 1251-1259	NS3	VLVLNPSVA	69829	DRB1*1101,DRB1*120,DRB1*0401, DRB1*1302
9	aa 1384-1401	NS3	VIKGGRHLIFCHSKKKCD	69007	DRB1*15
10	aa 1411-1426	NS3	GINAVAYYRGLDVSV	20375	DRB1*15
11	aa 1531-1550	NS3	TPAETTVRLRAYMNTPGLPV	65497	DRB1*0701
12	aa 1539-1554	NS3	LRAYMNTPGLPVCQD	39041	DRB1*15
13	aa 1581-1600	NS3	ENLPYLVAYQATVCARAQAP	13518	DRB1*1001
14	aa 1686-1705	NS4a	VVLSGKPAIIPDREVLYREF	71752	DRB1*0301
15	aa 1746-1765	NS4b	IAPAVQTNWQKLETFWAKHM	25372	DRB1*16 or DRB3*0202
16	aa 1767-1786	NS4b	NFISGIQYLAGLSTLPGNPA	43854	DRB1*1104
17	aa 1771-1790	NS4b	GIQYLAGLSTLPGNPAIASL	20417	DRB1*0404
18	aa 1806-1818	NS4b	TLLFNILGGWVAA	37286	DRB1*0101
19	aa 1907-1926	NS4b	GPGEGAVOWMNRLIAFASRG	72865	DRB1*1104,DQB1*0501
20	aa 2268-2282	NS5a	VSVPAEILRK SRRFA	14879	DRB1*1101
21	aa 2571-2590	NS5b	KGGRKPARLIVFPDLGVRVC	30946	DRB1*0404,DRB1*0407
22	aa 2841-2860	NS5b	ARMILMTHFFSVLIARDQLE	4199	DRB1*1101
23	aa 2941-2955	NS5b	CGKYLFNWAV RTKLK	6307	DRB1*1101
24	aa 2941-2960	NS5b	CGKYLFNWAVRTKLKLTPIA	6308	DRB1*1101

Table-2.4 CD8 + restricted epitopes

Protein	Amino Acid Position	Amino Acid Sequence	HLA Class I
CORE	35-44	YLLPRRGPRL	A2
CORE	131-140	ADLMGYIPLV	A2
CORE	178-187	LLALLSCLTV	A2
E1	220-227	ILHTPGCV	A2
E1	257-266	QLRRIDLLV	A2
E1	363-371	SMVGNWAKV	A2
E2	401-411	SLLAPGAKQNV	A2
NS3	1073-1081	CINGVCWTV	A2
NS3	1169-1177	LLCPAGHAV	A2
NS3	1287-1296	TGAPVTYSTY	A2
NS3	1406-1415	KLVALGINAV	A2
NS4	1789-1797	SLMAFTAAV	A2
NS4	1807-1816	LLFNILGGWV	A2
NS4B	1851-1859	ILAGYGAGV	A2
NS5	2252-2260	ILDSFDPLV	A2
NS5B	2578-2587	RLIVFPDLGV	A2
NS5B	2727-2735	GLQDCTMLV	A2
NS5	2588-2596	RVCEKMALY	A3
NS5B	2794-2802	HDGAGKRVY	A3
CORE	41-49	GPRLGFRAT	B7
CORE	111-119	DPRRRSRNL	B7
E1	235-242	ASRCWVAM	B35
CORE	88-96	NEGLGWAGW	B44
NS3	1436-1444	ATDALMTGY	A1
NS5B	2588-2596	RVCEKMALY	A3
CORE	2-9	STNPKPQK	A11
NS3	1292-1300	TYSTYGKFL	A24
CORE	28-36	GQIVGGVYL	B60
E2	541-550	NTRPPLGNWF	B57
E2	489-496	YPPKPCGI	B51
NS5B	421-429	ARMILMTHF	B27
NS3	1395-2003	HSKKKCDEL	B8
NS2	838-846	YISWCLWWL	A23
NS3	1383-1391	EVIKGGRHL	A26
NP9	81-100	YPWPLYGNEGLGWA	B44

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Chapter 3

Can this test be used as a diagnostic test for the detection of HCV infection, and for assessing response to treatment?

Introduction

Treatment with pegylated interferon alpha (PEG-IFN) plus ribavirin (RBV) is the current standard of care for patients with chronic hepatitis C (CHC). Data from pivotal trials demonstrated rates of sustained virological response (SVR) in 40–50% of genotype 1-infected and in about 70–80% of non genotype1-infected individuals(1-3).

The morbidity of Peg-IFN and Ribavirin therapy- fatigue, flu-like symptoms, depression, thrombocytopenia and neutropenia -is considerable(4). An accurate ability to predict response would allow both patients and clinicians to make more informed decisions regarding the risk-benefit of treatment, and the likelihood of success for any given individual(5).

Several variables for treatment success have been identified in trials of PEG-IFN plus RBV, including, as host factors, age >40 years, advanced degrees of liver fibrosis, male gender, increased body mass index, insulin resistance, and hepatic steatosis(6) and, most recently, IL-28 gene polymorphisms(7). Viral factors predictive of non-response response include genotype 1, lack of diversity in key genetic sequences, especially amino-acid mutations in the core and NS5A gene (6, 8-10). Patients who have a strong specific CD8+ T-cell response, as well as a sustained CD4+

response before starting treatment are more likely to present a rapid and sustained viral response(11-15).

This study was designed to assess whether these differences in T-cell response between responders and non-responders to treatment could be adequately evaluated using an interferon gamma release assay, using the Quantiferon platform.

Study design

This study was cross-sectional and non-interventional in design and conducted prospectively, enrolling patients and controls over a 12-month period at the University of Alberta Hospital. The Institutional review board approved the study (Pro 00004949). All patients were recruited from the University of Alberta Hospital, while healthy volunteers provided the control specimens.

This study was conducted in three parts-

1) Initial development of the QuantiFERON® assay for HCV peptides with comparison against a standard assay, and

2) A prospective test set comparing two T-cell specific assays (Quantiferon and ELISPOT) in predicting outcome of HCV infection after treatment.

3) A validation set, where blood samples are collected prospectively in patients infected with chronic HCV before commencement of interferon-

based anti-HCV treatment, to assess the predictive value of Quantiferon in predicting treatment outcome. This is a prospective, ongoing study, for which 13 patients have been recruited for testing before starting therapy so far.

Initial standardization set of blood tests was performed to determine appropriate peptide pools and optimal concentrations, as well as an assessment of co-stimulation to amplify specific response. The initial development and standardization was conducted using peripheral blood from 10 healthy volunteers, as well as blood from 17 HCV-infected individuals. Only those patients who had completed interferon-based treatment at least six months before the collection of the blood sample were included. Blood samples were collected for HCV-RNA quantification, and T-cell assays. Separate peptide pools were studied to assess CD4 and CD8 response. Interferon-gamma response was studied by two methods- ELISPOT assay and Quantiferon assay.

This was followed by a test set, during which patients and healthy controls were recruited to assess differences in elicited gamma-interferon response to assigned peptide pools. A third prospective group of patients continues to be enrolled before starting interferon-based therapy for HCV disease to assess the utility of the test in predicting response to anti-HCV treatment.

Methods:

Potential subjects were contacted by telephone or directly on a clinic visit by the author, or by the lead clinician or practice nurse in charge of the patients care. The study was discussed in brief, and patients who indicated interest, were invited to a special clinic, where the study was discussed in detail, consent sought and blood collected by venupuncture. A total of 20 millilitres of blood was collected by venupuncture – 8-10 ml in ACD (acid citrate dextrose- yellow cap) tube, 5 ml in a lithium-heparin (green top) tube and 5-7 ml in a serum collection tube. Whole blood was alliquoted within 2 hours of collection into the Quantiferon tubes, and blood from the ACD tube was used for PBMC extraction. This study was conducted on freshly extracted PBMCs alone.

Density gradient separation of Peripheral blood mononuclear cells (PBMC)

PBMC extraction was carried out using a Lympholyte density gradient. Lympholyte-Mammal is a density separation medium specifically designed for the isolation of viable lymphocytes and monocytes from the peripheral blood -it consists of Sodium Diatrizoate combined with Dextran. Standardization experiments performed in the lab (data not shown) found the PBMC isolation yield of Lympholyte to be comparable to the Ficoll-density gradient PBMC isolation.

Briefly, heparinized blood was mixed 1:1 volume with Phosphate-buffered saline, and 4 ml of the solution was layered slowly over 3 ml of Lympholyte. The tube was then centrifuged at 800 g for 20 minutes. The well-defined lymphocyte layer was removed, and diluted with complete RPMI before centrifugation at 800 g for 10 minutes. The cell pellet was re-suspended in a fixed volume of media, and a cell count performed. The suspension was re-centrifuged and the pellet re-suspended in a volume of media to give a final concentration of $2x10^{6}$ PBMCs/ml of media.

ELISPOT

ELISPOT assays were performed as previously described [Chapter-2, Page 52]. All asays were performed in triplicate, and the mean value of SFU/well was analyzed.

Quantiferon

The QuantiFERON test was performed according to the manufacturer's instructions (Cellestis Ltd, Melbourne, Australia). In brief, 1 ml aliquots of heparinized whole blood were collected in three QuantiFERON blood collection tubes. The tubes were shaken vigorously for 5 sec. Tubes contained either (i) pooled peptide antigens representing CD4 or CD8 specific peptides (ii) no antigens (negative control), or (iii) phytohemagglutinin (PHA; positive mitogen control). Pooled peptide antigens were added in 10µl DMSO to maintain a working concentration of

10 µg/ml of each peptide in the mix. The amount of DMSO was limited to 0.1% v/v of the total sample.

During the later part of the study, the use of IL-7 co-stimulation on the CD4 pool response of patients evaluated post-LT was also assessed. In such patients, an additional aliquot of 1ml of blood was incubated with the CD4 peptide pool of a similar peptide concentration (10 µg/ml) and 1µl of IL-7 solution to give a working concentration of 5ng/ml of IL-7.

The tubes were incubated for 16–24 hr at 37 degrees C. Following incubation; supernatants were harvested and analyzed for IFN-γ production using ELISA technology. Eight standards were included in each run and were assayed in duplicate. All samples were assayed in duplicate, and the coefficient of variation between wells was <15%. The IFN-γ results were calculated off a logarithmic curve. For the latter part of the study, the test range was expanded to 32IU/ml, and 10 standards were run, to establish a linear range of the IFN-γ response between 0.06 IU/ml and 32 IU/ml.

The optical density (OD) of each well was measured at 450nm. OD values were used to calculate results. The negative sample adjusts for background or nonspecific IFN-γ in samples. The IFN-γ result of the negative sample was subtracted from the result for the antigen tube.

Virological testing

The presence or absence of HCV antibodies was determined by third-generation ELISA, and the presence or absence of HCV RNA was quantified by commercially available qualitative polymerase chain reaction kits (COBAS® AMPLICOR HCV MONITOR Test, v2.0, Roche Diagnostics, Basel). This testing was performed by KMT Hepatech Inc. Clinical and laboratory data, including HCV genotype, was collected from patient records and interview.

Statistical methods

Clinical and assay details were maintained on a prospective database on SPSS 11.0 for Macintosh (SPSS Inc, Chicago, IL). ELISPOT results are presented as median spot-forming units (SFU) with the inter-quartile range, while Quantiferon data is presented as mean $\pm$ Standard error of mean, unless other wise specified. Statistical tests for significance include the chi-square test, Mann-Whitney 'u' test, the Kruskall-Wallis test, Pearson's coefficient of correlation as appropriate. Statistical significance was evaluated at an alpha of 0.05.

Results

Analysis – standardization set

Initial peptide testing was carried out on a cohort of 27 subjects, 7 of whom were healthy controls. Three patients had had a sustained virological response to treatment of HCV (SVR). Of the other patients, three had undergone a previous liver transplant for HCV associated liver disease, at least six months prior to the test, and had recurrent HCV disease.

Initial standardization experiments helped in the selection of the peptide pools and determination of the optimum concentration of peptides- we decided to use two sets of peptide pools, one of peptides with known CD4+ response and another of peptides with CD8+ response. Methods of amplifying specific response included increasing peptide concentrations; using pooled peptides and the use of IL-7 co-stimulation. Peptides were used at a final concentration of 10 μg/ml of solution. The use of IL-7 co-stimulation was determined to be effective at a concentration of 0.5ng/ml, consistent with published literature(16).

Analysis- test set

After standardization, the study was carried out on 50 test subjects. The demographic details are listed in Table-3.1. All treated subjects had completed anti-HCV treatment at least six months before inclusion. Ten

subjects were healthy, HCV antibody negative subjects, fourteen had a sustained virologic response (SVR) to treatment, 23 were non-responders to treatment. Three patients had no treatment before testing and were excluded from further analysis.

Demographics

Forty-seven patients were included in the test cohort. The demographic details were mentioned in Table-. The median age of the group was 53 years (Range 26-67 years); 46 (66.7%) were men. The results of the transplanted and non-transplanted cohort were considered separately. Genotype data was available for all non-transplant patients (total number – 48), and was not available for patients who had spontaneously cleared the disease (n=3) and 8 patients who had received a LT, as well as the 10 controls. 32 of 48 patients (66.7%) were Genotype 1.

Table-3.1. Table showing demographics of test population.

Demographic details	N(%)
Total number	47
- Controls	10 (14.5%)
- SVR/ SC	14 (20.3%)
- NR	23 (33.3%)
Male sex	29 (61.7%)
Median age	53 years (23-67 years)
Genotype (n=33)	
Genotype 1	20 (60.6%)
Genotype 2	5 (15.2%)
Genotype 3	8 (24.24%)
Genotype 4	1 (3.03%)

All values are expressed as number and percentage, except age which is expressed as median (range). [Genotypes not available for healthy controls or for SC].

ELISPOT and Quantiferon results for non-transplant subjects

The results of the 47 non-transplant recipients are discussed in Table -3.1. These included ten healthy controls, 14 patients who had either achieved a sustained virological response (SVR) to therapy or had evidence of spontaneous clearance of disease (SC), and non-responders to treatment. Patients in the NR group had significantly higher AST values than those in the SVR group (105.7 ± 17.9 vs. 23.5 ± 2.7; p<0..001). There were significantly more patients of HCV genotype 1 in the NR group (17 of 23 compared to 3 of 11; p=0.013). Ten patients had liver biopsy results and an additional six had fibrosis information on fibroscan suggestive of cirrhotic change. There was no significant difference in the activity levels on biopsy between SVR and NR (2 ±0.1 vs 1.66 ± 0.2; p=0.175) but patients in the NR cohort had significantly more fibrosis (3.1 +0.34 vs 1.7 ±0.25; p=0.01) . There was no significant association of ELISPOT or Quantiferon values with biopsy activity or fibrosis in non-transplanted subjects (Figures 3.1-3.4).

Table 3.2- ELISPOT and Quantiferon results compared between controls, patients with sustained viral response (SVR) and non-responders to treatment

	Healthy controls (n=10)	**SVR/SC (n=14)**	**NR (n=23)**	**'p' Value**
Mean AST values	-	23.5 +2.7	105.7 + 17.9	<0.001
Mean activity on biopsy	-	2 ±0.1	1.66± 0.2	0.175
Mean fibrosis		1.7 ±0.25	3.1 ±0.34	0.01
Genotype 1		3 (27.3%)	17 (73.1%)	0.013
ELISPOT				
CD4 pool	10.2 (3.8-37.9)	19 (5-23.5)	8 (5-16)	0.657
CD4+IL-7	10.2 (2.2-99.7)	37.5 (13-76.2)	16 (4-33)	0.34
CD8 pool	5 (2.4-14)	2(1.5-9.5)	3 (1-5)	0.271
CD8+IL-7	10 (3.25-38)	11 (5-28)	15 (1.5-26)	0.9
Mitogen response	80 (10-113)	92 (35-370)	66 (14-89)	0.113
Quantiferon				
CD4 Pool	0.16±0.06	2.8 ±0.19	0.06 + 0.02	0.039
CD8 Pool	0.02 + 0.01	1.8 + 0.07	0.04+0.02	0.013
Mitogen	19.5 + 2.8	27.4 + 2.3	18.1 + 2.8	0.07

ELISPOT values are listed as Median SFU (IQR). Quantiferon values are quantitative results of gamma interferon activity in IU/Ml. Listed as mean ± SEM. p Values computed by chi-square, Mann-Whitney or Kruskall- Wallis test as appropriate.

There is a specific response elicited by peptide pool in HCV infected patients for the CD4 but not the CD8 pools on ELISPOT

Specific ELISPOT responses were compared between healthy controls and HCV infected cases. The number of Spot-forming units in both the CD4, but not the CD8 peptide pools was increased as compared between healthy controls, SVR and NR patients, but this did not achieve statistical significance (CD4 pool 10.2 SFU (3.8-37.9) in healthy controls vs. 19 SFU (5-23.5) in the SVR group and 8 SFU (5-16) in the NR group; p=0.657: CD8 pool 5 SFU (2.4-14) in controls vs. 2 SFU (1.5-9.5) in SVR and 13 SFU (1-5) in NR ; p=0.27).

When the results of elicited response with co-stimulation (CD4 or CD8+IL-7) were compared, a higher degree of specific response was observed in the SVR group (SFU for CD4+IL-7 10.2 (2.2-99.7) in healthy controls; 37.5 (13-76.2) in SVR and 16 (4-33) in NR groups; p=0.34). The CD8 response revealed an amplification of response in all three groups (SFU CD8+IL-7 10 (3.25-38) in healthy controls; 11 (5-28) in SVR and 15 (1.5-26) in NR subjects; p=0.9). None of these differences achieved statistical significance.

Figure-3.1: ELISPOT responses for CD4 specific stimulation compared between healthy controls, patients with SVR and NR to treatment (p=0.65)

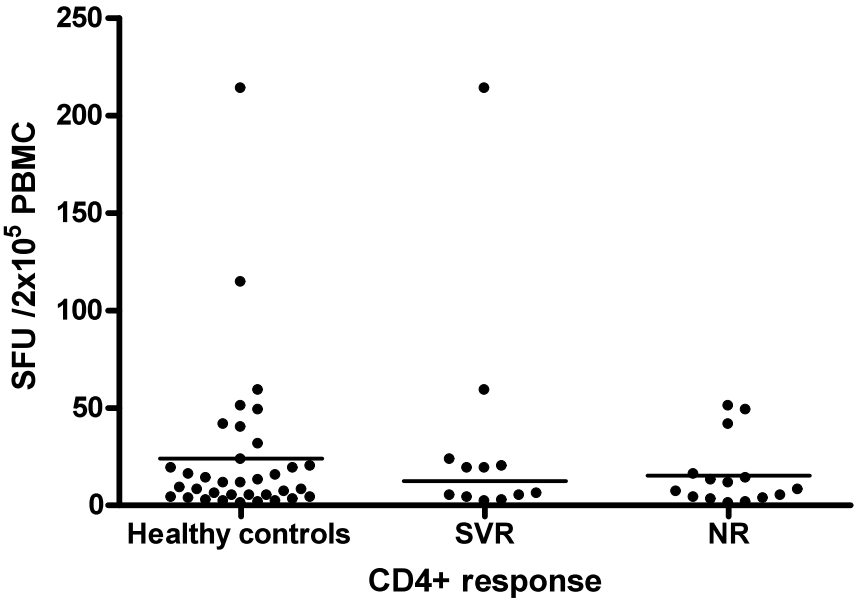


Figure 3.2: Effect of Interleukin-7 co-stimulation on ELISPOT results (CD4 pool) ; comparison between healthy controls, sustained response to treatment (SVR) and non-responders to treatment (NR) p=0.34.

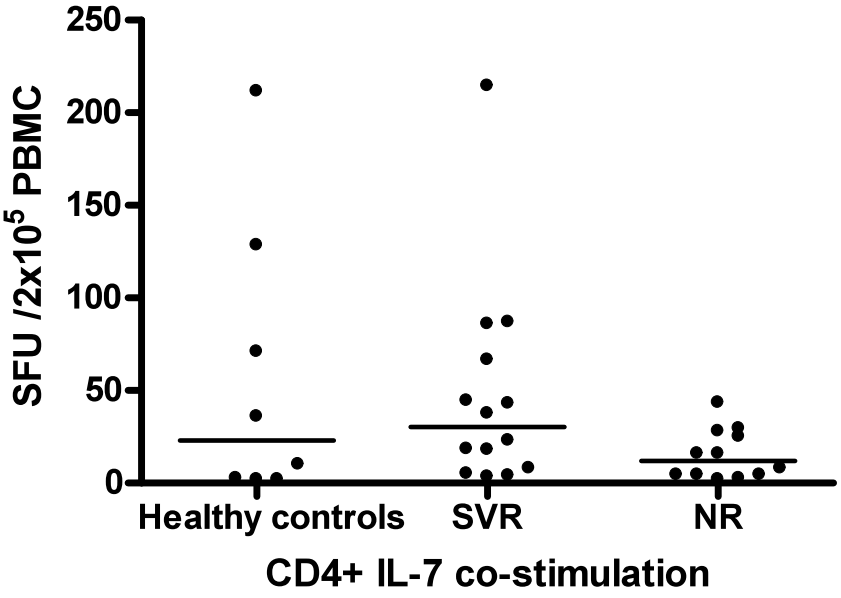


Figure -3.3: ELISPOT responses for the CD8 pool compared between healthy controls, patients with SVR and NR to treatment (p=0.271).

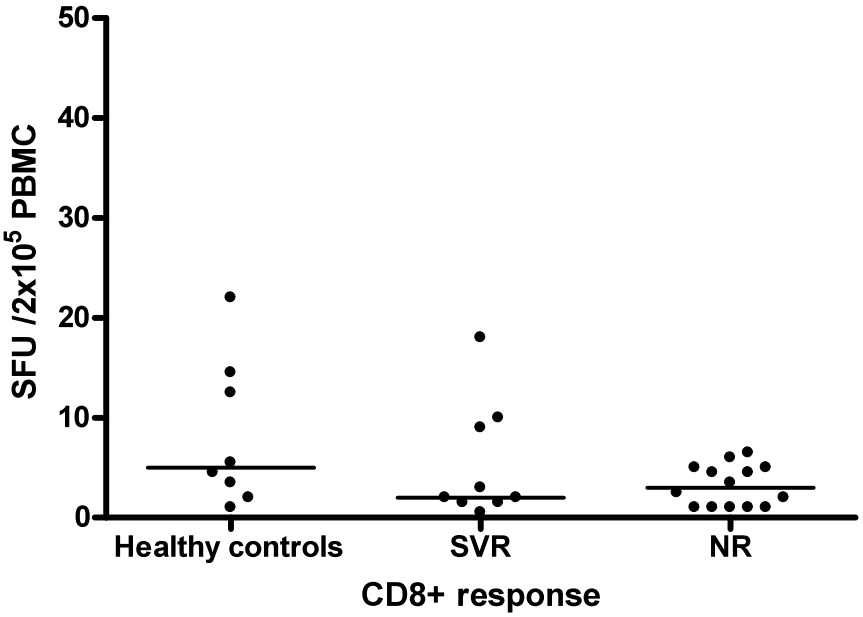
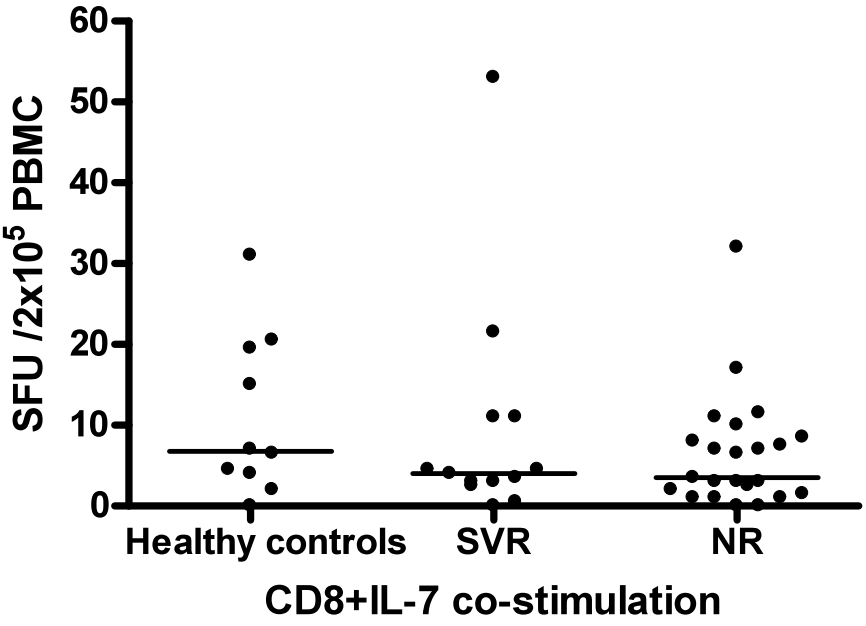


Figure- 3.4: Effect of Interleukin-7 co-stimulation on ELISPOT results (CD8 pool) ; comparison between healthy controls, sustained response to treatment (SVR) and non-responders to treatment (NR) (p=0.9).



There is a significant difference in the specific response elicited by the CD4 and CD8 pools and global response to mitogen stimulation by Quantiferon

Elicited CD4 and CD8 specific responses by Quantiferon demonstrated significant differences between controls, patients with SVR and non-responders to therapy. The average Quantiferon values for the CD4 pool were; 0.16±0.06 for the control group, 2.8 ±0.19 for the SVR group and 0.06 ± 0.02 IU/ml for the NR group respectively (p=0.039). The CD8 pool also elicited higher specific responses from patients who had SVR (0.02 ± 0.01 IU/ml for controls; 1.8 ± 0.07 IU/ml for the SVR group and 0.04±0.02 for the NR group; p=0.013) [Table-1]. The mitogen response was higher in the SVR group as compared to NR and controls, and trended towards statistical significance. (Average Interferon value 19.5 ± 2.8 IU/ml for healthy controls, 27.4 ± 2.3 IU/ml in the SVR group, 18.1 ± 2.8 in the NR group ; p=0.07) [Figures- 3.5 and 3.6].

Figure-3.5 : Interferon values elicited by CD4 and CD-specific peptide pools assessed by Quantiferon , compared between responders and non-responders .

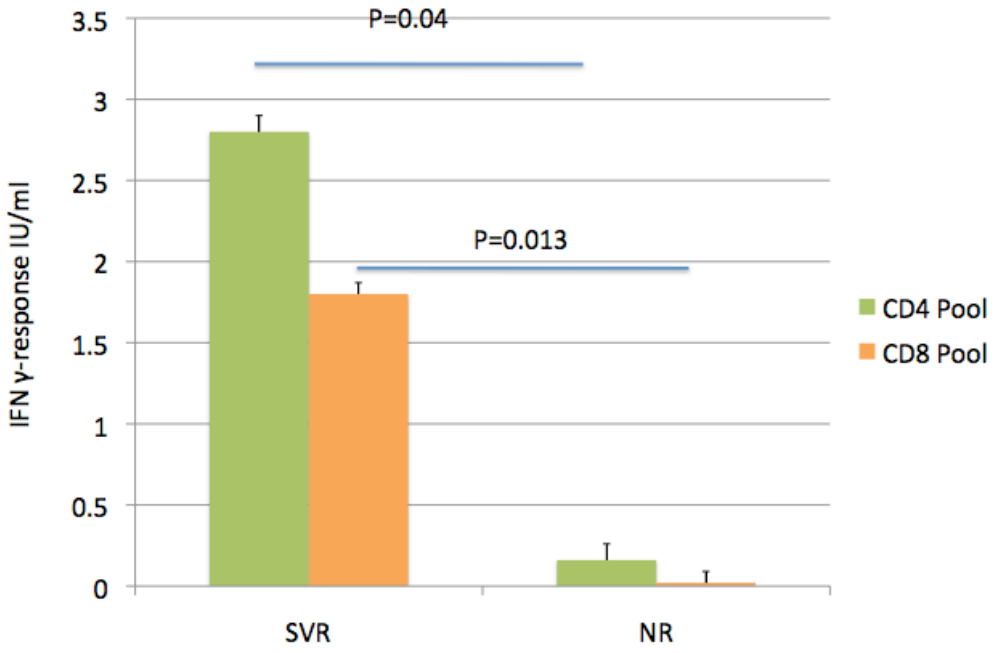
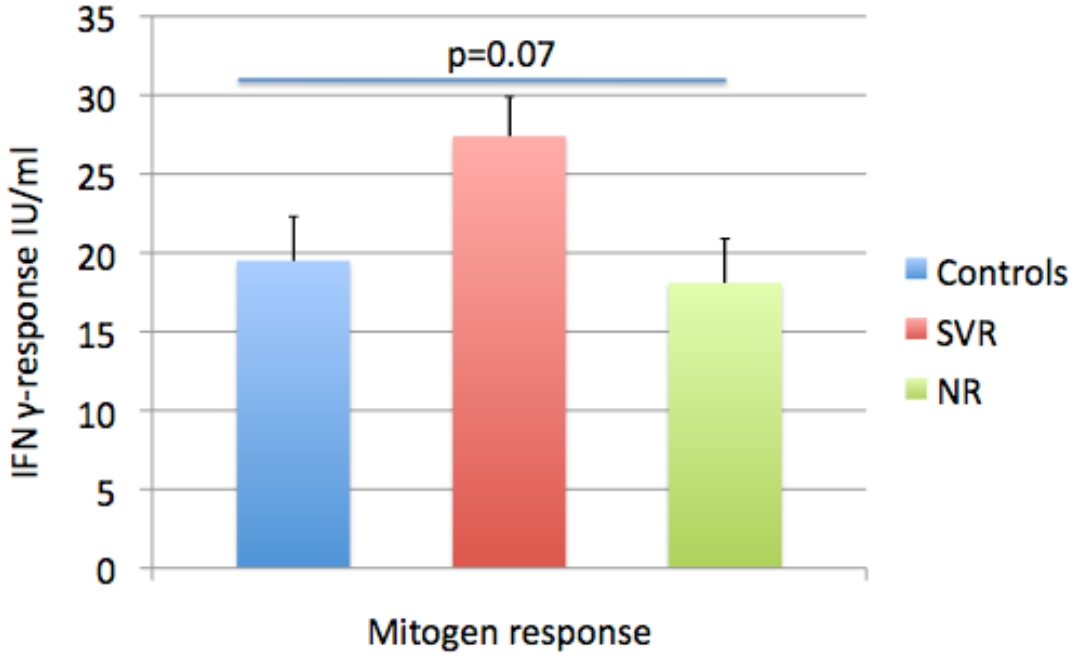


Figure-3.6: Mitogen-elicited IFN release compared between SVR, NR and controls



There was a negative correlation between the HCV viral titer and CD8 specific peptide response

CD4 and CD8 specific responses showed a negative correlation with the logarithmic HCV viral titer assessed by both ELISPOT and Quantiferon, however, these correlations did not achieve statistical significance, except for the CD8 Quantiferon response, which trended towards significance (correlation -0.35; p=0.053) [Table -3.2].

Table-3.2 Table showing correlation of peptide pool and mitogen responses elicited by Quantiferon and ELISPOT with the $\log_{10}$ HCV titer (Correlation by Spearman's test)

	Correlation (Rho value)	P value
ELISPOT		
- CD4 pool	-0.3	0.16
- CD4+IL-7	-0.18	0.4
- CD 8 Pool	-0.2	0.37
- CD8 + IL-7	-0.25	0.23
- Mitogen	-0.04	0.87
Quantiferon		
- CD4 Pool	-0.2	0.35
- CD8 Pool	-0.35	0.053
- Mitogen	-0.112	0.6

ELISPOT and Quantiferon results show poor correlation

The ELISPOT and Quantiferon values for non-transplant patients were compared by non-parametric correlation (Spearman's rho). Little direct correlation was observed between ELISPOT or Quantiferon values, except for a significant correlation between Quantiferon elicited CD8 response and an ELISPOT CD 8 response along with co-stimulation, suggesting that Spot-forming unit count may not accurately reflect interferon production.

ELISPOT	CD4	CD4+IL-7	CD8	CD8+IL-7	Mitogen
Quantiferon –CD4	0.14	0.18	0.17	0.27	0.21
Quantiferon-CD8	0.3	.07	0.1	0.44*	0.36
Quantiferon-mitogen	0.1	0.14	0.14	0.18	0.12

Table-3.3 Table showing correlation between ELISPOT and Quantiferon results in non-transplant test subjects (SVR, SC and NR). Values represent rho correlation; asterisk indicates p value<0.05.

There is a qualitative and quantitative difference in IFN production compared between SVR and NR groups.

The difference in total quantitative IFN production measured by Quantiferon and number of SFU as assessed by ELISPOT was assessed further. PBMCs from three patients with SVR and two patients with NR were isolated and suspended in 1 ml of complete RPMI media. Equal numbers of cells were incubated in Quantiferon-mitogen tubes in serial dilution ($1x10^5$/PBMCs, $0.75x10^5$PBMCs and $0.5x10^5$ PBMCs respectively). The mitogen responses in whole blood were comparable in both groups, but samples from the SVR group showed a higher and more sustained IFN production when standardized for the number of PBMCS through the serial dilution.

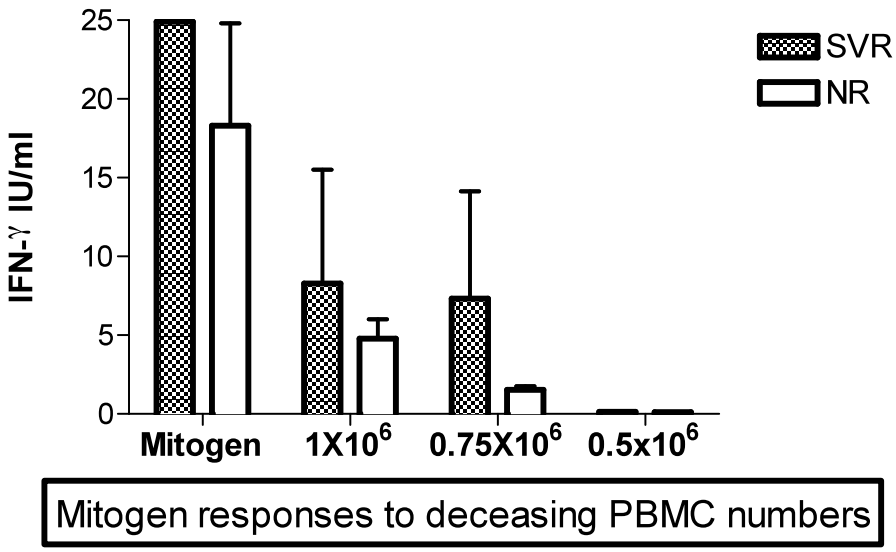


Figure-3.7 Graph showing difference in absolute mitogen response between SVR and NR patients when identical cell numbers were used. Mitogen response measures the mitogen response of 1 ml of whole blood, while numbers on the x-axis represent numbers of cells/ ml in each Quantiferon mitogen tube.

Quantiferon CD4 responses and mitogen responses could predict the difference between SVR and NR patients

A receiver-operating-characteristic curve (ROC) analysis of CD4 and mitogen response to SVR and NR states was performed [Figure 3.8]. The CD4 and mitogen response, predicted differences in the SVR and the NR states within statistical significance, while the CD8 response tended towards statistical significance (CD4+ response c=0.82 , p=0.003 (95% CI 0.67-0.97); CD8+ response c=0.69 , p=0.076 (95% CI 0.5-0.89) and mitogen c=0.75 p=0.02 (95% CI 0.57-0.94). Using a cutoff of 0.1IU/ml for the CD4 response conferred a sensitivity of 77% and specificity of 75% between diagnoses, while using the same cutoff for CD8+ response had a sensitivity of 38.6% and a specificity of 89%. A mitogen response cutoff of 23.13 had a sensitivity of 92.3% and a specificity of 69.1% in distinguishing between SVR and NR..

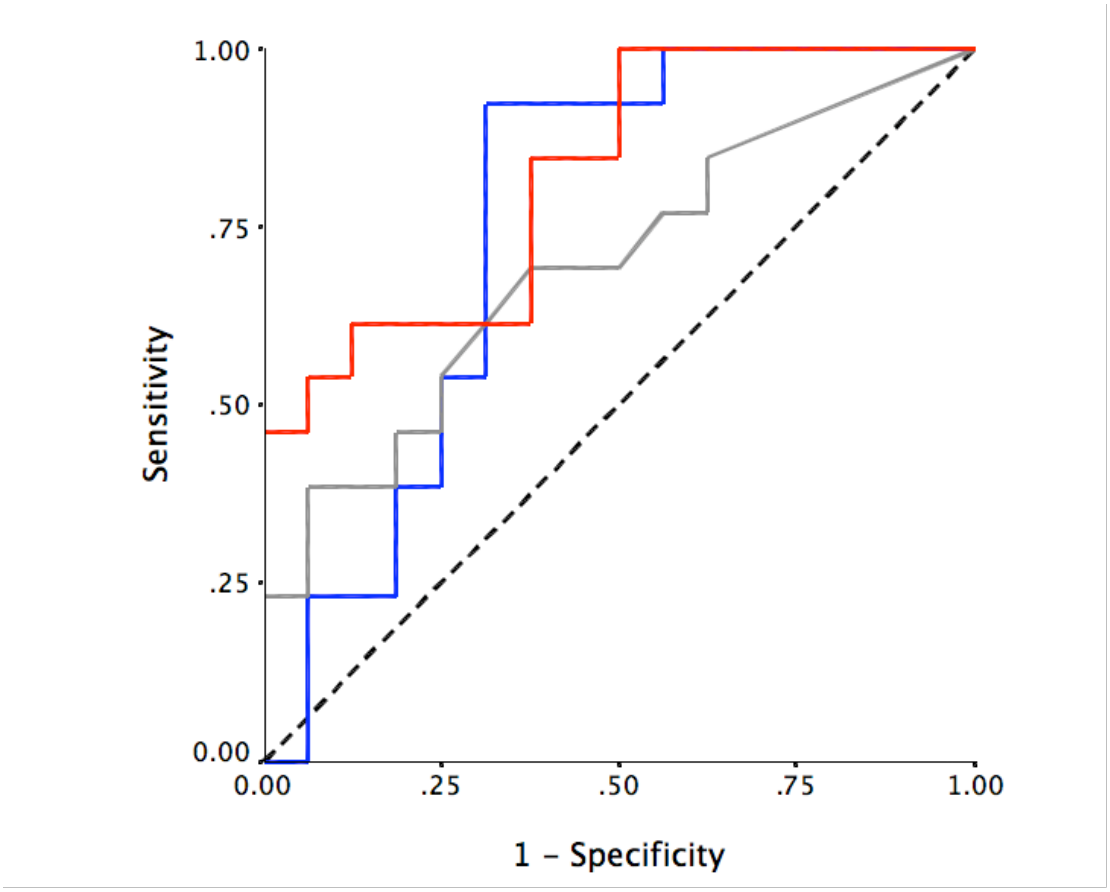


Figure- 3.8 Receiver –operating characteristic (ROC) curve showing difference between CD4+ (red line), CD8+, (grey line) and mitogen (blue line) in distinguishing between SVR and NR. (CD4+ response c=0.82, p=0.003 (95% CI 0.67-0.97); CD8+ response c=0.69 , p=0.076 (95% CI 0.5-0.89) and mitogen c=0.75 p=0.02 (95% CI 0.57-0.94).

Discussion:

This study evaluated two interferon –gamma release assays – Quantiferon and ELISPOT – in 47 subjects to assess whether the results could distinguish responders to peg- IFN and ribavirin for HCV infection from non-responders. Non-responders had significantly higher AST values, suggesting ongoing inflammation (105.7 ± 17.9 vs. 23.5 ± 2.7; $p<0.001$). Both tests did not accurately distinguish HCV patients from healthy controls. Quantiferon, but not the ELISPOT results showed a difference on CD4 or CD8 specific stimulation between SVR and NR subjects. (CD4 pool results: 2.8 ±0.19 for the SVR group and 0.06 ± 0.02 IU/ml for the NR group p=0.039), CD8 pool results 1.8 ± 0.07 IU/ml for the SVR group and 0.04±0.02 for the NR group; p=0.013). Interestingly, the mitogen response was higher in the SVR group as compared to NR and controls (19.5 ± 2.8 IU/ml for healthy controls, 27.4 ± 2.3 IU/ml in the SVR group , 18.1 ± 2.8 in the NR group ; p=0.07). There was a qualitative and a quantitative difference in the absolute mitogen response between SVR and NR. Quantiferon results from the CD4 and mitogen pool could identify the difference between SVR and NR groups with 82% and 75% accuracy respectively.

Previous studies have identified low-level HCV viremia, absence of cirrhosis, genotype other than genotype 1, elevated ALT, presence of diabetes, low cholesterol levels and black ethnicity as independent

positive predictors of an SVR(17-23). Enhancement of HCV-specific $CD4^+$ and $CD8^+$ T-cell responses is an important factor in determining the response to the IFN-α/ribavirin therapy and the outcome of the HCV infection, and high HCV-specific type 1 $CD4^+$ and $CD8^+$ T-cell responses before starting therapy and after completion of treatment have been shown to be associated with SVR, while chronic HCV infection is characterized by attenuated CD4+ and CD8+ HCV-specific T-cell responses (11, 12, 14, 15, 24-28). Biological reasons for the attenuation of these responses may include failure of antigen presentation, T-cell exhaustion and dysfunction, viral mutations, intrahepatic modulation or increased regulatory T-cell function(11, 12, 14, 15, 24-27). Studies of spontaneously recovered HCV patients have indicated a greater breadth of responses against multiple regions of the virus, compared with patients who could not clear the virus. These responses are often present before treatment and persist for several years after completion of treatment and attaining SVR status.

Interestingly, both ELISPOT and Quantiferon techniques showed T cell responses to HCV peptides even among healthy controls. Host responses to an infectious agent can be influenced by cross-reactive memory cells, or induced by past exposure to heterologous viruses, and cross reactivity between HCV and influenza-A specific cytotoxic T cells has been described. HCV NS3 and the IV NA peptide display a high degree of

sequence homology, and are recognized by cytotoxic T lymphocytes with similar affinity(29). Additionally, strong HCV specific T-cell responses have been described in antibody negative patients who have had contact with the virus, e.g., in spouses of HCV infected individuals.

ELISPOT assays using overlapping peptide libraries show good sensitivity but poor specificity in assessing HCV specific T-cell response(14). Additionally, the requirement for fresh blood collection, extraction of PBMCs and a relatively time-sensitive assay limit the potential for translational or commercial use.

Optimization of ELISPOT protocol has been employed to maximize signal-to-noise ratio by adjusting different co-stimulatory protocols, in order to achieve preferential amplification of the epitope-specific T cell responses. The use of Il-2, CD28 co-stimulation as well as IL-7 has been described in literature to amplify interferon-gamma response (16, 30-37). Martinuzzi et al described the use of IL-7 as a specific co-stimulator as it is thought to be an important co-stimulation factor for memory T cells (16, 38). Low dose (0.5 ng/ml) IL-7 induces a highly reproducible increase in specific signal, while increasing the basal levels of reactivity only marginally The median increase in net signal was of 50%, which was confirmed by our findings.

The Quantiferon platform using in-tube blood collection simplifies testing logistics, enabling remote location blood collection. Quantiferon assays

require a single patient visit to draw a blood sample, and results can be available within 24 hours.

Although both ELISPOT and Quantiferon levels were increased in SVR subjects, little direct correlation was observed between ELISPOT or Quantiferon values, except for a significant correlation between Quantiferon CD8 and the ELISPOT CD8 result with co-stimulation. The difference in measuring differences in the effector response of a T-cell assay may be related to the difficulty in quantifying spot-forming units- the number of spots offer at best a subjective assessment of degree of response, and not an absolute count. Similar differences have been documented in HIV-specific CD8+ responses, which show robust IFN-γ production in cytokine-based assay systems, whereas they do not show robust proliferative capacity.

Mitogen stimulation using identical numbers of PBMCs in serial dilution between SVR and NR subjects was undertaken to study the qualitative and quantitative difference of the interferon response. Absolute mitogen responses of subjects with SVR showed a sustained response,which persisted longer even after serial dilution, as compared to subjects with NR.

Interestingly, the absolute mitogen responses among subjects with SVR were higher than those with NR or healthy controls. HCV infection may cause subtle immunosuppression that goes beyond the attenuation of

HCV-specific responses. Having a higher global immune response may thus be beneficial. It has been suggested that treatment with Peg-IFN and ribavirin may have an *in vivo* immune modulator role (28) The mitogen response for NR subjects was lower than those who had not been exposed to the virus, which may suggest a more global immunosuppression as a consequence of the disease in non-responders. Assessment of the absolute T cell immune response may provide an assessment of global immunity, which may have consequences for predicting outcomes after anti-HCV treatment.

The CD4 and mitogen responses assessed by Quantiferon accounted for 82% and 75% of the difference between the SVR and the NR groups respectively. When a cut-off of IFN-gamma of greater than 0.1IU/ml for the CD4+ response was used, it conferred a sensitivity of 77% and a specificity of 75% for predicting SVR. CD8+ peptide responses did not have the same accuracy. While a strong HCV-specific CD4+ T-cell response is associated with viral clearance after therapy, the dynamics of CD8+ responses is not clear. Different studies have come to conflicting conclusions with some finding an increase in responses after treatment (39), while others noting a decrease or no change (40, 41).

In summary, patients with SVR to anti-HCV treatment demonstrated a strong response to CD4+ specific peptides and a high mitogen response, as measured by Quantiferon. No significant differences were noticed between Quantiferon results among healthy controls and HCV infected

individuals. These results support the prospective validation of Quantiferon-HCV as an inexpensive, reliable test, which may identify those patients likely to respond to treatment.

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Chapter 4

Will Quantiferon be a useful clinical tool for predicting timing and severity of HCV recurrence in HCV-infected patients who have undergone liver transplantation?

Introduction

Graft and patient survival after liver transplantation have improved for all indications except HCV-related cirrhosis, where they continue to be 10-15% lower when compared with non-HCV controls(1, 2). HCV re-infection usually occurs immediately after liver transplantation with a rapid increase in HCV-RNA peaking at 1–3 months; acute lobular hepatitis developing in 60–80% of patients at a median of 4–6 months and cirrhosis in 20% by 5 years (1, 3, 4). The presence of allograft rejection (AR) is strongly associated with recurrent HCV-related liver disease, particularly because of the use of higher doses of immunosuppression to treat episodes of AR. In HCV-positive LT recipients, there is a 3-fold increased risk of death if patients experience an episode of rejection, and a 5-fold increased risk if they have experienced steroid-refractory AR (5-7).

Differentiation of AR episodes from recurrent HCV infection can be difficult based on biochemical results or histology alone. Histopathological differentiation of recurrent HCV from ACR after LT has low inter-observer and intra-observer agreement rates (8, 9).

The lack of an optimal CD4+ T helper 1 response (characterized by the secretion of interferon gamma) is associated with fibrosis/cirrhosis development in patients with chronic HCV infection. Recent tests have focused on assessing the global immune response to distinguish between

recurrent HCV disease and AR. One such test is the Cylex ImmuKnow assay, which measures the ability of CD4+ T cells to respond to mitogenic stimulation. Low Immunknow values correlate with recurrent disease and fibrosis progression in patients who have undergone LT for HCV(10-12).

The global immune response can also be quantified by measuring interferon –gamma release on mitogen stimulation of T cells. The QuantiFERON® kit measures interferon-gamma release on stimulation of whole blood, and can easily be quantified by an ELISA. Quantiferon tests for the detection of latent TB infection and CMV disease post-transplantation are in widespread clinical use(13-18).

We hypothesize that interferon-gamma release after mitogen stimulation using the Quantiferon assay may serve as a good surrogate marker for global immune response in patients with recurrent HCV infection post-LT, and therefore help in distinguishing recurrent HCV from AR.

Methods:

Patients who had had a single-organ liver transplant (LT) for Hepatitis C associated liver disease, and had documented HCV recurrence on liver biopsy were included in this study. Patients, who had other viral co-infection such as Hepatitis B infection, were excluded. Patients were included at least six months after LT- if they had received anti-HCV

treatment after LT, then inclusion was deferred till six months after completion of interferon-based treatment.

The T-cell assays were correlated with other available clinical and biochemical data, including allograft biopsy if performed within six months of the blood draw. The necro-inflammatory state of HCV recurrence was reported according to the METAVIR classification. The necro-inflammatory histology of the included study subjects was consistent with HCV recurrence but none had cholestatic hepatitis. Scores for different stages of fibrosis were reported as follows: 0, no fibrosis; 1, minimal fibrosis without any septa; 2, portal fibrosis with rare septa; 3, numerous septa with bridging fibrosis without cirrhosis and 4, cirrhosis.

Potential subjects were contacted by telephone or directly on a clinic visit by the author, or by the lead clinician or practice nurse in charge of the patients care. The study was discussed in brief, and patients who indicated interest, were invited to a special clinic, where the study was discussed in detail, consent sought and blood collected by venupuncture. A total of 20 milliliters of blood was collected by venupuncture – 8-10 ml in ACD (Acid citrate dextrose- yellow cap) tube, 5 ml in a lithium-heparin (green top) tube and 5-7 ml in a serum collection tube. While blood was alliquoted within 2 hours of collection into the Quantiferon tubes, and blood

from the ACD tube was used for PBMC extraction. This study was conducted on freshly extracted PBMCs alone.

Density gradient separation of Peripheral blood mononuclear cells (PBMC)

PBMC extraction was carried out using a Lympholyte density gradient as described in Chapter-3 [Page 73].

ELISPOT

ELISPOT assays were performed according as previously described Chapter-2 [Page-52]. Assays were performed in triplicate, and results were analyzed as the mean of values.

Quantiferon

The Quantiferon test was performed according to the manufacturer's instructions (Cellestis Ltd, Melbourne, Australia), as described in Chapter-3 [Page 74-75].

During the later part of the study, the use of IL-7 co-stimulation on the CD4 pool response of patients evaluated post-LT was also assessed. In such patients, an additional aliquot of 1ml of blood was incubated with the CD4 peptide pool of a similar peptide concentration (10 µg/ml) and 1 µl of IL-7 solution to give a working concentration of 5ng/ml of IL-7.

The tubes were incubated for 16–24 hr at 37 degrees C. Following incubation; supernatants were harvested and analyzed for IFN-gamma production using ELISA technology. Eight standards were included in each run and were assayed in duplicate. All samples were assayed in duplicate, and the coefficient of variation between wells was <15%. The IFN-gamma results were calculated off a logarithmic curve. For the latter part of the study, the test range was expanded to 32IU/ml, and 10 standards were run.

The optical density (OD) of each well was measured at 450nm. OD values were used to calculate results. The negative sample adjusts for background or nonspecific IFN-gamma in samples. The IFN-gamma result of the negative sample was subtracted from the result for the antigen and mitogen tube.

Virological testing

The presence or absence of HCV antibodies was determined by third-generation ELISA, and the presence or absence of HCV RNA was quantified by commercially available qualitative polymerase chain reaction kits (COBAS® AMPLICOR HCV MONITOR Test, v2.0, Roche Diagnostics, Basel). Quantitative testing was performed by KMT Hepatech, Inc. Edmonton, AB. Clinical and laboratory data, including HCV genotype, was collected from patient records and interview.

Statistical methods

Clinical and assay details were maintained on a prospective database on SPSS 11.0 for Mac (SPSS Inc, Chicago, IL). ELISPOT results are presented as median spot-forming units (SFU) with the inter-quartile range, while Quantiferon data is presented as mean ± Standard error of mean, unless other wise specified. Statistical tests for significance include the chi-square test, Mann-Whitney 'u' test, the Kruskall-Wallis test, and Pearson's coefficient of correlation as appropriate. Statistical significance was evaluated at an alpha of 0.05.

Quantiferon and ELISPOT results after liver transplantation:

Quantiferon and ELISPOT determination of T-cell response was evaluated in 22 patients after single-organ liver transplantation for HCV related disease. All patients had evidence of recurrent HCV, with positive viral titers and were evaluated at least six months after the LT. Twelve of the 14 patients for whom genotype data was available had Genotype-1 infection. Some patients had undergone interferon-based treatment for HCV disease – patients were included at least six months after completion of treatment.

The median age of patients was 48.1 years; 17 (77.3%) were male. Four patients were on Sirolimus (SRL) based immunosuppression (IS), while

seventeen were receiving CNI-based treatment. One patient was maintained on mycophenolate –based IS because of CNI-associated renal dysfunction. One patient, assessed 16 years after LT had developed renal failure and was on dialysis waiting for a kidney transplant. Two patients had a negative mitogen result on Quantiferon assay – this was interpreted as a failed test, and these results were excluded from analysis.

The ELISPOT results for post-LT patients are shown in Figure-4.1. Median SFUs for each group were as follows CD4 pool: 56.9± 40.4; CD4+ IL-7: 77.9± 40.9; CD8: 15.8± 12.5; CD8+IL-7: 30.1± 19.2 and pooled mitogen 77.5± 33.6.

An assessment of Quantiferon showed IFN gamma values, which were significantly lower than non-transplanted patients – (mean values of CD4+ set; 0.08± 0.04, CD8+ pool 0,07± 0.03). However, the elicited mitogen values were comparable to non-transplanted controls (Mean value of mitogen response 20.8± 13.7 IU/ml).

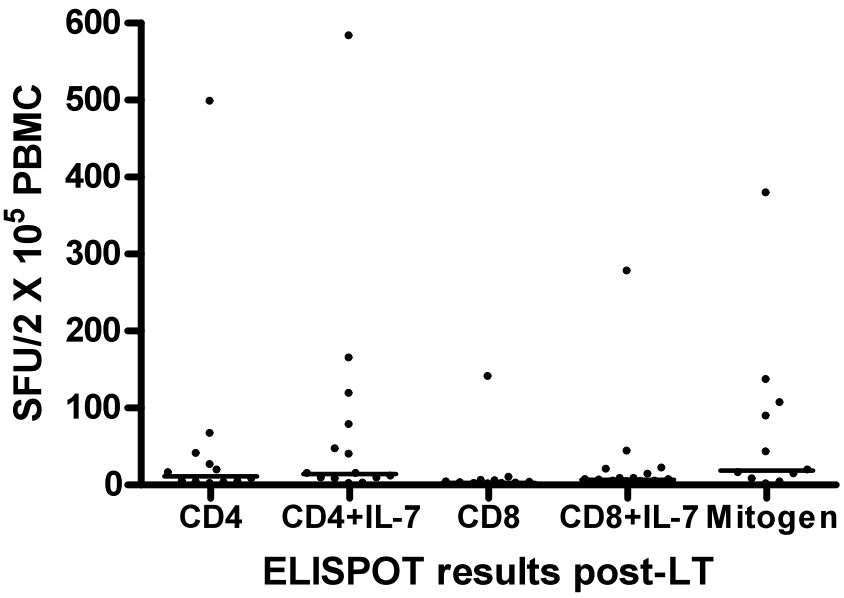


Figure- 4.1 Scatter plot showing distribution of CD4+, CD8+ specific peptides with and without co-stimulation, as well as mitogen response for all patients after LT.

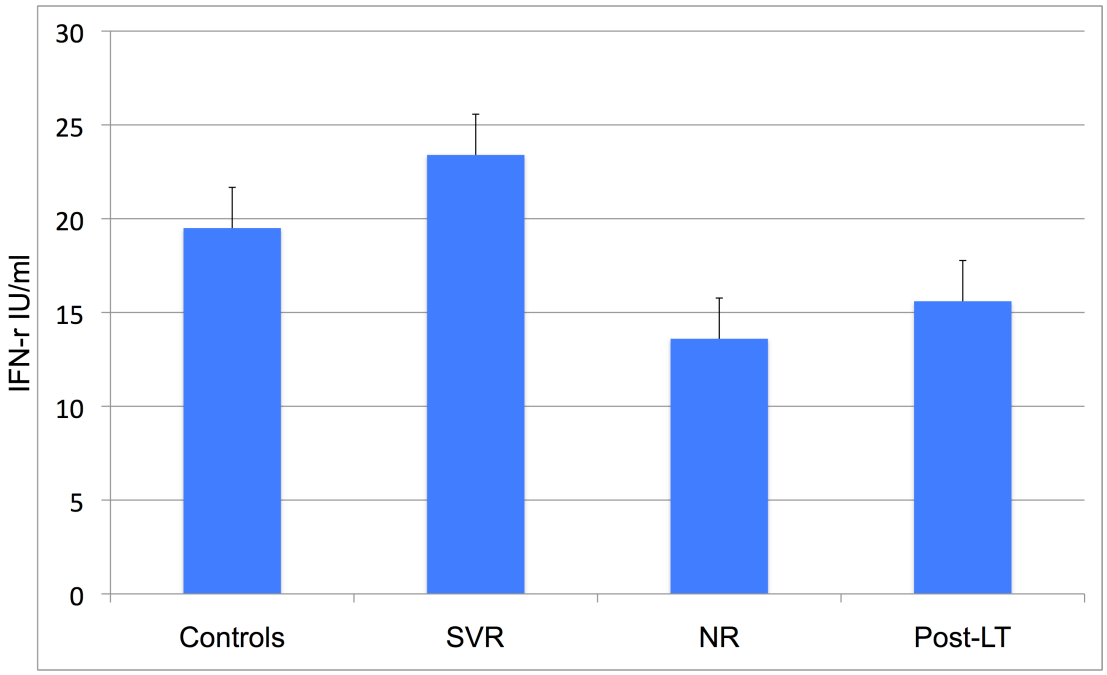
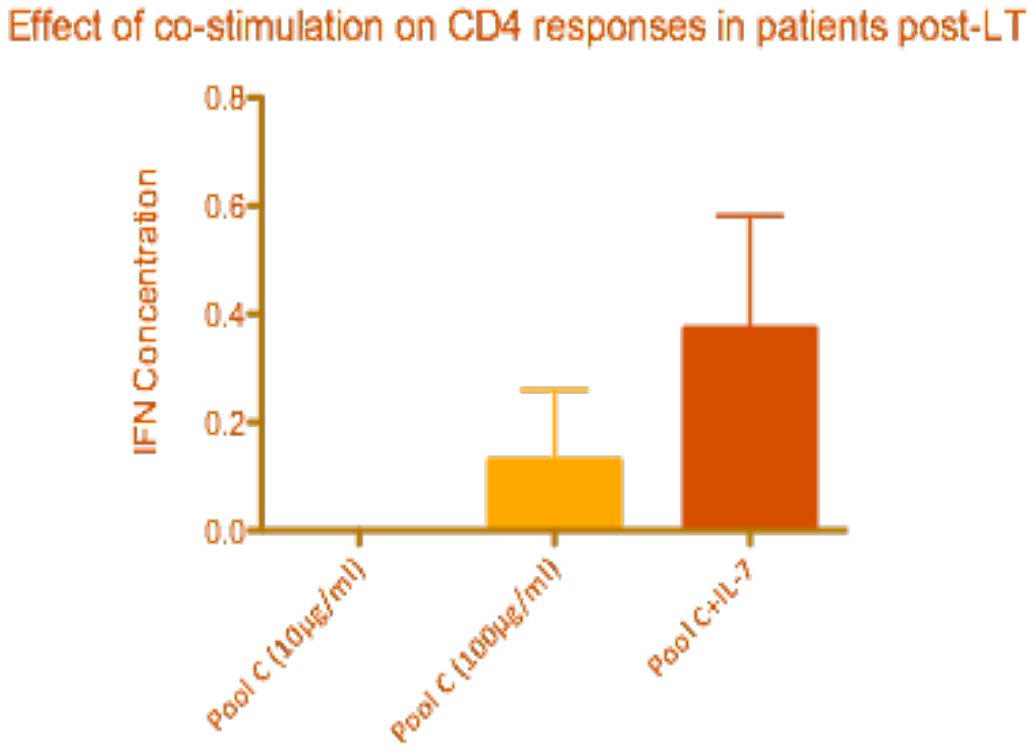


Figure-4.2 Bar graph showing difference in mitogen response for Quantiferon comparing healthy controls, patients with SVR, NR to treatment as well as those after LT (p value = 0.7).

Interleukin-7 co-stimulation significantly increased the CD4 specific response assessed by Quantiferon

Means of amplifying specific CD4 response post-LT were assessed on 5 patients. Patient samples were treated with standard peptide pool (10 μg/ml), increased peptide concentration (100μg/ml) or with 0.5ng/ml of interleukin-7 along with the standard peptide concentration. Peptides along with IL-7 increased the CD4 response in a specific fashion, more than an increase in the peptide concentration [Figure-4.3].

Figure- 4.3 Effect of co-stimulation on CD4 responses in patients post-LT



There is no correlation between viral titers and interferon response in the post-LT population

No correlation could be demonstrated between viral titers post-LT and interferon response, either elicited by ELISPOT or by Quantiferon. While CD8 responses measured by ELIPSOT and Quantiferon response from the CD8 pool showed significant correlation (Pearson's correlation 0.884; $p<0.001$), no similar correlation could be observed between CD4 responses measured by Quantiferon or by ELISPOT.

One patient (MP) had early HCV recurrence (within 3 months post-LT) , and had two Quantiferon assessments performed, one at 4 months post-LT and the second 11 months post-LT. A drop in viral titer correlated with reconstitution of the global immune response (Figure-4.4), although the specific CD4 and CD8 responses remained undetectable on serial follow-up.

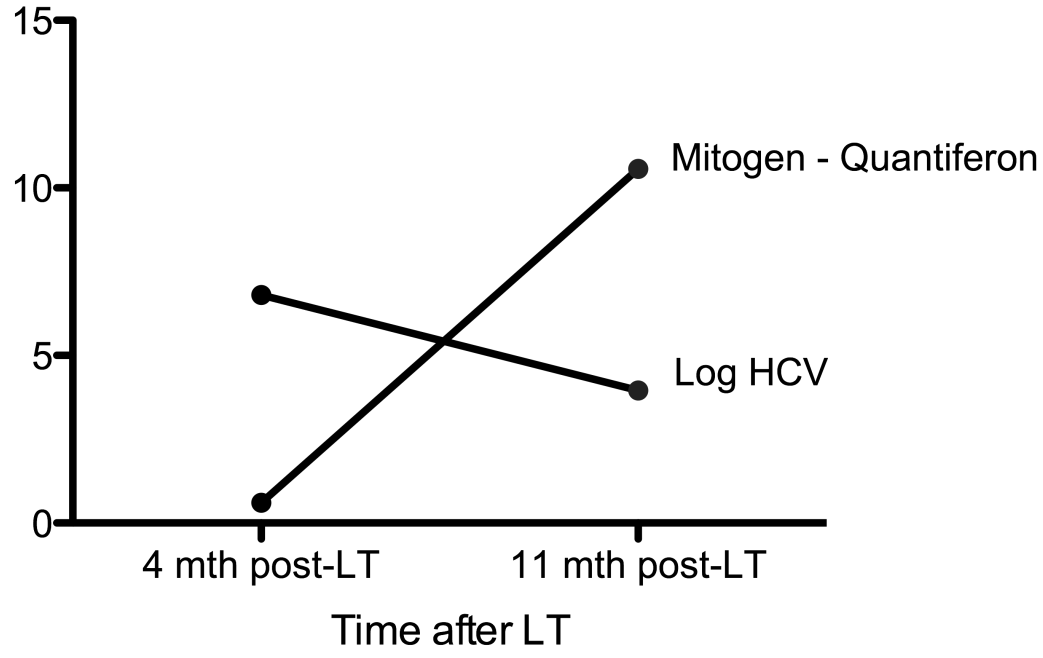


Figure-4.4: Figure showing negative correlation between reconstitution of global immune response, measured by Quantiferon-Mitogen and the log viral titer.

Lower Quantiferon values were significantly associated with advancing allograft fibrosis

Sixteen patients had results from allograft biopsies that were performed within three months of the test. One of these patients had a negative mitogen test and was therefore exclude from analysis. Quantiferon and ELISPOT tests were compared to the reported METAVIR score of the allograft, with an activity or a fibrosis level of 2 being taken as the cutoff. While CD4 responses were higher in the group that had a higher activity, no significant differences could be observed between median spot counts for CD8+ response by ELISPOT (Median Spot forming units for activity <2 and >=2; CD4+ pool 49.1 (5-327.4) vs. 5 (1-7.75) ; p=0.024 :CD8+ pool 7 (3.7-45) vs 2 (1-5) ; p=0.34.

The mitogen responses were comparable in the high and low-activity groups compared by ELISPOT. (Median counts 51 (13-280) vs 23.7 (10-92.1; p=0.8).

The mean IFN- gamma release measured by Quantiferon was non-significantly higher in the high activity group (CD4+ pool 0.06 $\pm$ 0.02 vs. 0.12 $\pm$ 0.1; p=0. 6, CD 8+ pool 0.06 $\pm$ 0.04 vs 0.1$\pm$ 0.06; p=1). The mitogen responses measured by Quantiferon were comparable in both groups (Mean IFN release 22.8 $\pm$3.1 vs 23.7 $\pm$ 6.4; p=0.9) [Table-4.1].

When Quantiferon and ELISPOT results were compared between the high and low fibrosis group (using a cutoff of a fibrosis score of 2 and above), marked differences were observed [Table-4.2]. There were more spot-forming units identified in the CD4+ group by ELISPOT (Median SFU 7.8 (6-81) in fibrosis<2 group vs. 2(0-41) in fibrosis=>2group; p=0.667); however differences could not be observed in the CD8 pool (SFU 3.5 (1.7-6) vs. 2 (1-5) p=0.48). Assessment of IFN release by Quantiferon showed a significant difference in CD4+ response based on allograft fibrosis (mean IFN value for CD4+ pool; 0.12 $\pm$ 0.1 for fibrosis< 2 vs. 0.02 $\pm$ 0.01 for fibrosis=>2; p=0.05). The CD8+ pool did not show a significant difference in elicited IFN response (0.08$\pm$ 0.05 for fibrosis <2 vs. 0.08$\pm$ 0.07 for fibrosis=>2; p=0.8). The difference in mitogen response between the groups was statistically significant. (Mean mitogen IFN value 27.6 $\pm$3.6 vs. 14.4 $\pm$ 2.6; p=0.019). (Figure 4.6 and 4.7)

	Activity <2(n=6)	**Activity≥ 2 (n=9)**	**'p' value**
Mean AST values	78.3 ± 39.8	83.1±15.7	0.38
Lymphocyte count	0.9 ± 0.17	1.1 ± 0.13	0.26
Genotype 1	6 (100%)	6 (66.7%)	
Sirolimus IS	1 (11.1%)	2 (22.2%)	
ELISPOT			
CD4	49.1 (5-327.4)	5 (1-7.75)	0.024
CD4+IL-7	150 (16-513)	9 (7-28)	0.012
CD8	7 (3.7-45)	2 (1-5)	0.34
CD8+IL-7	30 (17-108)	8 (6-13)	0.164
Mitogen	51 (13-280)	23.7 (10-92.1)	0.8
Quantiferon			
CD4	0.12 ± 0.1	0.06 ± 0.03	0.6
CD8	0.1± 0.06	0.06 ± 0.04	1
Mitogen	22.8 ±3.1	23.7 ± 6.4	0.9

Table-4.1: Table showing differences in ELISPOT and Quantiferon results between patients with low (<2) and high (≥2) activity on allograft biopsy. ELISPOT results are median SFU (IQR) while Quantiferon results are measured as mean IU/ml ± SEM.

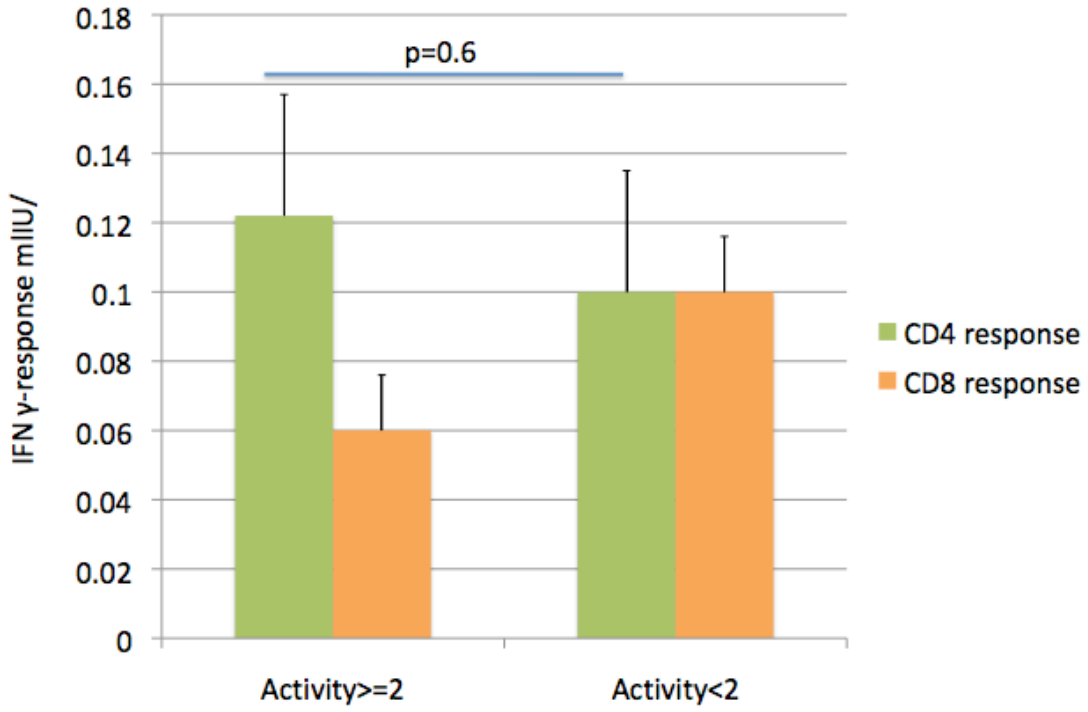


Figure-4.5 Figure showing differences in CD4 and CD8 pool elicited Quantiferon response compared by differences in allograft biopsy activity and fibrosis

	Fibrosis<2(n=10)	**Fibrosis≥ 2 (n=5)**	**'p' value**
Mean AST values	83.1 ± 25.2	77.2± 20.1	0.95
Lymphocyte count	1.1 ± 0.12	0.9 ± 0.2	0.26
Genotype 1	8(88.9%)	4(80%)	
Sirolimus IS	2 (20%)	1 (20%)	
ELISPOT			
CD4	7.8 (6-81)	2 (0-41)	0.667
CD4+IL-7	27 (7.8-200)	14 (7-98)	0.648
CD8	3.5 (1.7-6)	2 (1-5)	0.486
CD8+IL-7	13 (17.3-26)	9 (4.7-30)	0.788
Mitogen	23.7 (10-92.1)	4.7 (2-10)*	0.33
Quantiferon			
CD4	0.13 ± 0.08	0.02 ± 0.01	0.05
CD8	0.08± 0.05	0.08 ± 0.07	0.8
Mitogen	27.6 ±3.6	14.4 ± 2.6	0.019

Table-4.2 Table showing differences in ELISPOT and Quantiferon results between patients with low (<2) and high (≥2) fibrosis on allograft biopsy. ELISPOT results are median SFU (IQR) while Quantiferon results are measured as mean IU/ml ± SEM. *Indicates that two patients in the advanced fibrosis group had little to no mitogen response on ELISPOT.

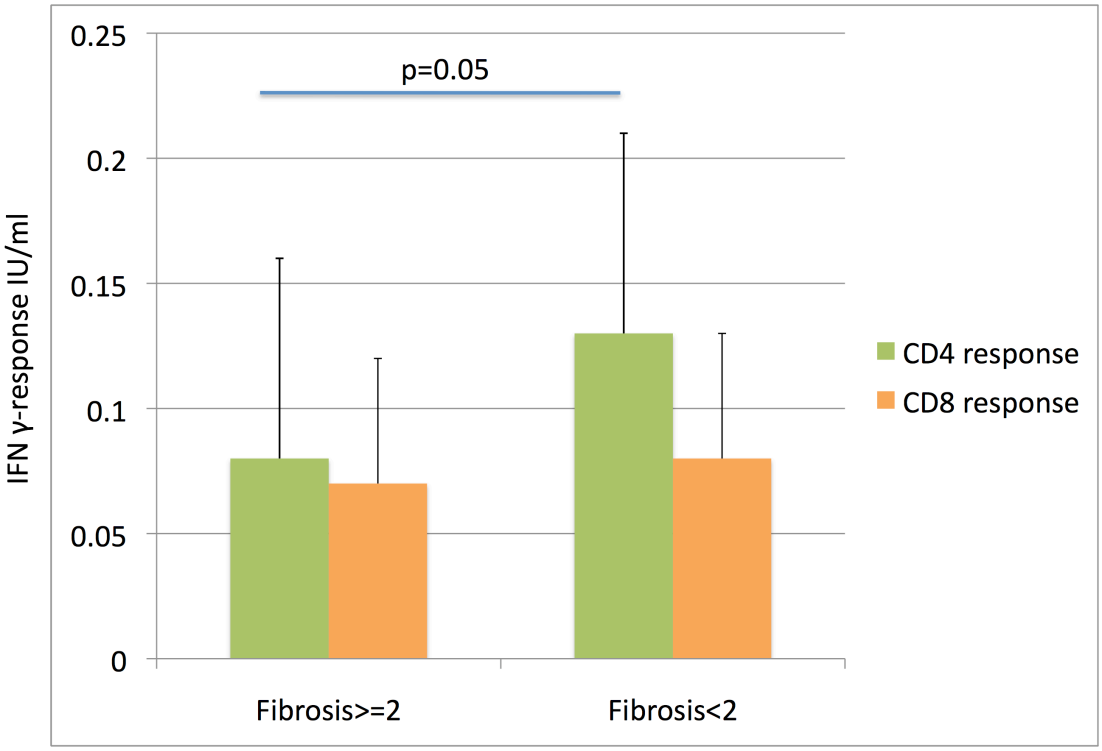


Figure-4.6 showing differences in Quantiferon response compared by differences in allograft biopsy fibrosis

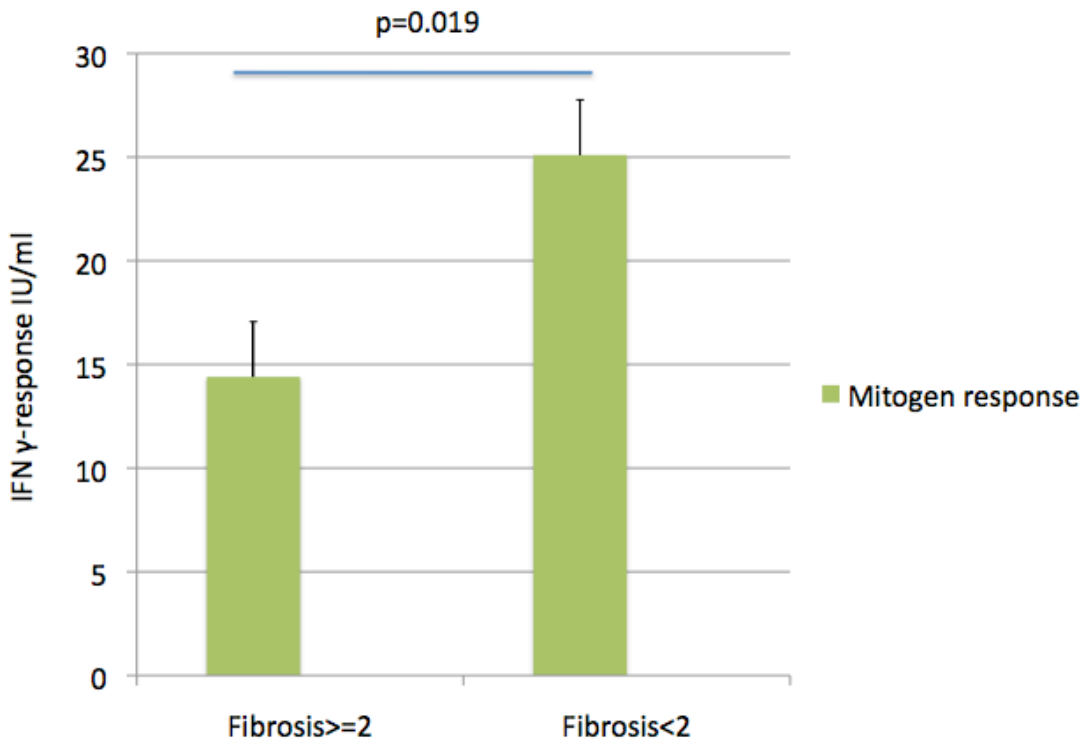


Figure-4.7 showing differences in Quantiferon mitogen response compared by differences in allograft biopsy fibrosis

Correction for lymphocyte count did not affect test correlation with either activity or fibrosis

To account for differences in the lymphocyte count among patients who had received a liver transplant, Quantiferon values were corrected to the lymphocyte count. The degree of correlation between activity and fibrosis with the corrected and uncorrected CD4+, CD8+ and mitogen elicited responses is shown in Table-3.

Table-4.3. Correlation of Quantiferon –IFN response between CD4, CD8 and mitogen groups with activity and fibrosis, with and without correction for lymphocyte count.

Test group	Activity>=2 (n=9)	Fibrosis>=2 (n=5)
CD4 pool	-0.07	-0.687*
CD8 pool	0.073	-0.06
Mitogen	0.15	-0.48^{+}
Corrected CD4	-0.1	-0.7*
Corrected CD8	0.08	-0.03
Corrected mitogen	-0.07	-0.07

* represents p value <0.05; $^{+}$ represents p value tending to significant (p=0.058) . Correlation values represent Spearman's rho coefficients

Discussion

When graft dysfunction develops, it is critical to determine the cause to guide appropriate management, as therapeutic strategies directed at ACR and recurrent HCV are diametrically opposite. A liver biopsy has been the only accepted method and is considered the gold standard to distinguish recurrent HCV from acute cellular rejection (ACR). However, it is sometimes quite difficult to distinguish these two conditions because of overlapping histopathologic features(9, 19).

Quantiferon and ELISPOT results for specific peptide stimulation were significantly lower in post-LT subjects compared to non-transplanted subjects, despite comparable mitogen results. Interestingly, the ELISPOT SFUs were marginally decreased as compared to the absolute IFN as measured by Quantiferon, suggesting that attenuation of this response post-OLT by the pharmacological suppression of cell-mediated immunity (CMI) was related to decrease in interferon production rather than the number of T cells with HCV specific immunity. Attenuation of donor specific signal on ELISPOT has been associated with recurrent HCV post –LT (20-24).

This was confirmed by the increased CD4+ specific signal observed with the use of IL-7 co stimulation. No such signal amplification of specific response was observed on increasing the available peptide concentration.

HCV viral titers did not correlate with the measured CD4+ or mitogen Quantiferon response in this study. HCV RNA levels begin to rise (doubling-time = 2.0 days) as soon as 15 hours after the anhepatic phase, and 96% of the doubling occurs in the liver(4, 25). High viral titers are associated with worse post-LT outcomes- however, viral titers do not stabilize till one year after LT, may not reflect the necroinflammatory state of allograft infection(9, 26-32).

Patients with relatively advanced fibrosis (>2 Metavir score) had significantly lower specific and mitogen responses as compared to those with less advanced disease. The correlation between poor cellular immunity and aggressive recurrent HCV post-LT has been previously described(12). An immune function assay provides an independent measure of the strength or weakness of the immune system by providing a standardized and objective measure of a recipient's immune response.

Stimulated T-lymphocytes first undergo an influx of ions and increased adenosine triphosphate (ATP) synthesis, which is followed by surface receptor clustering, RNA synthesis, cytokine production and release, and DNA replication. In 2002, the US Food and Drug Administration approved a global immune cell function assay (ImmuKnow)- this assay measures the concentration of ATP (ng/mL) released from CD4+ cells. However, the

Immuknow requires substantial laboratory support, in the form of lymphocyte isolation, CD4+ cell separation and measurement of ATP production (11).

Casanovas et al studied proliferative T-cell response and cytokine production (gamma-interferon and IL-10) after HCV specific and phytohemagglutinin (PHA) stimulation in cultured peripheral blood mononuclear cells (PBMCs) and compared results between patients with SVR, SC, NR and post-LT patients. They found liver post-transplantation patients with spontaneous clearance of HCV-RNA and those with sustained viral response after therapy showed an immune response despite immunosuppression. (33)

Quantiferon assays offer a number of significant advantages over the existing T-cell assays: there is no requirement for lymphocyte isolation; the logistics for large-scale use are more favorable, as it uses inexpensive and readily available laboratory equipment.

Since several patients have low lymphocyte counts post-LT, we attempted to standardize the IFN-gamma response by adjusting the IFN values for the absolute lymphocyte count. IFN-gamma values adjusted for lymphocyte count did not show a better correlation with adverse outcome as compared to unadjusted scores. These results are concordant with

serial dilution experiments performed with mitogen stimulation with patients with SVR and NR (Chapter -3). No appreciable difference was in IFN production was noticed within a normal range of lymphocyte count. Quantiferon tests used for TB in the presence of immunodeficiency show a decrease in sensitivity, when compared to ELISPOT, which uses standard PBMC numbers in culture(34, 35).

However, these results would be entirely consistent with the use of this test as a functional immune assay, suggesting that a low interferon response, either due to low lymphocyte number or T cell hyporesponsiveness, independently predicts aggressive disease. This use of Quantiferon as a functional assay, independent of lymphocyte counts, CD4+ levels, or pharmacokinetics may be as effective in predicting outcome, is consistent with other global immune assays(11).

In summary, specific responses to CD4+ and CD8+ peptides, but not mitogen responses, were attenuated in post-LT subjects. There was an increase in CD4+ specific signal upon co-stimulation with IL-7. CD4+ and global specific immune responses were significantly lower in patients with advanced allograft disease (fibrosis>2). These preliminary results indicate that there may be a significant difference in global immune response between those with mild and aggressive recurrent HCV disease. Low response to mitogen stimulation may be useful for detecting overly

immunosuppressed recipients vulnerable to allograft damage due to recurrent HCV.

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Chapter 5

General discussion

The incidence of chronic HCV infection has almost doubled in the last decade. HCV –associated liver disease is now the leading indication for liver transplantation in developed nations and will continue to pose an important health and economic burden during the next 10 to 20 years(1,2).

HCV infected patients have a higher mortality than the normal population due to disease morbidity and associated psychosocial factors- young patients with HCV aged between 20-29 years had 18.2-fold increased risk of death as compared to controls(3). The rate of progression to cirrhosis for those infected with hepatitis C virus (HCV) was 8.1 per 1000 person-years (95% CR, 3.9-14.7) in a recent meta-analysis- this corresponds to a 20-year cirrhosis prevalence of 14.8% (95% CR, 7.5-25.5). Among those with compensated cirrhosis, the estimated annual rate of death/transplantation is 4.58%, that of decompensation is 6.37% and that of HCC, 3.36%(4). Therefore, HCV infection has considerable economic and health costs for the society and the individual.

The standard of care for treatment for HCV infection is the use of pegylated-interferon and ribavirin for 48 weeks. Peg-IFN / RBV have a response rate of 70-80% for genotype 2 and 3, while viral eradication (sustained viral response (SVR) defined as the absence of virus 24 weeks after treatment completion), is achieved in <50% of patients with Genotype1 infection (5-9). Since antiviral therapy has several serious side

effects (10), the decision to treat HCV disease is based on risk-benefit analysis . An accurate ability to predict response would allow both patients and clinicians to make more informed decisions regarding treatment, and the likelihood of success.

Several factor influence the response to anti-HCV treatment- degree of HCV viremia, absence of cirrhosis, genotype other than genotype 1, elevated ALT, presence of diabetes, low cholesterol levels and black ethnicity are independent positive predictors of an SVR (11-14).

Enhancement of HCV-specific $CD4^+$ and $CD8^+$ T-cell responses is an important factor in determining the response to the IFN-α/ribavirin therapy and the outcome of the HCV infection, and high HCV-specific type 1 $CD4^+$ and $CD8^+$ T-cell responses before starting therapy and after completion of treatment have been shown to be associated with SVR, while chronic HCV infection is characterized by attenuated CD4+ and CD8+ HCV-specific T-cell responses (15-24).

This objective of this study was to develop an in-tube interferon gamma release assay specific to HCV infection using the Quantiferon platform. Strong, broad, and sustained CD4+ and CD8 + T cell responses, with a Th1 cytokine secreting profile, have been observed in 79–100% of individuals who were able to clear HCV. The frequency of CD4+ or the

Th1 cytokine secreting CD4+ T cells is much higher, (0.01–0.1%) in the peripheral blood mononuclear cells (PBMC) from patients with spontaneous or treatment-induced viral clearance; while it is only 0.001% or undetectable in persistently infected patients (25). While IFN- gamma responses to HCV-specific peptide stimulation have been extensively studied by ELISPOT, these assays have not been adapted to clinical practice because of the laboratory logistics and infrastructure, and inter-test variability.

The Quantiferon assay has several logistical advantages favoring widespread use. There is no requirement for lymphocyte isolation. Tubes with dried peptides can be stored at room temperature. Blood handling and the risks to laboratory staff are minimal. The incubation of whole blood preserves the in vivo cellular and biochemical environment for lymphocyte stimulation, maximizing cellular response. The interferon-gamma ELISA uses inexpensive and readily available laboratory equipment. Easy reproducibility of the test enables ready direct comparison of results from multicenter studies.

These advantages have led to the widespread adoption of the Quantiferon TB test for the detection of latent TB, active TB and for monitoring response to treatment(26-30). Quantiferon CMV, monitors CMI post transplantation, and may be useful for predicting late-onset CMV disease(31-34). The ease of the in-tube CMI assay has led to the

development of other Quantiferon-based diagnostic tests for infectious disease(35), but no such assay currently exists for HCV infection.

We selected 57 HCV-specific peptides, which had been reported to elicit an IFN-gamma response either by ELISPOT or by flow cytometry, for study. Peptides were selected from all major HCV proteins, and were assessed in two pools- one with peptides known to elicit CD4 response. CD8 specific peptides were used in a single pool to maximize the coverage across HLA specificities. DMSO is known to attenuate IFN-gamma release; hence pools were constituted to restrict the final DMSO concentration to 0.1% (v/v) per experiment.

Quantiferon tests performed on 47 non-transplanted individuals (10 healthy controls, 37 subjects) showed evidence of IFN- gamma release from samples from healthy controls, as well as from HCV –infected individuals. Some HCV peptides, notably HCV NS3 and the IV NA peptide display a high degree of sequence homology, and cross-reactivity between HCV and influenza-A specific cytotoxic T cells has been described. Additionally, all healthy control samples were collected from healthcare staff, who were sero-negative for HCV. Hepatitis C virus-specific T-cell immune responses in sero-negative populations has been well described by ELSIPOT, and some authors have hypothesized that exposure to HCV may lead to development of HCV-specific CMI without anti-HCV and ongoing viral replication(36, 37).

There were marked differences in IFN- gamma response between patients who responded to therapy as compared to those with no response or with cirrhosis. Quantiferon and ELISPOT responses were higher in SVR patients, but the Quantiferon-CD4 specific response and the absolute mitogen responses were significantly higher in the SVR group. Patients with SVR and spontaneous clearance have been shown to have identical IFN-gamma responses to peptide stimulation. Serial dilution studies of stimulated PBMCs demonstrated a qualitative and a quantitative difference in IFN –gamma production between subjects with SVR and NR, suggesting that an absolute number of IFN producing cells on stimulation, as measured by ELISPOT, may not accurately represent quantitative IFN production.

Some subjects with NR had high IFN gamma responses to CD4 specific peptide stimulation. Kaplan et all had reported discordant T cell responses for HCV infection, where some patients with robust CD4+ responses failed to develop neutralizing antibodies or clear the infection, suggesting that a robust T cell response, although important, may not be the only factor determining response to anti-HCV treatment(38). Recent studies on the IL28 genotype have identified a nucleotide polymorphism, which is highly associated with a favorable response to interferon therapy(39). Ge *et al.* reported that a favorable *IL28B* genotype variation (C/C, patients with two copies of the C allele at the discovery SNP rs12979860) was associated with a two- to threefold increase in SVR rate (40-42). Additionally a

favorable genotype also identified patients who went on to SVR despite failing to achieve RVR(43).

Quantiferon-CD4 and mitogen respectively could account for 82 and 75% of the variation between the SVR and the NR subjects. Quantiferon CD4 + response of greater than 0.1IU/ml, had sensitivity of 77% and a specificity of 75% for SVR subjects, while a strong mitogen response (>23) had a sensitivity of 92.3% and a specificity of 69.1% in distinguishing between SVR and NR. We speculate that a Quantiferon-HCV test could be useful in three situations- firstly, identifying subjects likely to respond to anti-HCV treatment. Secondly, rapid virological response still remains the most important predictor of a successful response to HCV treatment - a robust pre-treatment interferon gamma response to HCV peptides may identify those patients who are more likely to achieve SVR and would benefit from prolongation of therapy, even if RVR was not achieved. Thirdly, serial measurement of IFN- gamma production may help monitor the course of anti-HCV treatment, as successful treatment is often associated with a reconstitution of specific immunity to HCV. This has not been tested in the current study, and presents a potential direction for future study.

Post-LT recurrence of hepatitis C virus (HCV) infection, as defined by detection of HCV RNA, is nearly universal, but clinically apparent recurrent disease is present in less than half of all patients (44) . Twenty to forty percent of patients with recurrent HCV progress to allograft cirrhosis within

5 years after LT, as compared to less than 5% of non-transplant patients(45).

The gold standard for the diagnosis of recurrent HCV disease is allograft biopsy, however differentiation of recurrent HCV infection from acute rejection can be difficult, since recurrent HCV and AR can often co-exist. Histopathological differentiation of recurrent HCV from ACR after LT has low inter-observer and intra-observer agreement rates (46-48). A recent study at this institute, examining one hundred and eighty-one biopsies performed in 64 patients with presumed rejection after LT for HCV-related disease, found 32 biopsy results in 18 patients to be discordant on review.

Quantiferon and ELISPOT results for specific peptide stimulation were significantly lower in post-LT subjects compared to non-transplanted subjects, despite comparable mitogen results. Blunting of the T -cell response post-LT by the pharmacological suppression of cell-mediated immunity (CMI) may contribute to the accelerated disease progression observed after transplantation.

Patients with relatively advanced fibrosis (>2 Metavir score) had significantly lower specific and mitogen responses as compared to those with less advanced disease. The lack of an optimal CD4+ T helper 1 response (which is characterized by the secretion of interferon gamma) is associated with fibrosis/cirrhosis development in patients with recurrent HCV disease post-LT.

Assessment of the global immune response has been found helpful in distinguishing between recurrent HCV disease and ACR. One such test is the Cylex ImmuKnow assay, which measures the ability of CD4+ T cells to respond to mitogenic stimulation by phytohemagglutinin-L in vitro by quantifying the amount of adenosine triphosphate (ATP) produced in these cells after stimulation. Low Immunknow results correlate with recurrent HCV disease, as well as fibrosis progression in patients who have undergone LT for HCV disease. (49, 50)The Immunknow assay requires selective enrichment of CD4 cells and measurement of ATP production, which add time and expense to the procedure.

Interferon-gamma release after mitogen stimulation using the Quantiferon assay may serve as a surrogate for global immune response in patients with recurrent HCV infection post-LT, and therefore help in distinguishing recurrent HCV from ACR. Additionally, low CD4 or mitogen scores may help identify patients at risk of aggressive recurrent disease and select patients who could benefit from early anti-HCV treatment or modification of immunosuppression.

In summary, we have developed and standardized an in-tube interferon gamma release assay to detect specific responses to a pool of HCV peptides. Information on the specific and global immune response of HCV infected individuals may potentially help identify patients likely to respond favorably to anti-HCV treatment. Quantiferon HCV and mitogen responses

in patients with recurrent HCV disease post-LT may be helpful in ascertaining the cause of allograft dysfunction, and identifying those patients at risk of developing allograft fibrosis.

We speculate that Quantiferon-HCV will be useful in identifying subjects likely to respond to anti-HCV treatment, and help base treatment decisions upon improved information of individual risk- benefit. A prospective study measuring CD4 and CD8 responses by Quantiferon-HCV in patients before commencement of interferon-based anti-HCV treatment, to assess the predictive value of Quantiferon in predicting treatment outcome is an important future consideration. Quantiferon-HCV can be evaluated in patients transplanted for HCV-related disease assessed for allograft dysfunction, to help distinguish between acute rejection and recurrent HCV infection in the allograft.

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