EVALUATING
T-CELL IMMUNITY IN HCV/HIV
CO-INFECTION

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Due to shared routes of transmission, co-infection with Hepatitis C (HCV) and Human Immuno-deficiency Virus (HIV) has become prevalent worldwide, with approximately one-third of all HIV-infected individuals and about 10% of all HCV infected individuals in North America being co-infected with the other virus (1). Recent advances in HIV treatment have increased the life expectancy of HIV-infected patients, resulting in HCV-associated disease to develop into a major cause of morbidity and mortality among the co-infected population. HIV is consistently shown to have a negative effect on different aspects of HCV disease, from increased HCV RNA levels (2) to aggravated liver fibrosis and more rapid progression to cirrhosis and end-stage liver disease (3). The host immune responses play a major role in not only controlling HCV infection, but also in causing hepatic inflammation and damage. Despite major advances in the understanding of the pathogenesis of these two infections, the mechanisms underlying the role of immune responses in more rapid progression of HCV disease in HCV/HIV co-infection is not quite clear.

This thesis is generated based on an investigation to understand why HIV infection worsens HCV pathogenesis. This question is addressed throughout this thesis from different immunological aspects, with a focus on the function of T-cells. I have
demonstrated that in HCV/HIV co-infection, functional HIV-specific T-cells accumulate in the liver and produce an array of cytokines, including INF-γ and TNF-α, which may represent a bystander role for HIV in the aggravation of HCV-induced liver damage. My data also demonstrate that co-expression of two defined T-cell exhaustion markers, Tim-3 and PD-1 may play a significant role in HCV-specific T-cell dysfunction, in the setting of HIV co-infection. Both total and HCV-specific peripheral T-cells co-express Tim-3 and PD-1 in significantly higher frequencies, compared to HCV mono-infection. In co-infection, HCV-specific CD8+ T-cells showed greater frequencies of Tim-3/PD-1 dual-expression than those being HIV-specific, indicating a greater degree of exhaustion in the former. Additionally, I demonstrated that some HIV mono-infected individuals may contain CD8+ T-cells that cross-recognize two defined HLA-A2-restricted epitopes within the HIV and HCV proteome, the HIV-Gag: SLYNTVATL and HCV-NS5b: ALYDVVSKL. This T-cell cross-reactivity was further elaborated in the context of HCV/HIV co-infection, demonstrating that degeneracy of HIV-specific T-cells may play a role in the immuno-pathology of co-infection.

Altogether, these data could be integrated into the foundation of potential mechanisms involved in the immunopathogenesis of HCV/HIV co-infection, and be applied to further investigation in basic science and clinical studies in this field.
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List of Abbreviations

AIDS – Acquired Immunodeficiency Syndrome
ALT – Alanine Aminotransferase
ANOVA – Analysis of variance
APC – Allophycocyanin
bDNA – Branched Deoxyribose Nucleic Acid Assay
CCR5 – CC Chemokine Receptor 5
CD – Cluster of Differentiation
CFSE – Carboxyfluorescein Succinimidyl Ester
CMV – Cytomegalovirus
CTL – Cytotoxic T-cell
CXCR4 – CXC Chemokine Receptor 4
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribose Nucleic acid
EBV – Epstein Bar Virus
ECM - Extracellular Matrix
ELISPOT – Enzyme Linked Immunospot Assay
ENV – HIV envelope glycoprotein
FACS – Flow Cytometry
FBS – Fetal Bovine Serum
FCS – Fetal Calf Serum
FITC – Fluorescein isothiocyanate
GAG – Group Specific Antigen
GALT – Gut Associated Lymphoid Tissue
GP120 – Envelope Glycoprotein 120
GP41 – Envelope Glycoprotein 41
HAART – Highly Active Antiretroviral Therapy
HCV - Hepatitis C Virus
HIV – Human Immunodeficiency Virus-1
HLA – Human Leukocyte Antigen
HSC – Hepatic stellate Cells
IFN-γ-Interferon-gamma
Ig- Immunoglobulin
IL – Interleukin
IM- Infectious Mononucleosis
LCMV – Lymphocytic Choriomeningitis Virus
LTNP – Long Term Non-Progressor
LTR – Long Terminal Repeat
MHC – Major Histocompatibility Complex
MIP-1β – Macrophage Inflammatory Protein 1-beta
NEF – HIV Negative Factor
NK cell – Natural Killer cells
P24 – Gag Capsid protein
P17 – Gag Matrix protein
PD-1 – Programmed Death-1
PE – Phycoerythrin
PerCP – Peridinin Chlorophyll Protein

PHA - Phytohemaglutinin

REV – Regulator of Expression of Viral proteins

RPMI 1640 – Roswell Park Memorial Institute media

RNA – Ribonucleic Acid

SEB – Staphylococcus Enterotoxin - B

SFU – Spot Forming Unit

SIV – Simian Immunodeficiency Virus

SR-B1 – Scavenger Receptor Class B type-1

TCR – T-cell Receptor

TIM-3 – T-cell immunoglobulin and mucin domain-containing molecule-3

TNF-α – Tumor necrosis factor – alpha

VIF – Viral Infectivity Factor

VPR – Viral Protein R

VPU – Viral Protein U
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Chapter 1:

Introduction,
Hypothesis and Research Aims
1.1-Introduction

Hepatitis C virus (HCV) infects over 170 million people worldwide, is a major cause of end-stage liver disease and the primary reason for liver transplantation in North America (4). Human immunodeficiency virus type-1 (HIV) infects over 33 million people worldwide and has become a pandemic health problem (5). Unfortunately, co-infection with HCV and HIV is common, and HIV infection appears to adversely affect HCV disease. With the advent of highly active antiretroviral therapy (HAART) for HIV infection, end-stage liver disease due to HCV has emerged as an important cause of morbidity and mortality in this population. The current therapy for HCV disease in HIV infected individuals with type 1 genotype is abysmal with unacceptable response rates. Therefore, defining the mechanisms for HCV disease progression in the setting of HIV co-infection is necessary to improve therapeutic options.

1.2-Statement of Hypotheses and Research Aims

This thesis is generated based on three different hypotheses to address one general question: Why does HIV worsen the natural history of HCV disease? All three hypotheses focus on the role of T-cell immune responses towards these two viruses, but each hypothesis is directed at a specifically different aspect of the nature of T-cells.

The first hypothesis focuses on the bystander role of HIV-specific T-cells, and states that: ‘In HCV/HIV co-infection, HIV-specific $CD^{8+}$ T-cells are recruited to the liver and contribute to hepatic injury through bystander activation and cytokine production.’
To address the above hypothesis, two specific aims were considered in cohorts of HCV mono and HCV/HIV co-infected individuals:

1. Determine if HIV-specific T-cells are found in the liver in HCV/HIV co-infection.
2. Quantify, characterize and compare T-cell immune responses to HIV and HCV in the blood and liver.

Chapter 3 of this thesis is allocated to this part of the research, presenting my published findings addressing the above-mentioned aims.

The second hypothesis focuses on the aspect of T-cell exhaustion and states that:

‘The exhaustion phenotype of antigen-specific T-cells, and consequently their function, varies in the context of HCV mono and HCV/HIV co-infection.’

Two aims address this hypothesis:

1. Determine if T-cell exhaustion profile varies between HCV and HIV mono-infections compared to HCV/HIV co-infection.
2. Determine if this profile varies between HCV- and HIV–specific T-cells during co-infection?
3. Determine if viral-specific exhaustion profile of T-cells associate with HCV disease progression.

This is fully elaborated in chapter 4 of the thesis, presenting the published data.

The third hypothesis focuses on heterologous T-cell cross-reactivity and states that: ‘HIV co-infection induces responses from HIV-specific T-cells that are cross-reactive to HCV-specific epitopes, thus modulating the immunopathology of HCV disease.’

Two specific aims address this hypothesis:
1. Determine if any T-cell epitopes across HIV and HCV proteome share similar amino acid sequences.

2. Determine if T-cells would cross-recognize these similar heterologous epitopes in HCV/HIV co-infection.

Chapter 5 of this thesis contains analysis of findings related to this section of the research.

All three projects presented in this thesis provide fundamental data regarding the role of HIV-specific T-cells in affecting and modulating the pathogenesis of HCV disease during HCV/HIV co-infection.
Chapter 2:

Background and Literature Review
2.1-HIV

2.1.1-HIV: Epidemiology, Origins and Transmission

Infection with Human Immunodeficiency Virus-1 (HIV) is a global health problem. Almost three decades have passed since the identification of this viral infection (6) and an estimate of 25 million human lives are since lost as a consequence (5). As of the year 2007, a global approximation of 33 million people have been infected with HIV (5). The pandemic spread of HIV infection is disproportionate, with Sub-Saharan region of Africa being the most heavily affected and containing 22 million individuals living with HIV, as reported in 2007 (5). This number constitutes two thirds of all globally infected people.

It was in 1981 that health complications due to what we now know as Acquired Immunodeficiency Syndrome or AIDS, first became noticed in humans. At the time, the attention was drawn to a rise in the incidence of rarely occurring opportunistic infections such as *Pneumocystis carinii* type pneumonia, as well as Kaposi’s sarcoma type cancer, in American men with no known cause of immunodeficiency (7, 8). Because of its high mortality rate and its prevalence among clusters of homosexual men, AIDS soon drew intensive global attention to itself as an infectious disease. It was soon discovered that haemophiliacs, transfusion recipients and injection drug users were also susceptible to this infection (9). HIV was first identified as the infectious agent causing AIDS in 1983 by two different labs (6, 10). This discovery earned the French scientists, Drs. Montanier and Barre-Sinoussi a shared Nobel Prize in Physiology and Medicine in 2008 (11-13).
Despite the recent identification of the virus, the estimated existing date of the last common ancestor of the pandemic strains of HIV is shown to go back to as early as 1931 (14). HIV is thought to have entered the human population through exposure to non-human primates infected with Simian Immunodeficiency Virus (SIV) (15).

HIV transmission routes are now well defined. Unprotected sexual intercourse is considered as the most prominent transmission route, accounting for 75-85% of globally occurred infections, and with receptive anal sex producing the highest risk of infection (16). Interesting enough, the risk of sexual transmission of HIV is extremely small, with a probability of 0.01-0.32% per sexual act in receptive vaginal transmission and 0.01-0.1% for insertive vaginal transmission (16). Although HIV sexual transmission seems to be an inefficient process, it still accounts for the majority of HIV infections. Injection or transfusion of infected blood or blood products, sharing infected injection equipment and maternofetal transmission—during pregnancy, birth or through breastfeeding—are considered as the other routes of transmission for HIV infection (16).

2.1.2-HIV: Viral Structure and Host Interaction

2.1.2.1-Morphological Structure

HIV is a retrovirus from the family of lentiviruses. Lentiviruses typically show a long period of clinical latency and persistent viral replication followed by a chronic course of disease (17). HIV is a spherical viral particle with a diameter of 100 nm and surrounded by a lipoprotein membrane. A total number of 72 glycoprotein complexes are integrated into this membrane; each containing an external glycoprotein structure known
as gp120 and a transmembrane spanning protein referred to as gp41 (17). The loose binding between these two parts, may result in the shedding of the external gp120 protein within an infected environment. The viral particle consists of structural proteins, including the matrix protein p17 that is anchored to the inside of the viral lipoprotein membrane, as well as the p24 core antigen that contains two copies of HIV RNA. The HIV RNA is a part of a complex that contains the nucleo-protein p7 and the reverse transcriptase p66. The viral particle contains enzymes that are required for viral replication, including a reverse transcriptase (RT), an integrase (p32) and a protease (p11) (18) (Figure 2.1).

2.1.2.2-Viral Genome

Retroviruses in general are dependent on three genes for their replication. These genes are known as gag (group-antigen), pol (polymerase) and env (envelope) (19). The classic genome structure of retroviruses is defined as: 5’LTR-gag-pol-env-LTR 3’, in which LTR (long terminal repeat) represents the two end parts of the viral genome. These two regions attach to the host cellular DNA after integration, but they do not encode for any viral proteins. Gag, pol and env regions on the other hand, are responsible for encoding viral proteins, including the nucleocapsid, membrane glycoproteins, and reverse transcriptase and other enzymes. In addition to these genes, the HIV genome contains six more genes (vif, vpu, vpr, tat, rev and nef) which are referred to as accessory genes, since they are not necessary for in vitro viral replication (19) (Figure 2.1).
Figure 2.1- Schematic of HIV virion and genome organization.

A. Cross-sectional diagram of HIV virion with the viral envelope proteins (gp41 and gp120), the internal proteins (matrix and capsule proteins) and the viral RNAs.

B. The genome of HIV is encoded on a single-stranded RNA of approximately 9.6 kb in length. Each viral genome is composed of nine genes encoding three structural, two envelope, and six regulatory proteins.

Adapted from: Roe, B., Exp. Rev. Mol. Med. 2008 (10)
2.1.2.3-Cellular Entry

HIV enters a host cell through the binding of its gp 120 external glycoprotein with the CD4 molecule on the surface of the host cell, mainly helper T-cells and macrophages (20, 21). This binding results in conformational changes in the viral gp 120 molecule and a subsequent exposure of additional binding sites (22). Additionally, HIV uses two main co-receptors, CCR5 and CXCR4 chemokine molecules, to enter the host cell (23-25). Once inside the cell, HIV RNA undergoes reverse transcription in the cytoplasm before targeting the nucleus where it integrates into the host DNA. This integrated DNA further serves as a template for the transcription and translation of HIV proteins (26).

2.1.3-HIV: Natural Course of Infection

2.1.3.1-Acute Infection: Clinical and Immunological Events

Primary HIV infection is associated with a burst of HIV replication, reaching as high as 100 million copies of HIV RNA/ml of blood. Acute phase of infection presents as a transient illness with non-specific flu-like symptoms in about 40-90% of infected individuals (5). During this acute phase of infection the virus invades several tissue reservoirs and eradicates CD4+ T-cells within the gut lymphoid tissue. Acute HIV infection is accompanied with an abrupt drop in absolute CD4+ T-cell count in the blood. The level of CD4+ T-cell count usually rebounds with the resolution of primary infection, but it rarely returns to baseline levels in the absence of antiretroviral therapy (Figure 2.2). In addition to the decline in CD4+ T-cell counts, the function of CD4+ T-cells that are
specific for HIV, become impaired early in acute infection (27) which is shown to be related to the preferential infection of virus-specific CD4+ T-cells by the virus (28).

HIV antibodies with neutralizing capacities are rarely detectable during the acute phase. HIV-specific cellular immune responses on the other hand, have a crucial role in the initial control of viral replication during this stage of infection. A substantial expansion of CD8+ T-cell responses has been described during acute HIV infection (29), and the appearance of HIV-specific CD8+ T cells has been associated with the initial decline of viremia (30).

2.1.3.2-Chronic Infection and AIDS: Clinical and Immunological Events

The chronic phase of infection follows the first 6 months of acute infection and is accompanied by a rapid decline in HIV viremia from the peak to a somewhat stable level known as the “viral set point”, which is shown to associate with the rate of disease progression (31). This chronic phase is often asymptomatic and is accompanied by a gradual decline of the absolute CD4+ T-cell count. Eventually, the fall of blood CD4+ T-cell count to levels below the threshold of 200/mm³ marks the clinical demonstration of AIDS (Figure 2.2). Clinical progression to AIDS is demonstrated by a rapid rise in HIV viremia, extreme immunodeficiency and the appearance of opportunistic infections and malignancies (32). In the absence of antiretroviral therapy, the average time for adults to develop AIDS is approximately 10 years. Some individuals, referred to as rapid progressors, manifest clinical AIDS within 5 years after infection; while a limited number of individuals, referred to as long-term non-progressors or slow progressors, stay symptom-free for a longer period of time, without decline in CD4+ T-cell counts (33).
This variable HIV pathogenesis is shown to be associated with not only genetic factors, but also with host cellular immune responses. Studies have shown an inverse correlation between the frequencies of HIV-specific CTL activity and plasma viral load at all stages of HIV infection (34, 35). Data also suggest an association between the breadth of HIV-specific CD8+ T-cell responses and better clinical status (36).
Figure 2.2- Natural course of HIV disease progression.

Acute HIV infection is associated with a precipitous drop in absolute CD4+ T-cell counts and a rise in HIV viremia. After the initial acute phase of infection, HIV viremia drops to a lower level called the viral set-point. Establishment of a viral set-point typically results in an increase in absolute CD4 counts, which subsequently decline over the course of infection. A drop of CD4 counts below 200/mm3 is defined as clinical AIDS which is associated with a rapid rise in viremia and decline in absolute CD4+ counts.

(The x-axes is not on scale)

Adapted from: Langford, S.E., AIDS Res. Ther. 2007(4)
2.2-HCV

2.2.1-HCV: Epidemiology, Origins and Transmission

Hepatitis C virus (HCV) is a member of the Hepacivirus genus in the Flaviviridea family (37). HCV was identified in 1989 as the pathogen causing the non A-non B hepatitis when its RNA genome was molecularly cloned (38), and is now considered as the most common cause of liver disease and currently infecting 170 million people worldwide (39). HCV can only infect humans and chimpanzees. The majority of humans infected (60-80%) develop chronic HCV infection, which frequently leads to cirrhosis and hepatocellular carcinoma (40, 41). HCV transmission occurs mainly through exposure to infected blood, in the form of injection drug use, blood transfusion and organ transplantation (before 1992), occupational exposure to infected blood, and birth to infected mother. Sexual transmission of HCV occurs at lower rates (39). Currently, there is no effective vaccine for the prevention of HCV infection and the available medical treatments help achieve sustained virological response in only half of the treated individuals (42).

2.2.2-HCV: Viral Structure and Host Interaction

2.2.2.1-Morphological Structure and viral genome

HCV is a virus with a single positive-stranded RNA genome of approximately 9600 nucleotides that encodes a single polyprotein of 3000 amino acids (43). This polyprotein is further processed by cellular and viral enzymes to produce the three
Figure 2.3- Schematic of HCV viral structure and genome organization.

A. Cross-sectional diagram of HCV virion consisting of a single stranded RNA in a nucleocapsid surrounded by a lipid envelope with spike-like glycoprotein projections.

B. The genome of HCV is encoded on a single-stranded RNA of approximately 9.6 kb in length that comprises one long large protein of 3000 amino acids. The polyprotein precursor is co- and post-translationally processed by both cellular and viral proteases to produce ten structural and nonstructural proteins.

*Adapted from: Roe, B., Exp. Rev. Mol. Med. 2008 (10)*
structural HCV proteins, core (C), envelope glycoproteins (E1 and E2), and seven non-
structural proteins designated as (P7, NS2/3, NS3, NS4A, NS4B, NS5A and NS5B) 
(Figure 2.3). HCV has 6 major genotypes which differ in geographical distribution and 
response to alpha interferon therapy (44). HCV generally exists as quasispecies 
populations of related RNA genomes that could differ in host cell range and susceptibility 
to immune recognition (45).

2.2.2.2-Host Interaction

Hepatocytes are the major target cells for HCV. It has been suggested that 
approximately less than 10% of hepatocytes become infected with HCV (46). HCV enters 
these cells through a receptor-mediated endocytosis. Several receptors and co-receptors 
are identified to be involved with cellular entry of HCV, including the tetraspanin CD81 
(47), the scavenger receptor class B type-I (SR-BI) (48), the tight junction proteins 
claudin-1 (49) and occludin (50), the LDL receptor, and the lectins DC-SIGN and L-
SIGN (51). Once inside the cell, HCV uncoats and releases its RNA into the cytoplasm. 
The released RNA acts as a template to generate the viral proteins. These proteins 
alongside the newly replicated viral RNA are assembled to form new virions (52).
2.2.3-HCV: Natural Course of Infection

2.2.3.1-Acute Infection: Clinical and Immunological Events

HCV RNA can be detected in blood within 1 to 3 weeks after exposure. Antibodies to HCV are detected in only 50 to 70% of patients at the onset of symptoms. Within a period of 8 to 12 weeks, elevation in the level of serum alanine aminotransferase (ALT) is detected. It is during this stage that T-cells against HCV become detectable in the blood. This delayed immune response, despite an increase of viremia, is one of the characteristics of HCV infection. Symptoms usually subside after several weeks as ALT levels decline (53). Some HCV infected patients spontaneously resolve the infection during the acute phase, while the majority (around 75%) continue to maintain persistent viremia that leads to chronic infection (54).

Antibody responses against HCV are mounted during the acute phase of infection; however, these responses are delayed and limited by the rapid emergence of viral escape mutants. Unlike neutralizing antibodies which fail to clear HCV in this stage, HCV-specific T-cells are essential for viral clearance. Early studies signified the role of HCV-specific CD4+ T-cells in controlling the infection (55, 56). Although resolution of the HCV infection during the acute phase was shown to be associated with sustained HCV-specific CD4+ T-cell response (57), follow-up studies demonstrated that these responses are not absent in all acute infections that persist (58). Those infected individuals who are unable to mount an HCV-specific CD4+ T cell response developed chronic disease (Figure 2.4). Some individuals on the other hand, who initially clear the virus from their blood, show strong HCV-specific CD4+ T cell responses. However, these responses
contract just before a rebound in viremia and the infection continues to become chronic (58). Expansion of HCV-specific CD8\(^+\) T-cells in the blood is also associated with the clearance of acute HCV infection (59). In those individuals who are unable to control the virus during the acute phase, HCV-specific CD8\(^+\) T-cells show impaired proliferation and cytokine production (60). Studies on CD4\(^+\) T-cell depletion in chimpanzees, signified the importance of CD4\(^+\) T helper cells for the generation and maintenance of CD8\(^+\) T-cells and successful HCV control (61).

### 2.2.3.2-Chronic Infection: Clinical and Immunological Events

Persistence of HCV infection is defined as detection of HCV RNA in the blood for at least 6 months (39). As shown by prospective studies, 60-85% of HCV-infected individuals develop chronic infection. The clinical outcome of chronic HCV infection is manifested by progressive liver fibrosis, cirrhosis and end-stage liver disease (39). Chronic HCV infection is associated with a substantial, permanent loss of HCV-specific CD4\(^+\) T-cells. The reasons behind the lack of CD4\(^+\) T-cell responses during the chronic phase of HCV infection is still a topic of speculation. Escape mutations take place among some MHC class II HCV epitopes, which could be one of the mechanisms behind the weak CD4\(^+\) T-cell responses (62). However, available data suggest that CD4\(^+\) T-cell escape mutations are rare and may not play an important role in the silencing of CD4\(^+\) T-cells in chronic phase of infection (63). CD8\(^+\) T-cell activity against HCV is also impaired in this stage and insufficient to control the virus (Figure 2.4). The inability of CD8\(^+\) T-cells to control the virus could also be related to escape mutations in MHC class I epitopes (64), but effector anergy of these cells is also an involved mechanism (65).
These mechanisms however, are probably secondary to the failure of help from HCV-specific CD4$^+$ T-cells (66).
Figure 2.4- Models of Immune Responses to HCV Infection.

(A) An immune response to HCV is considered successful when robust virus-specific CD4\(^+\) and CD8\(^+\) T-cells are generated. The maintenance of these T cells beyond this point results in permanent clearance of the infection.

(B) A transient immune response becomes unsuccessful. Partial control of viral replication happens through a short-lived generation of virus-specific CD4\(^+\) and CD8\(^+\) T-cells. Sudden loss of the CD4\(^+\) responses coincides with the recurrence of viremia, which also leads to inadequacy of virus-specific CD8\(^+\) T-cells and the persistence of viremia.

Adapted from: Shoukry, N.H., Annu. Rev. Microbiol. 2004 (58)
2.2.3.3- Immunological Events in the Liver

Studies of the liver during the acute phase of HCV infection demonstrate that the virus is present in the liver for a period of time without any inflammation and tissue damage (59, 61). This suggests that the virus, at least in short term, is not cytopathic, which explains the lack of association between HCV viral load and liver disease.

Liver is comprised of a variety of cell types, including hepatocytes which constitute about two thirds of the total cell population in the liver. The remaining population of non-parenchymal cells includes sinusoidal endothelial cells, macrophages (known as Kupffer cells), biliary cells, stellate (Ito or fat-storing) cells and intrahepatic lymphocytes (67). About 30% of the total blood passes through the liver every minute, carrying about $10^8$ peripheral blood lymphocytes in 24 hours. The average human liver contains a population of approximately $10^{10}$ lymphocytes, which are shown to consist of cells involved in both innate (NK, NK T-cells) and adaptive (B and T cells) immunity (68). Circulating lymphocytes come in contact with antigens that are displayed by endothelial cells, kupffer cells and liver dendritic cells. T-cell mediated protection against liver-trophic viruses such as HCV depends on the constant supply of activated, effector T-cells. Naïve, CD8$^+$ T-cells are preferentially located in secondary lymphoid compartments. In order to become activated, these naïve T-cells require signals provided by the binding of specific T-cell receptors to the peptide-MHC class I complex, as well as co-stimulation signalling. The presence of so many subsets of antigen presenting cells in the liver has raised this question of whether T-cells could be primed at the site of the liver. Several lines of evidence support that liver is a secondary lymphoid organ and a site of primary T-cell activation. Nevertheless, only up to about 10% of the liver lymphocyte
population in patients with chronic hepatitis C are shown to recognize HCV-specific epitopes (69). This suggests that T-cells that are not HCV-specific may also induce damage from bystander activation. It has been postulated that the liver can non-selectively trap activated T-cells during any infection, and thus act as a ‘sink’ or ‘graveyard’. It is demonstrated that in influenza-A virus infection, a significant percentage of activated influenza-A virus-specific T-cells are not only found in the lung, but also in the liver, where influenza virus is not found (70). In chronic HCV infection, viral-specific T-cells home to the liver and produce an array of antiviral cytokines. By doing this, the T-cell infiltrate in the liver contributes to not only controlling the virus, but also the induction of hepatic inflammation.
2.3-HIV and HCV Co-infection

2.3.1-Epidemiology and Natural History

Due to shared routes of transmission, approximately one third of HIV infected individuals in north America are also infected with HCV (71), with this prevalence being as high as 65% among intravenous drug users (72). Globally, about one in 10 of those infected with HIV are also HCV infected; which means 4-5 million of global population are co-infected with HIV and HCV (4). HCV-related liver disease is increasingly becoming a major cause of morbidity and mortality among HIV infected individuals (73). The effects of HCV infection on HIV disease progression are reported to be controversial and still not clear. Although co-infection with HCV was shown to be associated with HIV disease progression in a few studies (74-76), not all studies on HCV/HIV cohorts support these findings (77). On the contrary, the negative effect of HIV infection on the natural history of HCV disease is well established. HCV RNA levels are shown to be significantly elevated in HCV/HIV co-infection compared to HCV mono-infection (78, 79). The clinical course of HCV disease is also accelerated during co-infection with HIV, with more advanced stage and higher rates of progression to liver disease. The estimated mean interval from the time of HCV infection to development of cirrhosis is shown to be significantly shorter for patients co-infected with HIV (7 years vs. 30 years) (80-84). A meta-analysis of 8 HCV/HIV co-infected cohorts, demonstrated that co-infection with HIV increased the risk of histological hepatic cirrhosis by 2.1 and clinically decompensated liver disease by 6.1 (3). HIV co-infection is also associated with decreased response rates to HCV treatment (85, 86). All these findings demonstrate that HIV clearly has a negative impact on HCV pathogenesis.
2.3.2-Antigen-Specific T-Cell Immune Responses

Potent and broad CD4$^+$ and CD8$^+$ T-cell immune responses are important for virologic control in both HCV and HIV infections. HCV-specific CD8$^+$ T-cell responses in blood are generally weak in HCV mono-infected individuals when measured \textit{ex vivo}, with frequencies ranging from 0- 1.2% of total CD8$^+$ T-cells and being detectable in only about 65% of HCV infected individuals (87). In contrast, HIV-specific CD8$^+$ T-cell responses are easily detectable in the blood of HIV infected individuals with high frequencies ranging from 1.6- 18.4% of total CD8$^+$ T-cells (88). Similarly, HCV-specific CD4$^+$ T-cell responses in HCV mono-infection were shown to be weaker than those with HIV specificity in HIV mono-infection (89). Peripheral HCV-specific CD8$^+$ T-cell responses are shown to be somewhat weaker in HCV/HIV co-infected individuals, with HCV-specific CD4$^+$ T-cell responses being absent or at strikingly low frequencies in co-infection with HIV (90, 91). Alatrakchi et al., demonstrated that intra-hepatic CD8$^+$ T-cell responses to HCV antigens were similar in frequency between HCV mono and HCV/HIV co-infected individuals (92). Intra-hepatic CD4$^+$ T-cell responses were also comparable and correlated with liver CD8$^+$ T-cell responses (92). Neither HCV-specific intrahepatic CD8$^+$ nor CD4$^+$ T-cell responses were shown to correlate with the peripheral CD4$^+$ T-cell counts in HCV/HIV co-infected subjects (92). This was in contrast with a previous report demonstrating that the magnitude and breadth of circulating HCV-specific CD8$^+$ T-cell responses depend on the absolute CD4$^+$ T-cell count in co-infection (93).
2.3.3- Pathogenesis of Hepatic Damage in HCV/HIV co-infection

Viral infections like any other acute or chronic stimuli can induce tissue damage. The repair process of the damage involves two distinct stages: a regenerative phase, in which the damaged cells are replaced by cells of the same type and there is no lasting injury, and a fibroplasias phase, in which the connective tissue replaces the normal tissue. After any acute liver injury, parenchymal cells regenerate and replace the necrotic or apoptotic cells. This process is associated with an inflammatory response and a deposition of the extracellular matrix (ECM) proteins. Chronic damage to the liver and the accumulation of ECM proteins distort the hepatic architecture by forming fibrous scar in the liver tissue. Liver fibrosis is a characteristic of many chronic liver diseases, including HCV infection (94). Persistence of hepatic injury results in the failure of liver regeneration and substitution of hepatocytes with abundant ECM. Eventually, nodules of regenerating hepatocytes form and cirrhosis develops. Cirrhosis results in hepatocellular dysfunction, increased intrahepatic resistance to blood flow, portal hypertension, hepatic insufficiency, jaundice and ascites (95).

The pathogenesis of hepatic damage associated with HCV infection is not clearly elucidated. HCV was originally suggested not be directly cytopathic to hepatocytes. However, some histopathological evidence suggest that fatty liver might be a consequence of direct cytopathic effect of HCV (96, 97). Although HCV might be directly involved, the hepatic damage is thought to be mainly caused by host immune-mediated responses directed against HCV-infected hepatocytes (98-100). HCV-specific CTL responses have been detected in the liver of HCV infected individuals, and were shown to be associated with a lower level of HCV viremia, but a greater degree of
inflammation in the liver (101, 102). This suggests that although HCV-specific T-cell activities are required for viral control, they also contribute to hepatic damage. At present, the prevailing hypothesis is that HCV-specific T-cell responses mediate liver damage while being ineffective in clearing the virus.

Specific mechanisms that could be involved in the acceleration of hepatic injury in the setting of co-infection with HIV are currently unclear. Although HCV viral loads are generally higher in co-infected individuals compared to those being HCV mono-infected, there is no clear association between HCV viral load and the degree and progression of liver disease (79, 103). If T-cell-mediated inflammation is assumed to be responsible for HCV-related liver damage, then it would be paradoxical that liver disease is aggravated in the immuno-compromised state of HIV infection.

2.3.3.1-Role of Host Immune Responses and Inflammation in Hepatic Damage

Hepatic injury induced by cellular immune responses in the liver are either the result of a direct cytolytic effect of perforin and Fas/Fas Ligand pathway (104, 105) or by the release of cytokines. In chronic HCV infection, intra-hepatic CD4+ and CD8+ T-cells that express cytokines such as IFN-γ, TNF-α, IL-10, and IL-2 have been identified (106). The two cytokines IFN-γ and TNF-α, which are released from the activated lymphocytes, are suggested to play important roles in HCV-mediated liver injury. The effect of tissue-specific expression of IFN-γ on the induction of liver damage was demonstrated in transgenic mice (107). This was further shown in humans (108, 109), exhibiting a positive correlation between IFN-γ expression levels in the liver and the degree of liver injury (110). The antiviral capacity of IFN-γ is related to its role as a growth factor for the
proliferation and differentiation of T-cells which eventually attack and lyse the infected hepatocytes (111). IFN-γ has been shown to up-regulate nitric oxide synthase (iNOS) in monocytes and macrophages, resulting in the production of nitric oxide which plays a dual pro-inflammatory and antiviral role in HCV infection (109). On the other hand, anti-fibrogenic effects of IFN-γ has also been demonstrated in humans (112), which makes IFN-γ a controversial cytokine in liver pathology. Studies on animal models of hepatitis have also shown the effect of TNF-α on inducing hepatocytes to undergo apoptosis and cause liver injury (113, 114). Levels of TNF-α and its receptor have also been shown to be increased in sera and liver of patients with chronic HCV infection and correlate with the degree of liver disease (115-117).

The process of fibrogenesis is considered to be more complex in HCV/HIV co-infection compared to what is observed in HCV mono-infection, due to interplay of an array of immunological and viral factors that contribute to the pathogenesis of liver damage. The immune dysfunction associated with HIV infection, is not only due to loss of CD4+ T-cells, but also to a cytokine imbalance that affects the global immune regulation. The current mechanisms considered responsible for the accelerated liver diseases in HCV/HIV co-infection, focus on alterations of cytokine networks and shifts between Th1 and Th2 responses. These mechanisms mainly rely on two principal causes: the persistence of HCV, which depends on alterations of cell-mediated immunity, and the general activation of the immune system and secretion of pro-inflammatory cytokines.

2.3.3.2- Role of HIV in Hepatic Damage

Liver is known to efficiently clear many foreign pathogens, including RNA viruses (118, 119). Several lines of evidence suggest that different types of liver cells
could be infected with HIV. Liver Kupffer cells in HIV infected individuals were shown to stain positive for antibody to HIV p24 (120). The same study demonstrated the detection of HIV RNA in liver mononuclear and sinusoidal cells (120). In another report, HIV proviral DNA was amplified from the liver samples of patients with AIDS (121). HIV antigen and mRNA were also detected in both Kupffer cells and hepatocytes in the same study, suggesting potential HIV replication in the liver. In addition, it has been shown that the hepatocyte-derived cell line HepG2 expresses the CD4 receptor and produces infectious HIV particles in vitro (122). More recently, HIV has been shown to replicate in hepatic stellate cells (HSC) (123), which suggests a potential role of HIV in liver damage as mediated through HSCs. The literature however, is limited on the productivity of HIV infection of primary hepatocytes. Since hepatocytes are the primary cells for HCV replication, the use of other liver cells for HIV replication could still place both viruses at the proximity of each other. This could potentially result in virus-virus interaction that might have an effect in liver pathology. On the other hand, the role of T-cell immune responses against HIV in the liver may have been underestimated.

2.4- T-Cell Exhaustion in HIV and HCV Infections

The inability of the T-cell immune responses to control persistent viral infections like HIV and HCV has been correlated with impairment in the ability of viral specific T-cells to produce cytokines, proliferate and survive. This dysfunction, termed “T-cell exhaustion”, allows continuing viral replication in infected individuals and chronic progression of the disease.

T-cell exhaustion was first described in the lymphocytic choriomeningitis virus (LCMV) murine model. In this model, LCMV was shown to induce virus-specific CD8+
T-cells that failed to produce effector cytokines upon antigen stimulation (124). The decline of the CD8$^+$ T-cell response demonstrates a hierarchical pattern of dysfunction in which proliferative capacity, cytotoxic potential, and the ability to produce IL-2 are lost early, followed by a loss of TNF-α production, while the production of IFN-γ lasts longer. At later stages of exhaustion, IFN-γ production is also diminished and virus-specific CD8$^+$ T-cells are lost (124). High levels of antigen and absence of CD4$^+$ T-cell help, were shown to correlate with greater levels of exhaustion in this model.

A similar T-cell phenotype is observed in chronic progressive HIV infection, in which HIV-specific T-cells are defective in their proliferative capacity and IL-2, TNF-α and IFN-γ production (125). In contrast, T-cells from HIV-infected, non-progressive individuals are shown to be poly-functional in response to cognate antigen (126). Poor functional capacity of HCV-specific CD8$^+$ T-cells has also been reported at the acute stage of HCV infection. T-cell exhaustion is one of the possible mechanisms considered responsible for this phenomenon, initially sustained by the rapid kinetics of HCV replication and later on by the persistent exposure to high loads of antigen (65).

In recent years, T-cell exhaustion in persistent viral infections has been emphasized to be modulated through several pathways, including the PD-1, CTLA-4, and more recently Tim-3 signaling pathways (127-130).

2.4.1-Role of PD-1 in T-Cell Exhaustion

Several lines of evidence are demonstrating the importance of pathways that may affect and regulate T-cell exhaustion during chronic viral infections. Recently, signalling through PD-1/PD-L1 pathway was shown to play an important role in T-cell exhaustion in several models of chronic viral infection, including LCMV in mice, SIV in rhesus
macaques, and HIV and HCV in humans (127, 130-133). PD-1 (Programmed death-1 or CD279) is a 55 kD member of the CD28 immunoglobulin super-family of transmembrane proteins, and mainly expressed on the surface of T- and B-cells. Pathways in the B7–CD28 family regulate the balance between the stimulatory and inhibitory signals needed for defence against infections and for self-tolerance. These pathways provide second signals that can regulate the activation, inhibition and fine-tuning of T-cell responses (134). PD-1 is composed of an extracellular IgV domain, a transmembrane domain, and an intracellular signalling domain with tyrosine-based signalling motifs (Figure 2.5). PD-1 is expressed at low levels by naïve T-cells, presumably to modulate activating stimuli that might result in autoreactivity (135). Its ligands, PD-L1 and PD-L2 are expressed on a large variety of cells, including hepatocytes (136). PD-L1 (B7-H1:CD274) is more broadly expressed on both professional and non-professional antigen-presenting cells (APCs) (134). CD80 (B7-1) has also been identified as an additional binding partner for PD-L1 (137). Ligation of PD-1 causes a cascade of cellular activities that results in the dampening of T-cell receptor signalling and the eventual inhibition of T-cell activation (138, 139). Blockade of the PD-1/PD-L1 interactions in vivo is shown to enhance T-cell responses and viral control in a murine model of LCMV infection (131).
Figure 2.5- PD-1 Structure and Signalling Pathway.

T-cell activation requires TCR-mediated signalling, that is modulated by co-inhibitory molecules. In approximity to the TCR signalling complex, PD-1 which is expressed on T-cells, delivers a co-inhibitory signal upon binding to either of its ligands, PD-L1 or PD-L2. These ligands become upregulated on antigen presenting cells by cytokines produced after T-cell activation. Engagement of PD-1 to its ligand results in tyrosine phosphorylation of PD-1 cytoplasmic domain and recruitment of phosphatases particularly SHP2. This results in dephosphorylation of TCR proximal signalling molecules and attenuation of TCR signalling.

Adapted from: Freeman, G., PNAS 2008 (105)
2.4.1.1- PD-1 and HIV Infection

In HIV infection, PD-1 expression is up-regulated on HIV-specific CD8\(^+\) T-cells, but not on CMV-specific ones from the same individual (127, 140). A large proportion of HIV-specific CD8\(^+\) T-cells are also shown to express CD27 and CD45RO, which indicates their previous activation. These CD8\(^+\) T-cells have also lost expression of the co-stimulatory receptor CD28 and perforin and expressed only low levels of CCR7 and CD127 (IL-7 receptor \(\alpha\)), which are important molecules for the maintenance of memory T-cells (132). This phenotype suggests that these T-cells are poorly functional, are not transiting into memory cells, and are particularly receptive to inhibitory signals. The expression of PD-1 on HIV-specific CD4\(^+\) and CD8\(^+\) T-cells also correlates with HIV viral load and HIV disease progression (127). Blocking the engagement of PD-1 with its ligand is shown to result in enhanced survival and proliferation of HIV-specific CD4\(^+\) and CD8\(^+\) T-cells \textit{in vitro} (127, 140). Although total levels of cytokine production and the frequencies of cytokine-producing T-cells are increased in response to HIV antigens in short-term \textit{in vitro} cultures treated with anti-PD-L1 (127, 140), no direct relationship is documented between the levels of PD-1 expression of antigen-specific CD8\(^+\) T-cells and the ability of those cells to produce cytokine upon \textit{ex vivo} stimulation (133). This suggests that PD-1 expressing T-cells could still produce cytokines and may be a marker of earlier stages of T-cell exhaustion, representing a stage of impaired cellular proliferation, but sustained T-cell function. In this regard, recent studies show that PD-1 expressing HIV-specific T-cells demonstrate polyfunctional cytokine profiles, a feature usually associated with viral control (126, 141).
2.4.1.2- PD-1 and HCV Infection

During the early period of HCV infection, PD-1 is up-regulated on all HCV-specific peripheral CD8\(^+\) T-cells, irrespective of the final outcome (130, 142). After the acute stage, however, PD-1 expression is modulated depending on the progression. During self-limited infection HCV-specific CD8\(^+\) T-cells down-regulate PD-1 expression, while in persistent infection these cells continue to maintain high levels of PD-1 expression. Studies demonstrate that high levels of PD-1 expression on CD8\(^+\) T-cells in chronic HCV infection is associated with impaired capacity of these cells to proliferate and express IFN-\(\gamma\), TNF-\(\alpha\), IL-2, perforin and granzyme B (143, 144). Not all T-cell effector functions are altered at the same time though. Proliferation and IL-2 production are lost early, while the production of other cytokines and the cytolytic capacity of T-cells are gone at later time points. This progressive impairment could be related to the level of PD-1 up-regulation. Blocking the interaction between PD-1 and its ligand is shown to increase the proliferative capacity of HCV-specific peripheral CD8\(^+\) T-cells in some chronically infected individuals, but not all (145, 146). This suggested the involvement of other potential mechanisms in T-cell exhaustion in HCV infection.

2.4.1.3- PD-1 Expression in the Liver during HCV Infection

Liver is known to be an immuno-tolerant organ that deals with a wide range of antigens transferred from the gut. PD-1 and its ligand PD-L1 that are expressed on resident or infiltrating cells are involved with this task (147). Since liver is the main site of HCV replication, the hepatic tolerance could contribute to the incompetence of local immune responses against HCV. The anergy of HCV-specific T-cells in the liver could be modulated through several mechanisms. One potential mechanism is the up-regulation
of PD-1 on liver T-cells (144, 148) and the expression of its ligand PD-L1 on hepatocytes, kuppfer cells and sinusoidal endothelial cells (136). The increased expression of PD-1 on total intra-hepatic T-cells (142) may indicate that some non-specific, HCV-dependent stimulus is acting upon liver infiltrating T-cells to favour PD-1 up-regulation. Data suggests that this could be related to HCV-core protein (149). In addition to the non-specific T-cell stimulation, PD-1 expression is also induced by persistent TCR stimulation. Since PD-1 is shown to be more expressed on intra-hepatic than peripheral HCV-specific CD8\(^+\) T-cells (144), it is suggested that the intense TCR activation in the liver, accompanied with high levels of HCV-core protein are responsible for the high PD1 expression.

2.4.2-Role of Tim-3 in T-Cell Exhaustion

T-cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3) is a member of the immunoglobulin (Ig) superfamily, that was identified as a specific cell surface marker on murine Th1 CD4\(^+\) T-cells (150). In humans, Tim-3 is expressed on a subset of activated CD4\(^+\) and CD8\(^+\) T-cells, and at lower frequencies on Th17 cells. A defect in up-regulation of Tim-3 on IFN-\(\gamma\) producing CD4\(^+\) T-cells in humans, has been demonstrated to contribute to the pathology of multiple sclerosis (151). Based on these data, Kuchroo et. al., proposed a model in which CD4\(^+\) T-cells differentiate into Th1 cells, which in turn induces the up-regulation of both soluble and membrane-bound Tim-3 (152). Surface Tim-3 up-regulated by the Th1 cells will interact with Tim-3 ligand, inducing apoptosis and shutting down the response. Given that a Th1 response is critical
for control of viral infections, the role of Tim-3 in chronic viral infections has recently been emphasized.

Tim genes encode cell surface glycoproteins with an N-terminal immunoglobulin (Ig)-like domain, a mucin domain, a single trans-membrane domain and a cytoplasmic region (153). The Tim-3 Ig domain has two sites for ligand binding, one that is glycosylated and binds to galectin-9, and another non-glycosylated site which likely binds an unknown ligand (154) (Figure 2.6). Galectin-9 is expressed broadly on immune cells and gut epithelium. Interaction of murine Tim-3 with its ligand galectin-9, is shown to regulate Th1 responses by promoting the apoptosis of IFN-γ-producing Th1 cells (155). Galectin-9 production is up-regulated by IFN-γ, which is produced by Th1 cells, thus involved in the negative feedback that results in the death of Tim-3⁺ Th1 cells. Tim-3 expression is shown to be regulated by the transcription factor T-bet as shown by reduced levels of Tim-3 expression in mice deficient in T-bet (156).
Figure 2.6- Schematic Representation of Tim-3 Structure.

Each of the *Tim* genes encode a cell-surface glycoprotein with a common structure, consisting of an N-terminal immunoglobulin (IgV-like domain), a mucin-like domain with O-linked glycosylations and N-linked glycosylation sites close to the membrane, a transmembrane region and an intracellular, cytoplasmic tail with tyrosin phosphorylation motifs.

*Adapted from: Freeman, G., Immunol. Rev 2010 (235)*
2.4.2.1- Tim-3 and HIV Infection

Recently a novel population of dysfunctional T-cells was identified as marked by surface expression of the glycoprotein Tim-3 (128). The frequency of this population is shown to be increased in the blood of HIV-infected chronic progressors compared to uninfected individuals. Levels of Tim-3 expression on T-cells from HIV-infected individuals correlate positively with HIV viral load and CD38 expression (marker of ongoing T-cell activation) and inversely correlate with CD4\(^+\) T-cell count (128). In progressive HIV infection, Tim-3 expression is up-regulated on HIV-specific CD8\(^+\) T-cells. This Tim-3–expressing T-cell population in HIV-infected individuals is shown to be distinct from the PD-1–expressing population. Tim-3–expressing T-cells also demonstrate a failure to produce cytokine or to proliferate in response to antigen. The suppression of T-cell function by Tim-3 expression seems to contribute not only to the loss of virus-specific T-cell responses but also to the impairment of responses to other antigens. This is supported by the report that a subset of CMV and EBV-specific CD8\(^+\) T-cells in chronic HIV-infected individuals also expresses high levels of Tim-3 (128), and is consistent with observations that HIV-infected individuals generally have reduced responses to recall antigens and vaccinations (157).

The initiation of HAART in progressive HIV infection is shown to generally result in a decrease in T-cell Tim-3 expression (128). However, a subset of chronically infected individuals on HAART continues to sustain high levels of tim-3 expression, even with decreased HIV viral load. This is shown to be associated with persistent T-cell activation (128). Blocking the Tim-3 signaling pathway successfully restores proliferation and enhances cytokine production in HIV-specific T-cells in culture (128).
and seems to be a novel target for the therapeutic reversal of T-cell dysfunction in HIV infection.

### 2.4.2.2- Tim-3 and HCV Infection

Analysis of both peripheral and intra-hepatic T-cells in individuals chronically infected with HCV, has demonstrated an increase in the frequency of Tim-3-expressing CD4\(^+\) and CD8\(^+\) T-cells in comparison to uninfected controls (158). HCV-infected individuals were shown to have significantly greater frequencies of Tim-3 expression on HCV-specific CD8\(^+\) T-cells compared to CTLS specific for CMV.

Although the Tim-3 ligand, galectin-9 is widely distributed in various tissues, it is shown to be particularly abundant in the liver (155, 159). Consistent with the general hypothesis that the liver is enriched for functionally exhausted T-cells, Golden-Mason et al., found that a significantly higher percentage of total T-cells and HCV-specific CTLs in the liver express Tim-3 (158). The Tim-3/galectin-9 pathway is known to negatively regulate Tc1/Th1 cytokine secretion (160), which would hypothetically contribute to a failure to contain hepatic viral control, but also affect T-cell immunopathology. Accordingly, Golden-Mason et al. demonstrated that in vitro production of TNF-\(\alpha\) and IFN-\(\gamma\) was decreased among CD4\(^+\) and CD8\(^+\) T-cells that expressed Tim-3 compared to their Tim-3 negative counterparts (158). Tim-3 expression was shown to correlate with a dysfunctional and senescent phenotype (CD127\(^{low}\) CD57\(^{high}\)) and central rather than effector memory profile (CD45RA\(^{negative}\) CCR7\(^{high}\)). Blocking the Tim-3/Tim-3 ligand pathway was also shown to enhance T-cell proliferation and IFN-\(\gamma\) production in response to HCV-specific antigens in culture (158).
2.4.3-Role of Tim-3 and PD-1 Co-expression in T-Cell Exhaustion

Co-regulation of CD8+ T-cell exhaustion by PD-1 and Tim-3 was recently demonstrated during chronic LCMV infection (161). The majority of LCMV-specific CD8+ T-cells (up to 80%) in lymphoid and non-lymphoid organs of the LCMV mouse model were shown to co-express Tim-3 and PD-1 (161). This subset of CD8+ T-cells (Tim-3+/PD-1+) demonstrate the phenotype and functional characteristics of more severely exhausted T-cells than those expressing only PD-1 (161). This was shown as lack of proliferation and reduced secretion of effector cytokines including IFN-γ, TNF-α and IL-2. This subset was also shown to produce the suppressive cytokine IL-10. Simultaneous in vivo blocking of PD-1 and Tim-3 pathways in the LCMV mouse model results in a synergistic restoration of antiviral immunity and viral control, compared to blockade of either pathway alone (161). Hence, Tim-3 and PD-1 seem to cooperate and independently contribute to the negative regulation of CD8+ T-cell responses during chronic viral infections (Figure 2.7).
Figure 2.7
Figure 2.7- Tim-3 and PD-1 co-operate in T-cell Exhaustion during Chronic Viral Infections.

Tim-3 and PD-1 are both upregulated on antigen-specific T-cells during HIV and HCV infection, which leads to T-cell dysfunction. CD8 T-cell upregulation of Tim-3, leads to impaired proliferation, survival and cytokine production. Upregulation of PD-1 happens during earlier stages of exhaustion, which affects T-cell proliferation but not necessarily T-cell function. Blocking of each pathway restores the survival and functional capacity of antigen-specific T-cells.

Adapted from: Hafler, D., and Kuchroo, V., J. Exp. Med. 2008 (205)
2.6- Heterologous Immunity in Viral Infections

Large pools of memory T-cells that are specific for a variety of pathogens are developed during the lifetime of an individual. Memory T-cells are responsible for providing enhanced immunity upon re-exposure to the original pathogen. Re-exposure to the original virus initiates a rapid antigen-driven proliferation and activation of memory T-cells, which are at a higher activation state than naïve T-cells (162, 163). The memory T-cell population has a diverse T-cell receptor (TCR) repertoire and their network continually evolves, as immune responses to any new pathogen changes the frequencies and activities of these cells. This modulation of immune responses by the activated memory T-cells to an unrelated pathogen is referred to as heterologous or cross-reactive immunity.

2.6.1- T-cell Receptor Repertoire and Cross-reactivity

TCRs belong to the immunoglobulin super family of proteins that are expressed on the surface of T-cells and are responsible for recognizing foreign antigens. Germ line DNA contains four TCR multi-gene families, each encoding one of the four T-cell receptor chains (\(\alpha\), \(\beta\), \(\gamma\), \(\delta\)). The two T-cell receptors currently identified are each a heterodimer, made up of either one \(\alpha\) and one \(\beta\) chain (the \(\alpha\beta\) T-cell receptor) or one \(\gamma\) and one \(\delta\) chain (the \(\gamma\delta\) T-cell receptor). The \(\alpha\beta\) receptor is expressed on over 95% of peripheral blood T-cells. Each of the chains make up the heterodimer through the combination of four polypeptides chains; the variable (V), diversity (D), joining (J) and constant (C) regions. All the regions are encoded on separate gene segments that recombine, which results in increased potential diversity of each of the chains (164).
The TCR of CD8$^+$ T-cells recognize peptides of 8-10 amino acids that are embedded in a major histocompatibility complex class I (MHC-I) molecule. T-cells are both specific and “poly-specific” in this recognition. They are specific due to their recognition of a small fraction of the whole pool of MHC-peptide complexes, and poly-specific, due to the large size of this pool. Given that there are 20 amino acids and an average of 9 residues within a class I MHC-restricted peptide, this potential pool of MHC/peptide complexes would be as high as $20^9$. However, the actual number of T-cell clonotypes present in an individual is much lower than that and estimated to be about $10^6$ (165). Evidence suggests that T-cells are much more degenerate in recognition of antigens than what it was previously believed. TCR diversity and degeneracy are important factors in preventing pathogen escape mutations. Since only a few TCR contact residues on the peptide ligand are required for T-cell stimulation, certain amino acid substitutions in the peptide sequence can still result in the activation of T-cells (166). This recognition which is referred to as ‘molecular mimicry’ happens when a variant of an original peptide ligand interacts with the cognate TCR, inducing a total or partial T-cell activation.
2.6.1.1-Molecular Mimicry and Alternative Recognition of TCR

One of the best explained mechanisms underlying the degeneracy of T-cells refers to the potential capacity of a TCR -that recognizes a given MHC-peptide complex- to cross-recognize other peptides that contain the appropriate MHC motif and amino-acid side-chains that are able to stimulate the TCR. This degenerate quality of T-cells defines why a given TCR could potentially recognize a million different peptide-MHC combinations (165). Structural studies have demonstrated that peptides do not necessarily have to share similar amino-acid sequences to be able to stimulate TCR (167). Indeed, this molecular mimicry is only one of the mechanisms behind T-cell cross-reactivity. The ‘alternative recognition’ explains when different determinants of the TCR would recognize different peptides without any amino-acid similarities (168). This potential mechanism makes it very difficult to predict when cross-reactivity may occur. By contrast, cross-reactions that involve the same TCR determinants –as in case of molecular mimicry- are easier to predict. A third explanation for T-cell degeneracy describes when a given T-cell expresses two different TCRs as a result of incomplete allelic exclusion of the second TCR $\alpha$-chain (169) (Figure 2.8). This situation makes the prediction of cross-reactivity virtually impossible. Based on these potential mechanisms, the occurrence of T-cell cross-reactivity is probably more common than expected and more difficult to identify.
**Figure 2.8- Potential Mechanisms of T-cell Cross-reactivity.**

A. A T-cell receptor (TCR) recognizes and binds with a peptide presented by an MHC molecule. During molecular mimicry, a different peptide with similar determinants interacts with the same TCR in the same manner. During the alternative recognition of peptides, different determinants of the same TCR interact with a different peptide.

B. A single T-cell could potentially express two different TCR α chains that results in the recognition of two different peptides by the same T-cell.

2.6.2- T-cell Cross-reactivity and Immuno-dominance

The epitope-specific memory CD8+ T-cell repertoire is determined at the peak of the acute response to a viral infection. After the down-regulation phase, the frequencies of memory T-cells remain stable over time as long as the host is not exposed to a subsequent infection. If the host is exposed to sequential heterologous viral infections, memory T-cell pool to the original virus gradually shrinks. The pool of memory CD8+ T-cells that are created after a viral infection in a naïve host, demonstrate a distinct hierarchy of epitope-specific responses. Within this hierarchy, some epitopes become dominant and stimulate strong T-cell responses, while others become sub-dominant and stimulate weaker responses (170). Epitope immuno-dominance is regulated by several factors, including the efficiency of antigen processing and presentation, the affinity between peptide and MHC molecule and the availability of the specific TCR (171). When a pool of memory T-cells encounters a cross-reactive antigen, the higher frequency of those cells and their higher activation state give them an advantage over naïve T-cells. This can result in a preferential expansion of the cross-reactive T-cell population, which could, in turn, change the hierarchy of T-cell responses. This phenomenon has been observed in such sequential heterologous viral infections as LCMV and Pichinde virus (PV) in mice (172). These two distantly related viruses encode an epitope with a high amino acid sequence similarity. This epitope is normally subdominant for either virus. However, when LCMV-immune mice are infected with PV, or PV-immune mice are infected with LCMV, T-cell immune responses to that epitope become dominant (172). This is assumed to be due to the selective expansion of the epitope-specific cross-reactive memory CD8+ T-cells, and might partly explain the individual variability in the hierarchy
of immune responses with human viral infections. This could be seen in individuals with identical MHC-I haplotypes infected with either HIV or HCV, demonstrating different T-cell immuno-dominance repertoire (173). These data support the idea of cross-reactive T-cells contributing to the hierarchies of T-cell immune responses.

2.6.3- T-cell Cross-reactivity and Disease Pathology in Viral Infection

Experimental models of viral infections have shown that T-cell cross-reactivity to a heterologous virus can be partially protective, with a difference of life and death in the infected subject (174, 175). In a mouse-model of respiratory infection, heterologous immunity to LCMV infection prevented death in acute vaccinia virus (VV) infection, with marked changes in lung immunopathology (174). The development of severe immuno-pathology has also been demonstrated in sequential heterologous infections. Overall, studies indicate that prior viral infections can either augment or inhibit clearance of a subsequent infection with a heterologous virus (175). Even if protective, heterologous immunity may not be as protective as an original homologous immunity that results in high-affinity T-cell responses. Cross-reactive encounter elicits T-cell responses of lower affinity and could be more successful in enhancing pathologic rather than protective immunity. In this regard, low affinity cross-reactive memory T-cells might interfere with the development of more effective responses from high-affinity T-cells. This is the reminiscent of a phenomenon referred to as “Original Antigenic Sin”.

2.6.3.1- Original Antigenic Sin

“The deliberate sin of the first man is the cause of original sin”
The phenomenon of original antigenic sin was first proposed by Thomas Francis in 1950s (177). Francis described in his “Doctrine of Original Antigenic Sin” that: “antibody forming mechanisms appear to be oriented by the initial infections of childhood so that exposures later in life to antigenically related strains result in a progressive re-enforcement of the primary antibody.”

It has been the discovery of Influenza virus in the early 1930s that initiated an exploration to understand the epidemiology of pandemic viral infections. Studies on serologic data from flu-infected individuals demonstrated that the descendants of the flu virus of the 1918th pandemic were still circulating and that different human cohorts were displaying fundamentally different infection experiences. A Flu-vaccine trial in 1946 demonstrated higher sero-conversion titres to older virus strains that had infected the vaccine recipient (178). This was further confirmed in both animals and humans as a stronger antibody response to a secondary influenza infection rather than to the original viral variant that had infected the individual (179, 180) and subsequently in dengue, HIV and Malaria infections (181-183). More recently, the phenomenon of original antigenic sin has also been described with the involvement of cytotoxic T-cells (184). In this report, mice initially infected with LCMV-WE strain, responded to a subsequent infection of a WE-derived epitope with CTL responses mostly directed against the original epitope rather than the new variant (184).
2.6.4- Examples of T-cell Cross-Recognition of Distant Viral Antigens

2.6.4.1-HCV and Influenza Virus

Analysis of CTL responses to HCV-specific epitopes in the blood of HCV negative donors was first described by Cerny et. al. (185). Different hypotheses were proposed to explain this observation. It was suggested that these individuals might have been infected with HCV in the distant past and spontaneously cleared the virus, lost humoral responses but maintained cellular immune responses (186). Another proposed explanation was that these individuals could have been exposed to HCV and generated HCV-specific memory T-cells without viremia (187). It was also suggested that HCV-specific CD8\(^+\) T-cell responses could have been induced \textit{in vitro} by the prolonged stimulation with HCV peptides. This last hypothesis however, could not explain why these HCV-specific responses were isolated from only a subset of healthy donors and not all. All together, these findings suggested another hypothesis, that these supposedly HCV-specific T-cell responses may represent cross-reactive memory responses to other pathogens.

Wedemeyer et al. (188) were the first to examine this idea by the analysis of CD8\(^+\) T-cell responses of healthy individuals to HCV antigens and another pathogen, the Influenza virus. They narrowed down their analysis to an immuno-dominant HCV epitope, the HLA-A2-specific NS3-1073 epitope. The CD8\(^+\) T-cells specific to this epitope were shown to be expanded from the blood of 60% of studied healthy individuals and displayed the phenotype of memory T-cells (188). Their database search for the highest degree of amino-acid homology to this HCV peptide resulted in the identification of a new T-cell determinant in influenza proteome. The HLA-A2-specific flu-
neuraminidase (NA-231) peptide was shown to have a high degree of amino-acid similarity with HCV-NS3 epitope. Direct *ex-vivo* analysis demonstrated the presence of Flu-NA-specific T-cells in the blood of those healthy donors with the HCV-NS3-specific responses (188). They were also able to show the induction of HCV-NS3-specific responses after the Flu-IV infection in HLA-A2-transgenic mice (188). Later, Urbani, et al., demonstrated that HCV-infected patients with severe fulminant hepatitis, but not those with milder disease, had CD8<sup>+</sup> T-cell responses in their blood that were narrowed down to only one single HCV epitope, the NS3-1073. These HCV-specific T-cells were then shown to cross-recognize the Flu-NA epitope, previously described (189). The authors suggested that this T-cell cross-reactivity could have influenced the severity of the HCV-associated disease, since hypothetically the focusing of T-cell responses to a single immuno-dominant epitope may have severe pathological consequences. They argued that although sensitization to Flu is common, the individual repertoire of memory T-cells might play a role in the induction of cross-reactivity between Flu and HCV.

More recently, Kasprowicz et al. (190) demonstrated that although FLU-NA response was not prevalently seen in their studied cohort, HCV-NS3 specific CD8<sup>+</sup> T-cells were identified from HCV-infected individuals that cross-reacted with the FLU-NA epitope. These authors showed that the cross-reactive T-cells only weakly responded to the FLU-NA epitope, indicating that the FLU-NA peptide was a weak agonist, and was mainly a consequence of the presence of a pre-existing response to the HCV-NS3 epitope.
2.6.4.2- EBV and Influenza Virus

The role of heterologous cross-reactivity in the variable disease outcome of Epstein-Barr Virus (EBV)-associated Infectious Mononucleosis (IM) was reported by Clute et al. (191). They focused their study on the influence of the most commonly encountered virus, Influenza and its immuno-dominant epitope M1-58 from the matrix protein. They demonstrated a cross-reactive response specific for the two dissimilar epitopes, Flu-M1 and EBV-BMLF1, both in bulk T-cell cultures and T-cell clones. The frequencies of cross-reactive T-cells specific for M1 and BMLF1 were shown to correlate with IM disease severity (191).

2.6.4.3- HIV and Influenza Virus

The matrix protein of the influenza virus and the matrix and capsid proteins of HIV were shown to share some degree of structural similarities (192). When the influenza and HIV matrix proteins were compared by aligning selected stretches of their amino acid chains the HLA-A2-restricted FLU-M1 peptide (GILGFVFTL) was almost perfectly aligned with the sequence SLYNTIAVL which is a variant of the HIV p17 gag epitope SLYNTVATL, despite lack of amino-acid sequence similarities. Following this report, Acierno et al. demonstrated cross-reactivity between Flu-M1 and HIV-gag p17 specific CTL in HIV seropositive donors (193). They also showed that in vitro stimulation of PBMCs from seronegative donors with either Flu-M1 or Gag-p17 also resulted in the generation of measurable cross-reactive T-cell responses to either epitope. Their analysis of the β-chain TCR gene in a seronegative donor suggested that the cross-recognition of these two epitopes is due to the in vitro expansion of flu-specific memory T-cells.
Chapter 3:

Role of HIV-Specific T-cells in the liver during

HCV/HIV co-infection
3.1- HIV-Specific T-Cells Accumulate in the Liver in HCV/HIV Co-Infection

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3.1.1-Abstract

**Background and Aims:** Hepatitis C Virus (HCV)-related liver disease progresses more rapidly in individuals co-infected with Human Immunodeficiency Virus-1 (HIV), although the underlying immunologic mechanisms are unknown. We examined whether HIV-specific T-cells are identified in the liver of HCV/HIV co-infected individuals and promote liver inflammation through bystander immune responses. **Methods:** Ex-vivo intra-hepatic lymphocytes from HCV mono-infected and HCV/HIV co-infected individuals were assessed for immune responses to HIV and HCV antigens by polychromatic flow cytometry. **Results:** HCV/HIV liver biopsies had similar frequencies of lymphocytes but lower percentages of CD4+ T-cells compared to HCV biopsies. In co-infection, intra-hepatic HIV-specific CD8+ and CD4+ T-cells producing IFN-γ and TNF-α were detected and were comparable in frequency to those that were HCV-specific. In co-infected individuals, viral-specific CD8+ T-cells produced more of the fibrogenic cytokine, TNF-α. In both mono- and co-infected individuals, intra-hepatic HCV-specific T-cells were poorly functional compared to HIV-specific T-cells. In co-infection, HAART was not associated with a reconstitution of intra-hepatic CD4+ T-cells and was associated with reduction in both HIV and HCV-specific intra-hepatic cytokine responses. **Conclusion:** The accumulation of functional HIV-specific T-cells in the liver during HCV/HIV co-infection may represent a bystander role for HIV in inducing faster progression of liver disease.
3.1.2-Introduction

Approximately 25% of Human Immunodeficiency Virus-1 (HIV) infected individuals are also infected with Hepatitis C Virus (HCV) (72). HIV adversely affects each stage of the natural history of HCV infection. Fewer individuals recover spontaneously from HCV infection when also infected with HIV (194). Among those with persistent HCV infection, HIV co-infection is associated with higher HCV viremia and more rapid progression to cirrhosis and hepatocellular carcinoma (195). A recent meta-analysis showed that HIV co-infection increased the risk of histological hepatic cirrhosis by two-fold and clinically decompensated liver disease by six-fold (3). In addition, HCV co-infection is associated with increased incidence of HAART (highly active antiretroviral therapy) related liver injury (196).

The mechanisms for hepatic damage in HCV/HIV co-infection are poorly defined. Although intra-hepatic T-cell immune responses are necessary for HCV clearance, they have also been shown to play a central role in mediating hepatocellular injury by direct cytotoxicity or indirectly by releasing cytokines. In this regard, IFN-γ has been shown to be anti-fibrogenic, whereas, TNF-α activates hepatic stellate cells, which induce fibrosis, and likely contributes to progression to cirrhosis (197, 198).

Potent and broad CD4+ and CD8+ T-cell immunity are important for virologic control in both HCV and HIV viral infections. Ex-vivo HCV-specific CD8+ T-cell responses in peripheral blood mono-nuclear cells (PBMCs) from mono-infected individuals are generally weak (199). Although, peripheral HCV-specific CD4+ and CD8+ T-cell responses are somewhat weaker in HCV/HIV co-infected individuals (91), similar
frequencies of intra-hepatic HCV-specific responses appear to be obtained in HCV versus HCV/HIV co-infection (92, 200). However, ex-vivo HIV-specific CD8+ T-cell responses in PBMCs from HIV mono-infected individuals are about one log higher than ex-vivo HCV-specific responses in HCV mono-infection. In addition, impairment in cellular immune responses to HCV compared to HIV has been shown in HCV/HIV co-infection (89). HIV-specific CD8+ T-cells are easily detectable in blood of untreated HIV infected individuals (88). Such high frequencies of HIV-specific T-cells circulating in peripheral blood led us to question whether these cells could also migrate to the liver in HCV/HIV co-infection and through bystander responses add to the inflammation induced by HCV-specific T-cells.

3.1.3-Materials and Methods

3.1.3.1- Study participants

HCV mono-infected and HCV/HIV co-infected individuals who required liver biopsies for work up of liver disease were recruited for the study (see Results and Table 3.1). All study participants provided informed, written consent and the study protocol was approved by the research ethics board at the University of Toronto and St. Michael's Hospital. Both blood and liver biopsy samples were received from each participant.
3.1.3.2-Isolation of intra-hepatic lymphocytes from liver biopsy

Liver biopsy samples were washed in RPMI-1640 to remove contaminating blood lymphocytes, manually homogenized with a plastic plunger, and treated with DNase (0.002%, Sigma) and collagenase IV (0.02%, Sigma) for 30 minutes, stirring at 37°C. The digested cell suspension was filtered through a 70 µm strainer, washed and re-suspended in R-10 medium (10% fetal calf serum).

3.1.3.3-IFN-γ ELISPOT epitope mapping in PBMCs

In order to identify candidate epitope-specific responses to be detected in ex-vivo liver samples, we first mapped antigen-specific T-cell responses in blood against the entire HIV-1 clade-B and HCV-1a proteome using the matrix approach by IFN-γ ELISPOT assay as described previously (201). Mapped peptides were then pooled to evaluate hepatic responses. In order to address the possibility that differing epitopes were only targeted in the liver, we also used four peptide pools that previously were shown to target a majority of responses. These pools spanned HIV-Gag and HCV-NS3, HCV-NS4 and HCV-Core protein (2 µg/peptide/ml, from National Institute of Health Reagent Program). Of the HCV pools, the pool that gave the strongest ELISPOT response in PBMCs was used for hepatic cell stimulation (see below).

3.1.3.4-Ex-vivo stimulation and intracellular staining

All the extracted cells from each liver biopsy were split in three wells and stimulated on the same day as PBMCs. 1×10⁶ PBMCs and liver- isolated cells were stimulated with either DMSO, HIV or HCV peptide pools as described previously (201).
HIV pools consisted of peptides that were screened by the matrix approach in that individual plus the HIV-Gag pool. Likewise, HCV pools consisted of mapped peptides plus an HCV pool that gave the strongest response in PBMCs. CD107a antibody (PE-Cy5, BD Pharmingen) was added at the time of stimulation. The following antibodies were used for staining: CD8-PE Texas-Red (Beckman Coulter), CD4-Pacific Blue (e-Bioscience), CD3-APCCY7, IFNγ-FITC, TNFα-PECY7, IL2-APC, MIP-1β-PE (BD Bioscience), PD-1 FITC (Biolegend) and dead cell stain Aqua (Invitrogen, Molecular Probes).

3.1.3.5-Flow cytometry

Cells were analyzed on a multi-color FACSaria flow cytometer (BD Biosciences). For Blood samples between 500,000 to 1,000,000 total events and for liver biopsy samples between 50,000 to 200,000 total events were collected. Data analysis was performed using FlowJo version 8.6 (Treestar Inc., San Carlos, CA). Polychromatic FlowJo data were analyzed with PESTLE software, and pie-chart graphs were generated using SPICE software (obtained from M. Roederer, National Institutes of Health, Bethesda MD).

3.1.3.6-Tetramer Staining

Multi-parameter analysis of HIV-Gag: 77–85 (SLYNTVATL: SL9) specific CD8+ T-cells was conducted in both blood and liver of co-infected individuals initially identified with a positive ELISPOT response to the 15-mer HIV peptide including SL9 epitope, using the corresponding tetramer (iTAg MHC Class-I tetramer, Beckman
Coulter). Tetramer staining was performed prior to peptide stimulation, at room temperature for 20 minutes. Tetramer stained cells were then washed and stimulated with 10 µg/ml of SL9 peptide followed by ICS staining as mentioned above. Additional HIV, HCV and CMV-specific pentamer staining (Pro5 MHC class I Pentamers -Proimmune) was conducted followed by PD-1 staining.

3.1.3.7-Statistical analysis

Data were analyzed by performing two-tailed non-parametric Mann-Whitney test using GraphPad Prism version 4.00. P-values ≤0.05 were considered significant.

3.1.4-Results

3.1.4.1-Subject Characteristics

Three groups of individuals were studied as depicted in Table 3.1; HCV mono-infected (n = 6), HCV/HIV co-infected who were not receiving HAART (n = 8) and HCV/HIV co-infected who were receiving HAART for greater than one year at the time of evaluation (n = 12). All individuals never received prior treatment for HCV and underwent liver biopsies for staging and evaluation for pegylated-interferon/ribavirin treatment. HCV/HIV co-infected individuals had higher HCV viral loads. On average, CD4 T-cell counts of HIV infected individuals were >400/µl in both groups. Of note, the mean hepatic fibrosis scores were higher in the HAART treated and mono-infected groups in this cohort, indicating that individuals in these groups had more advanced disease at the time of biopsy in this study.
3.1.4.2-Characterizing intra-hepatic lymphocytes

Although similar frequencies of intra-hepatic lymphocytes were obtained in dual versus mono-infection, HAART-treated individuals showed a trend towards greater percentages of lymphocytes in their biopsies (Figure 3.1a). The percentage of intra-hepatic \( \text{CD4}^+ \) T-cells was significantly reduced in dual infection [31.6\%\±13.8 for HCV vs 6.5\%\±2.9, for HCV/HIV therapy naïve, \( p<0.01 \)], and was not associated with any improvement in HAART-treated individuals, as previously shown in the gut (202) (Figure 3.1b). However, compared to HCV mono-infected individuals the percentage of intra-hepatic \( \text{CD8}^+ \) T-cells was higher in both co-infected groups [33.8\%\±5.5\% for HCV vs 67.3\%\±15.5\% for HCV/HIV therapy naïve vs 59.5\%\±15.1\% for HCV/HIV on HAART, \( p<0.01 \)] (Figure 3.1b).

3.1.4.3-Intra-hepatic viral specific immune responses

To determine the presence of intra-hepatic viral specific T-cell responses, liver isolated cells were stimulated with HCV and HIV peptide pools. Summary data of viral specific responses are depicted in Figure 3.2. In response to stimulation with HIV peptide pool, untreated co-infected individuals showed significantly higher frequencies of intra-hepatic \( \text{CD4}^+ \) T-cells producing IFN-\( \gamma \), compared to HCV mono-infected [0.16\%\±0.05\% vs 0.02\%\±0.01\%, \( p<0.05 \)], and HAART-treated co-infected individuals [0.16\%\±0.05\% vs 0.03\%\±0.05\%, \( p<0.05 \)] (Figure 3.2a). Untreated co-infected individuals showed a trend towards lower frequencies of intra-hepatic IFN-\( \gamma \) producing \( \text{CD4}^+ \) T-cells in response to HCV peptides. Surprisingly, HAART-treated co-infected individuals had significantly
reduced HCV-specific IFN-γ producing CD4+ T-cells when compared to untreated co-infected individuals [0.02±0.01% vs 0.46±0.11%, respectively, p<0.01] (figure 3.2a).

Therapy naïve co-infected subjects had greater IFN-γ producing CD8+ T-cells in response to HIV peptides compared to HCV mono-infected individuals [1.39±0.37% vs 0.02±0.0%, p<0.05], and HAART was associated with a significant reduction in the frequencies of these cells [1.39±0.37% vs 0.30±0.26%, p<0.05] (figure 3.2b). Although there was a trend for enhanced intra-hepatic CD8+ T-cells producing IFN-γ in response to HCV peptides in therapy-naive co-infection compared to HCV mono-infection, this was not found to be statistically significant. HAART on the other hand, was associated with a significant reduction in HCV-specific, intra-hepatic CD8+ T-cells producing IFN-γ [1.3±0.37% vs 0.03±0.01%, p<0.05] (figure 3.2b).

Similarly, co-infected individuals had significantly greater intra-hepatic TNF-α expressing CD4+ T-cells after HIV peptide stimulation compared to HCV mono-infected [0.2±0.05 vs 0.02±0.01, p<0.01], although HAART had no significant effect on their frequencies (Figure 3.2c). HCV mono-infected individuals showed significantly higher frequencies of HCV-specific TNF-α producing CD4+ T-cells compared to HAART-treated co-infected individuals [0.91±0.25% for HCV vs 0.23±0.20 for HCV/HIV on HAART, p<0.01] (figure 3.2c), but did not show significant differences with the untreated co-infected group.

The therapy-naïve co-infected group showed significantly higher frequencies of intra-hepatic TNF-α producing CD8+ T-cells in response to both HIV-1 and HCV antigens. Both types of responses were shown to be reduced in the HAART group [HIV-
specific: 0.02±0.01% vs 1.08±0.21% vs 0.11±0.06%; HCV vs HCV/HIV vs HCV/HIV on HAART, p<0.01 for all], [HCV-specific: 0.59±0.2% vs 1.16±0.19% vs 0.20±0.07%; HCV vs HCV/HIV vs HCV/HIV on HAART, p<0.05 for all] (figure 3.2d).

3.1.4.4-Multi-parameter analysis of T-cell functions

To study the functional profile of virus-specific T-cells in HCV/HIV co-infection, simultaneous expression of 5 distinct CD8^+ T-cell markers were analyzed in 3 individuals within each cohort using a previously developed multicolor flow cytometry method (126). Expression levels of degranulation marker CD107a and cytokines IFN-γ, TNF-α and IL-2, as well as the chemokine MIP-1β were simultaneously measured in response to HCV or HIV peptides in both blood and liver of each individual. Figure 3 depicts a representative multi-parameter analysis of CD8^+ T-cell responses in liver and blood of a therapy-naïve, co-infected subject in response to HIV and HCV peptide pools. These data indicate that both HCV and HIV specific CD8^+ T-cells expressing one or more functions are detectable in the liver and blood. Compared to blood, the frequency of HIV-specific T-cells producing CD107a and IL-2 was shown to be significantly higher in the liver of therapy-naïve, co-infected individuals. Consistent with previously reported data (92), HCV-specific responses were compartmentalized to the liver and stronger than peripheral HCV-specific responses (Figure 3.3c).
3.1.4.5-Recognition of functional CTL, specific for the HLA-A0201-restricted HIV-SL9 epitope in HCV/HIV co-infected liver

To determine if T-cells specific for an HIV immuno-dominant epitope are present in HCV/HIV co-infected liver, we quantified CD8+ T-cells specific for HLA-A*0201-restricted SLYNTVATL (SL9) epitope in the liver of individuals with positive SL9 responses in their blood. We identified 3 co-infected HLA-A*0201 individuals, among them one showed a response to the SL9 epitope of HIV-Gag antigen. Figure 4 shows the multi-parameter analysis of tetramer positive CD8+ T-cells in blood and liver of this therapy-naïve, co-infected individual. The tetramer cytokine response pattern was shown to be different in the liver compared to blood of the same individual, with diminished intra-hepatic tetramer-specific IFN-γ responses and an increase in both CD107a and TNF-α responses, with the majority of SL9 tetramer positive cells expressing these two markers.

We also included CMV as a non-hepatotropic control virus in our liver analysis. Using a pool of HLA-A*0201- and HLA-A*2402-restricted, CMV-specific pentamers, we did not detect any CMV-specific CD8+ T-cells in HCV/HIV co-infected liver, although we could readily detect them in blood (Figure 3.4c).

3.1.4.6-Hierarchy of viral-specific CD8+ T-cell function during HCV mono and HCV/HIV co-infection

Using the panel of markers TNF-α, IFN-γ, MIP-1β, IL-2, and CD107a, we characterized the ability of CD8+ T-cells to simultaneously exert these ‘functions’ in
response to both HCV and HIV peptides. Figure 5 depicts a representative functional profile of virus specific CD8$^+$ T-cells in blood and liver during HCV/HIV co-infection (Fig. 5a) and HCV mono-infection (Fig. 3.5b). Analysis of co-infected subjects demonstrates a very limited functional hierarchy of HCV-specific T cells in the blood, with majority of T-cells producing one function. HIV-specific T-cells from blood had a more expanded functional hierarchy. In accordance with previous reports on HIV mono-infected individuals (126), cells expressing all 5 functions were absent in the blood of co-infected subjects, mainly due to lack of IL-2 production. In co-infection, intra-hepatic CD8$^+$ T-cells responding to HCV peptides were within the single-responding and 2+ populations. Intra-hepatic CD8$^+$ T-cell responses to HIV peptides produced a larger spectrum of responses. CD107a responding cells were represented in nearly all of the different HIV-specific populations in the liver of co-infected individuals.

As expected, HCV-specific responses in the blood of HCV mono-infected subjects were mainly single functional. The profile of HCV-specific responses in the liver of HCV mono-infected individuals showed the appearance of a very small population of 4+ responding cells in the liver.

We and others have considered a cutoff point of more than 2 simultaneously expressed markers to demonstrate poly-functional characteristic of responding T-cells (126, 203). Figure 3.5c shows a comparison between average frequency of intra-hepatic viral-specific responses within the pool of CD8$^+$ T-cell populations expressing 2 markers or less, and those within the pool of populations expressing more than 2 markers simultaneously. For both HCV and HIV-specific CD8$^+$ T-cells the majority of responses
had two or less functions. However, intra-hepatic HIV-specific responses demonstrated more poly-functionality, compared to HCV-specific responses either within co-infected or mono-infected individuals [0.05±0.01 vs 0.007±0.00, p<0.05; HIV-specific responses vs HCV-specific responses in HCV/HIV co-infected group]; [0.05±0.01 vs 0.01±0.00, p<0.05; HIV-specific responses in HCV/HIV co-infected group vs HCV-specific responses in HCV mono-infected group]. In summary, although viral-specific T-cells, simultaneously expressing all 5 measured markers were rarely found in the liver, intra-hepatic HIV-specific T-cells showed greater functional capacity when compared to those being HCV-specific.

Based on the recently highlighted role of PD-1 contributing to the dysfunction of T-cells in chronic viral infections, we also determined whether HIV and HCV-specific intra-hepatic T-cells differ in the degree of PD-1 expression. In an HCV/HIV co-infected liver, we found that 100% of intra-hepatic HCV-specific CD8\(^+\) T-cells were PD-1 positive, compared to 48.8% of those cells that were HIV-specific (Figure 3.5d).

### 3.1.5-Discussion

This is the first study to demonstrate the presence of HIV-specific T-cells within the liver of HCV/HIV co-infected individuals. The finding of HIV-specific T-cells within liver of co-infected individuals may not altogether be surprising, given the high frequencies of HIV-specific CD8\(^+\) T-cells found in the peripheral blood in untreated HIV infection. Nevertheless, it is surprising to find functional T-cells of such viral specificities to be accumulating in liver. In contrast, we could not detect CMV-specific T-cells in co-infected liver despite their abundance in blood indicating that different viruses target T-
cells to the liver. Recent studies have demonstrated that systemic viral infections may recruit viral-specific T-cells to the liver. The significance of non-hepatotropic viral-specific T-cells that are found in liver is unclear. It has been postulated that the liver can non-selectively trap activated T-cells during any infection, and thus act as a ‘sink’ or ‘graveyard’ (204), however it is unclear whether these cells are rendered anergic while traveling in the liver or contribute to inflammation and damage as a result of bystander activation. Of note, is that hepatitis has been observed in measles (205), SARS (206) and in 20% of individuals with acute HIV infection (207). Polakos et. al. (208) found that some individuals infected with influenza-A develop transaminitis and showed in a murine influenza model that influenza-specific CD8+ T-cells migrate to the liver and induce hepatic damage from by-stander activation. Non-hepatotropic viruses such as HIV, CMV and EBV, in general do not induce chronic hepatitis, thus, it is possible that the co-existence of hepatotropic viruses may alter the hepatic environment to allow recruitment of activated T-cells non-specifically. This could be due to an up-regulation of integrins such as ICAM-1 and VCAM-1 in hepatic sinusoids as previously shown during HCV infection (209) that could enhance T-cell recruitment. In this regard, Spangenberg et. al. (210) demonstrated the presence of influenza-specific T-cells in about 50% of liver biopsies from HCV mono-infected individuals.

There are several lines of evidence demonstrating that the liver efficiently clears many foreign pathogens, including RNA viruses. It is shown that liver is a major organ for clearing Simian Immunodeficiency Virus in rhesus monkeys (119). There is also evidence for the detection of HIV RNA in the liver of HIV infected individuals (121). These findings support the identification of HIV-specific T-cells in the liver. In
HCV/HIV co-infection, it is possible that intra-hepatic HCV-specific CD4\(^+\) T-cells become infected with HIV and recruit HIV-specific immune responses to this site. Evidence for these potential mechanisms will need further analysis on liver biopsies of co-infected individuals.

Our analysis of liver biopsies from HCV/HIV co-infected individuals not only demonstrate that HIV-specific T-cells producing IFN-\(\gamma\) and TNF-\(\alpha\) are detected in the liver, but also exhibit comparable frequencies of responses to those that are HCV-specific. This observation may explain the added contribution of HIV-specific immune responses to the ongoing intra-hepatic damage induced by HCV-specific T-cell responses that are inefficient in clearing the virus. Therapy naïve co-infected individuals demonstrated a higher frequency of intra-hepatic CD8\(^+\) T-cells that produce TNF-\(\alpha\) in response to both HCV and HIV antigen stimulation compared to HCV mono-infected individuals. In addition, we identified CD8\(^+\) T-cells specific for an immunodominant HIV epitope in co-infected liver, demonstrating high frequency of TNF-\(\alpha\) expression. Intra-hepatic TNF-\(\alpha\) has been previously associated with liver fibrosis, and the accumulation of cells expressing this marker may explain in part the faster rate of liver disease progression found in HCV/HIV co-infection. Further comparisons of TNF-\(\alpha\) responses between immunodominant HCV and other HIV epitopes in a larger cohort of individuals are warranted. Contrary to our expectation, viral-specific, intra-hepatic levels of IFN-\(\gamma\) were also higher in the therapy-naïve co-infected group, which would be against the expected protective role of IFN-\(\gamma\). However, we interpret this observation as a potential effect of the fibrogenic TNF-\(\alpha\) to mask IFN-\(\gamma\) protection. On the other hand, viral-specific T-cells are composed of several major populations with unique functional patterns.
Therefore, measurement of only one or two T-cell functions may not provide a comprehensive picture of the quality of T-cell responses.

Recent lines of evidence demonstrate the importance of the qualitative rather than quantitative characteristics of CD8\(^+\) T-cell responses to efficient viral control (88, 211). The significance of T-cell populations simultaneously representing 5 different functions has been discussed as a hierarchical functional model in viral infections such as CMV and EBV which are effectively controlled by respective CD8\(^+\) T-cells (126). HCV-specific CD8\(^+\) T-cells were not poly-functional which is consistent with the notion that although HCV-specific T-cells are found in hepatic tissue, their loss of poly-functionality may be associated with inefficient control of HCV replication. HIV-specific T-cells in the liver of co-infected individuals however, simultaneously could express 4 and 5 of the measured markers. Recently, T-cell exhaustion has been related to signaling pathways through PD-1 (127, 130). Our analysis of PD-1 levels of antigen-specific CD8\(^+\) T-cells from co-infected liver demonstrates higher expression of PD-1 on HCV-specific T-cells, compared to those specific for HIV, supporting the notion that the former are less functional. The observed poly-functionality of intra-hepatic HIV-specific T-cells, should have little effect on HCV replication but would further enhance the cytokine milieu induced from bystander activation, and contribute to liver damage during co-infection with HCV. In this regard, we found that the degranulation marker CD107a dominates the HIV-specific CD8\(^+\) T-cell responses in the liver, with the majority of the responding cells expressing CD107a, a surrogate marker for the cytotoxic function of CD8\(^+\) T-cells. Activated HIV-specific CD8\(^+\) T-cells with the potential to degranulate could induce bystander damage. In addition, the release of chemokines such as MIP-1\(\beta\) by the same
cells could also attract further lymphocytes without HCV specificity to the liver. Bystander function of these non-specific T-cells could expand the tissue damage triggered by HCV infection and ultimately activate fibrogenesis.

We found that the frequency of CD4\(^+\) T-cells within livers of co-infected individuals was reduced compared to HCV mono-infection. Surprisingly, HAART did not appear to reconstitute the CD4\(^+\) T-cell population within liver. Despite this defect of CD4\(^+\) T-cell help, comparable frequencies of HCV-specific-CD8\(^+\) T-cells were found in co-infected livers. HAART-treated biopsies showed further reduced frequencies of HCV-specific responses. These data support previous findings that show HAART induces CD4\(^+\) T-cell recovery but not any restoration of HCV-specific T-cell responses peripherally (90). Further investigation is needed to clarify the role of CD4\(^+\) T-cell help in affecting the frequencies of HCV-specific CD8\(^+\) T-cells in HCV/HIV-1 co-infection. HAART was also associated with a reduction in frequencies of HIV-specific T-cell responses within liver, indicating that removing the HIV antigenic load may also reduce the opportunity for such cells to accumulate within hepatic tissue.

Here, we propose a novel mechanism for enhanced HCV-related liver disease progression in HIV co-infection; that of bystander activation and induced inflammation from HIV-specific T-cells accumulated in the liver. Our data however are limited in the cross-sectional nature of our cohort, the low number of analyzed liver biopsies and the narrow range of CD4\(^+\) T-cell counts among the studied individuals. We should also acknowledge that ex-vivo functional T-cell capacity may not exactly reflect the situation in-vivo. Further studies, particularly, those which are prospective are warranted in order
to understand the role that HIV-specific T-cells play in contributing to fibrosis and in particular how HAART modulates these responses.
Table 3.1-Characteristics of HCV mono-infected and HCV/HIV co-infected individuals, untreated for HCV.

<table>
<thead>
<tr>
<th></th>
<th>HCV mono-infected (n = 6)</th>
<th>HCV/HIV-1 Co-infected</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>not on HAART (n = 8)</td>
<td>on HAART (n = 12)</td>
<td></td>
</tr>
<tr>
<td>Age (y, mean)</td>
<td>51(±3.5)</td>
<td>42(±5.7)</td>
<td>41(±2.8)</td>
</tr>
<tr>
<td>Sex (no. male)</td>
<td>5</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>HCV genotype (no. type 1)</td>
<td>5</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>HCV plasma VL (log10 IU/mL, mean)</td>
<td>5.03(±0.51)*</td>
<td>6.22(±0.12)*</td>
<td>6.3(±0.08)*</td>
</tr>
<tr>
<td>HIV plasma VL (log10 copies/ml, mean)</td>
<td>na</td>
<td>6.5(±0.15)</td>
<td>1.43(±0.37)</td>
</tr>
<tr>
<td>CD4 count/µl (mean)</td>
<td>na</td>
<td>492(±34.4)</td>
<td>599(±56.9)</td>
</tr>
<tr>
<td>ALT (mean)</td>
<td>65(±13)*</td>
<td>70(±16)</td>
<td>109(±52)*</td>
</tr>
<tr>
<td>Liver Biopsy Inflammation Grade (mean)</td>
<td>1.7(±0.16)</td>
<td>1.9(±0.25)</td>
<td>1.7(±0.15)</td>
</tr>
<tr>
<td>Liver Biopsy Fibrosis Score (mean)</td>
<td>3(±0.6)*</td>
<td>1.5(±0.3)*</td>
<td>2(±0.5)</td>
</tr>
<tr>
<td>Lymphocytes count/liver biopsy1 (mean)</td>
<td>43884(±11007)</td>
<td>47095(±7410)</td>
<td>59414(±5165)</td>
</tr>
</tbody>
</table>

1. Normalized to 100,000 events count.
na = not applicable.
no = number of participants.
HAART individuals were treated >1 year.
doi:10.1371/journal.pone.0003454.t001
Figure 3.1-Characteristics of intra-hepatic lymphocytes from HCV and HCV/HIV co-infected individuals

Frequencies of lymphocytes obtained from liver biopsies normalized to total live mononuclear cell count from FACS analysis are illustrated in (a) with percent composition of intra-hepatic CD4 and CD8 expressing T-cells shown in (b).
Figure 3.2

A

Intra-Hepatic Antigen-Specific IFN-γ Responses

% of Total CD4+ T-Cells

HCV Mono  HCV/HIV Naive  HCV/HIV on HAART

P < 0.01
P < 0.05

B

Intra-Hepatic Antigen-Specific IFN-γ Responses

% of Total CD8+ T-Cells

HCV Mono  HCV/HIV Naive  HCV/HIV on HAART

P < 0.05
P < 0.01
P < 0.05

C

Intra-Hepatic Antigen Specific TNF-α Responses

% of Total CD4+ T-Cells

HCV Mono  HCV/HIV Naive  HCV/HIV on HAART

P < 0.01
P < 0.01

D

Intra-Hepatic Antigen Specific TNF-α Responses

% of Total CD8+ T-Cells

HCV Mono  HCV/HIV Naive  HCV/HIV on HAART

P < 0.05
P < 0.01
Figure 3.2- Antigen-specific cytokine production by intra-hepatic T-cells in HCV mono-infection and HCV/HIV co-infection

Figure (a) represents the summary data of the percentage of intra-hepatic CD4$^+$ T-cells expressing IFN-γ in response to either HCV or HIV peptide stimulation in all the three studied cohorts, with figure (b) representing the same responses by CD8$^+$ T-cells. Figures (c) and (d) represent the percentage of viral-specific, intra-hepatic TNF-α producing CD4$^+$ and CD8$^+$ T-cells respectively.
Figure 3.3

A

![Graph showing data for different conditions and peptides over CD8, IFN-γ, IL-2, and TNF-α](image)

B

![Graph showing data for different conditions and peptides over CD8, IFN-γ, IL-2, and TNF-α](image)

C

![Bar charts comparing HIV- and HCV-specific CD8+ T-cells](image)
Figure 3.3- Polychromatic FACS analysis of viral-specific T cells in HCV/HIV co-infection

Shown are representative FACS data of the HIV and HCV specific multi-parameter CD8+ T-cell responses from (a) liver and (b) blood of subject OM 405, a therapy-naïve HCV/HIV co-infected individual, after in vitro stimulation using pool of HIV and HCV peptides. Initial gating on forward scatter area (FSC-A) versus height (FSC-H) was used to remove doublets. The events were further gated on forward scatter (FSC) versus the dead cell marker to remove dead cells. Lymphocytes were gated on the remaining live cells on a FSC versus SSC plot. Gates on CD3+/CD8+ cells were then generated. All responses are gated on a CD3+/CD8+ population and presented against TNF-α on the x-axis. Figure (c) shows a comparison of the frequency of HIV and HCV-specific CD8+ T-cells in the liver and blood of therapy-naïve, co-infected individuals. All intra-hepatic HCV-specific responses are significantly stronger than peripheral HCV-specific responses.
Figure 3.4

A

SL9 Tetramer

B

SL9 Tetramer

C

Blood

Control (No Pentamer)

HCV Pentamers

HIV Pentamers

CD 8

Liver
**Figure 3.4-** Tetramer positive cells specific for HIV are identified in the liver in HCV/HIV co-infection

Representative multi-parameter FACS plots of HIV-SLYNTVATL (SL9) tetramer positive/CD8\(^+\) T-cells are shown in a) blood and b) liver of a co-infected individual (OM 403). Gating scheme on tetramer positive responses are based on initial gating on responses from the tetramer negative population. Figure (c) represents the frequency of CD8\(^+\) T-cells specific for HCV, HIV and CMV in HCV/HIV co-infected liver (OM 385). Liver isolated mononuclear cells were stained with pools of HLA-A*0201 and HLA-A*2402-restricted pentamers (Pro5 MHC class I Pentamers, Proimmune), followed by staining for cell surface markers CD3 and CD8. The following pentamers were used for each group: HCV pentamers: NS3-CINGVCWTV and NS3-KLVALGINAV; HIV pentamers: Pol-ILKEPVHG and Gag p24-TLNWVKVV; CMV Pentamers: pp65-NLVPMVATV and pp65-QYDPVAALF. No CD8\(^+\) T-cells specific for CMV were detected in this co-infected liver sample. Similar findings were found in another individual (data not shown).
Figure 3.5

A

HIV Peptides HCV Peptides

% Responding Cells

PBMCs

HIV Peptides HCV Peptides

% Responding Cells

Liver

HIV Peptides HCV Peptides

% Responding cells

PBMC Liver

B

C

% of Total CD8+ T-Cells

HCV mono HCV/HIV HCV/HIV

HCV-Specific HIV-Specific

≤ 2+ Responses > 2+ Responses

P < 0.05

D

Control CD 8 HCV Pentamers CD 8

HIV Pentamers

HIV Pentamers

PD-1

PD-1

100%

49.8%
Figure 3-5. Characterization of the functional hierarchy of viral specific CD8\(^+\) T-cells in HCV/HIV co-infection

Representative functional profiles of virus specific CD8\(^+\) T-cells in blood and liver are depicted from an individual with (a) HCV/HIV co-infection (OM 405) and from an individual with (b) HCV mono-infection (OM 428). For multi-parameter analysis, the Boolean gating platform was used to create all of the possible combinations of functions, generating 32 response patterns for 5 of the different functions analyzed. All data are reported after background correction. Nonspecific background is shown to become extremely low when examining combinations of functions, nearly reaching 0 events for multiple functions simultaneously. This permits a very low threshold for detection of positive responses from multiple combinations. Consequently, for multi-parameter analysis, the results were thresholded based on a minimum criterion of positivity, as calculated by SPICE software and presented as the 90\(^{th}\) percentile of negative values for each analysis. Each pie chart generated by SPICE software, represents the hierarchy of responses to either HCV or HIV antigen stimulation. For simplicity, responses are grouped by number of functions and matched to the colored bars, with black bars representing the percentage of responding cells to HIV peptides and gray bars representing the percentage of responses to HCV peptide stimulation. In all pie charts, color red represents the 5+ responding population and the colors blue, green, turquoise, and yellow representing the 4+, 3+, 2+, and 1+ populations respectively. Color-coded arcs represent the dominant marker within each pie slice, with color blue representing TNF-\(\alpha\), red for CD107-\(\alpha\), green for IFN-\(\gamma\) and black for MIP-1\(\beta\). Although IL-2 is included in the presentation and demonstrated by bar graphs, the software would not
allow for arc colors for more than 4 responses. As a result there is no arc representative for IL-2. Figure (c) represents the average frequency of intra-hepatic viral specific responses within the pool of CD8+ T-cell populations simultaneously expressing 2 functions or less, compared with those within the pool of populations expressing more than 2 functions simultaneously; as analyzed in 3 subjects within each cohort of HCV mono and HCV/HIV co-infected individuals. The cutoff point of simultaneous expression of more than 2 measured markers is considered to show CD8+ T-cell poly-functionality. Figure (d) represents PD-1 levels on HIV-1 and HCV-specific T-cells from HCV/HIV Co-infected liver (OM 385). Liver cells were stained with two pools of HLA-A*0201-restricted pentamers (Proimmune): HCV pentamers: NS3-CINGVCWTV, NS3-KLVALGINAV and HIV pentamers: Pol-ILKEPVHGV, Gag p24-TLNAWVKVV. PD-1 gating was based on FMO (fluorescence minus one) of the control sample.
Chapter 4:

Role of T-cell Exhaustion during HCV/HIV Co-infection
4.1- HCV-Specific T-cells in HCV/HIV Co-infection Show Elevated Frequencies of Dual Tim-3/PD-1 Expression that Correlate with Liver Disease Progression

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4.1.1-Abstract

Co-infection of Hepatitis C Virus (HCV) with Human Immunodeficiency Virus-1 (HIV) has been associated with more rapid progression of HCV-related disease. HCV-specific T-cell immune responses, which are essential for disease control, are attenuated in co-infection with HIV. T-cell exhaustion has recently been implicated in the deficient control of chronic viral infections. In the current study we investigated the role of PD-1 and Tim-3 expression in T-cell exhaustion during HCV/HIV co-infection. We show that in HCV/HIV co-infection, both total and HCV-specific T-cells co-express Tim-3 and PD-1 in significantly higher frequencies, compared to HCV mono-infection. Co-expression of these two markers on HCV-specific CD8+ T-cells positively correlated with a clinical parameter of liver disease progression. HCV-specific CD8+ T-cells showed greater frequencies of Tim-3/PD-1 co-expression than those being HIV-specific, which may indicate a greater degree of exhaustion in the former. Blocking Tim-3 or PD-1 pathways restored both HIV- and HCV-specific CD8+ T-cell expansion in the blood of co-infected individuals. These data demonstrate that co-expression of Tim-3 and PD-1 may play a significant role in HCV-specific T-cell dysfunction, especially in the setting of HIV co-infection.
4.1.2- Introduction

Cell mediated immunity plays an important role in the clearance of Hepatitis C Virus (HCV) infection. Vigorous responses from both CD4\(^+\) and CD8\(^+\) arms of T-cells are necessary for the successful control of HCV replication (45, 59, 212, 213). Hepatitis C viral loads are higher in those individuals co-infected with Human Immunodeficiency Virus-1 (HIV) (72, 78, 79). Additionally, HCV-specific T-cell immune responses are attenuated in HCV/HIV co-infected compared to HCV mono-infected individuals (89, 194).

Attenuation of viral-specific T-cell responses in chronic viral infections has been related to a phenomenon known as T-cell exhaustion (125, 214). In recent years, T-cell exhaustion in persistent viral infections has been emphasized to be modulated through the “Programmed Death-1” (PD-1) signaling pathway (127, 130). Studies on chronic HIV infection suggest that PD-1 might be a marker of early T-cell exhaustion, representing a stage of impaired cellular proliferation and enhanced apoptosis, but sustained T-cell function, including cytokine production (133). More recently, “T-cell Immunoglobulin and Mucin Domain-Containing Molecule-3” (Tim-3) has been shown to be involved in the suppression of T-cell effector function in chronic HIV infection, defining a population of exhausted T-cells that is distinct from the PD-1-expressing population (128). The role of the Tim-3 pathway on the progression of HCV infection was also recently reported (158). However, the relative status of Tim-3 and PD-1 expression in HCV/HIV co-infection and its role in HCV-related disease progression is unclear.
In the current study, we profiled the expression of Tim-3 and PD-1 on total and antigen-specific T-cells within cohorts of HCV infected individuals, in the presence or absence of HIV infection.

4.1.3- Materials and Methods

4.1.3.1- Study Participants

Blood samples were obtained from 20 HCV/HIV co-infected, 8 HCV mono- and 5 HIV mono-infected individuals and cryo-preserved until use. All participants were chronically infected with either virus, as defined by being infected longer than 6 months. Informed consent was obtained in accordance with the guidelines for conduction of clinical research at the University of Toronto and St. Michael’s hospital institutional ethics boards. Co-infected individuals were also selected from participants in the Canadian Co-infection Cohort (CTN 222) under the Canadian HIV Trials Network (CTN). Peripheral blood mononuclear cells (PBMCs) were isolated as previously described (215). All HLA-A2+ subjects were identified using HLA-A2 antibody staining (BD Pharmingen) and subsequent flow cytometry analysis. Clinical data of the individuals are presented in Table 4.1.

4.1.3.2- Tetramer Staining and Analysis

Fluorochrome-conjugated, HLA-A0201 tetramers used included: HCV-NS3: 1073 (CINGVCWTV), HCV-NS3: 1406 (KLVALGINAV), HIV-Gag p17:76 (SLYNTVATL), HIV-Pol: 476 (ILKEPVHGV), CMV-pp65:495 (NLVPMVATV) and EBV-BMLF1: 259 (GLCTLVAML). All tetramers (iTAG™ MHC Class I) were obtained from Beckman Coulter. PBMCs were stained with tetramers in 1% FBS in PBS with 2mM EDTA for 20
minutes at room temperature, followed by staining against CD3 (BD), CD8 (BD), Tim-3 (MAb, R&D Systems) and PD-1 (BioLegend). Events were obtained using either a FACSARia flow cytometer, or a FACSCalibur instrument (BD Biosciences). Further analysis was performed using FlowJo version 7.5 (Tree Star Inc.).

4.1.3.3- Proliferation and Functional Assay

PBMCs were labelled with 1 mM of carboxyfluorescein diacetate succinimidyl ester dye (CFSE, Invitrogen) for 15 minutes at 37°C. Labelling was quenched by further incubation in 10% FBS in RPMI. CFSE-labelled cells were stimulated for 5-6 days with either DMSO or pools of HIV-derived Gag or HCV-derived NS3 peptides, in the presence or absence of a monoclonal Tim-3–blocking antibody 2E2 (kindly provided by V. Kuchroo, Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA), and/or purified anti-human CD274 (PDL-1) (Biolegend) or an IgG1 isotype control, as previously described (128). This was followed by cell surface staining for CD8, CD4, Tim-3, PD-1 and intracellular staining for IFN-γ (BD). Cells were fixed in PBS with 2% paraformaldehyde and acquired on FACSCalibur, with more than total of 100,000 events collected. All peptides were obtained from the National Institute of Health AIDS Research and Reagent Program, and each pool was used at the final concentration of 2µg/peptide/ml.

4.1.3.4- Statistical Analysis

Data were analyzed by performing two-tailed student’s t-test or Mann-Whitney test for non-parametric data using GraphPad Prism version 4.00 (GraphPad Software
Correlations were tested for significance by Spearman rank analysis. P-values of <0.05 were considered significant.

### 4.1.4- Results

#### 4.1.4.1- Subject Characteristics

The three groups of studied individuals are depicted in Table 4.1; HCV/HIV co-infected (n=20) (of whom 10 were untreated and 10 were receiving HAART for greater than one year at the time of evaluation) and HCV mono-infected (n=8). HIV mono-infected individuals were included in the study as a comparison group (n=5). None of the individuals received prior pegylated-interferon/ribavirin treatment for HCV infection. No significant difference in HCV viral load was observed between studied HCV/HIV co-infected and HCV mono-infected individuals [median 1,780,000 ± SE 848,760 IU/ml for HCV/HIV co vs. 2,690,000 ± SE 5,613,000 IU/ml for HCV mono, P=0.84]. No significant difference in the duration of HCV infection was observed between HCV/HIV co-infected and HCV mono-infected individuals [median 8.00 ± SE 2.62 years for HCV/HIV co vs. 5.00 ± SE 2.22 years for HCV mono, P=0.29]. HCV/HIV co-infected individuals had a median CD4 T-cell count of 510/mm$^3$ (± SE 59). Among co-infected subjects, there was a trend towards lower APRI values in individuals that were on HAART [median 0.65 ± SE 0.21 for on HAART vs. 1.15 ± SE 0.34 for off HAART, P=0.075]. Co-infected individuals, in general had significantly higher APRI values than those being HCV mono-infected [median 0.95 ± SE 0.17 vs. 0.32 ± SE 0.10, P=0.012].
4.1.4.2- Tim-3 and PD-1 are co-expressed at elevated levels on T-cells in HCV/HIV co-infection

Tim-3 and PD-1 expression frequencies on total T-cells were determined by flow cytometry on PBMCs obtained *ex vivo* from HCV/HIV co-infected, HCV mono-infected and HIV mono-infected individuals (See Table 4.1 for clinical details). Representative data from one HCV mono-infected and one HCV/HIV co-infected individual are shown in Figures 4.1a and 4.1c, and summary data of all studied individuals are depicted in Figures 4.1b and 4.1d, for CD8$^+$ and CD4$^+$ T-cells, respectively. Overall, HIV mono-infected and HCV/HIV co-infected individuals demonstrated significantly higher PD-1 and Tim-3 expression on total CD4$^+$ and CD8$^+$ T-cells compared to HCV mono-infection. Compiled data analysis on CD8$^+$ T-cells (Fig 4.1b) demonstrated that HCV mono-infected individuals had lower expression of PD-1 on CD8$^+$ T-cells [median %: 2.75 ± SE 1.31] compared to both HIV mono-infected [median %: 22.00 ± SE 3.72, P=0.0016] and HCV/HIV co-infected individuals [median %: 10.06 ± SE 3.20, P=0.002]. Compared to HCV mono-infected subjects, a trend towards increased frequencies of CD8$^+$ T-cell expression of Tim-3 was observed among HIV mono-infected [median %: 26.90 ± SE 3.38, P=0.057], and HCV/HIV co-infected individuals [median % 18.97 ± SE 1.64, P=0.103], [HCV mono-infected median %: 14.20 ± SE 2.76]. Of note, is that HCV/HIV co-infected individuals showed a distinct population of CD8$^+$ T-cells co-expressing Tim-3-and PD-1, which was not as prominent in HCV mono-infected individuals (Fig 4.1a). Significantly higher frequencies of dual Tim-3/PD-1-expressing CD8$^+$ T-cells were observed in both HIV mono-infected [median %: 5.50 ± SE 0.45, P=0.031] and HCV/HIV co-infected [median %: 2.97± SE 1.03, P=0.038], compared to HCV mono-
infected individuals [median %: 1.15 ± 0.32]. Also, CD4\(^+\) T-cells (Fig. 4.1d) demonstrated a significantly higher frequencies of dual Tim-3/PD-1 positive CD4\(^+\) T-cells among HIV mono-infected [median %: 5.90 ± SE 0.9, P=0.005] and HCV/HIV co-infected individuals [median %: 4.20 ± SE 1.4, P=0.01] compared to HCV mono-infected subjects [median %: 1.01 ± SE 0.6]. When looking at the frequencies of dual Tim-3/PD-1 expressing CD8\(^+\) and CD4\(^+\), no significant difference was observed between “On HAART” and “Off HAART” subcategories of co-infected subjects (data not shown).

4.1.4.3- HCV-specific CD8\(^+\) T-cells co-express Tim-3 and PD-1 at higher frequencies compared to HIV-specific CD8\(^+\) T-cells in HCV/HIV co-infection

Co-expression of Tim-3 and PD-1 on antigen-specific CD8\(^+\) T-cells was examined in HLA-A2\(^+\) individuals, chronically co-infected with HIV and HCV, using MHC class I tetramers presenting different epitopes of HIV, HCV and also CMV as a comparison control. Within our cohort of 20 co-infected individuals, we identified 12 individuals that demonstrated distinct antigen-specific CD8\(^+\) T-cells detected by all three categories of tetramers that also included CMV. Figure 4.2a shows representative flow cytometry plots of the frequencies of these cells in three chronic HCV/HIV co-infected subjects (with CMV-specific T-cells detected in two of them). We were also able to look at the frequencies of PD-1/Tim-3 expression on an EBV epitope among 8 co-infected individuals and include as a second control. A Large degree of individual variability of Tim-3 and PD-1 expression on EBV-specific CD8\(^+\) T-cells was observed among co-infected individuals (supplement figure 4.1s). Compiled data analysis of the frequencies of PD-1 and Tim-3 expression among co-infected subjects (Fig 4.2b upper) demonstrated
that although there was a considerable heterogeneity in HCV- and HIV–specific CD8\(^+\) T-cells, cells of both specificities displayed greater frequencies compared to CMV specific T-cells. HCV specific CD8\(^+\) T cells tended to show greater PD-1 or Tim-3 expression compared to HIV specific CD8\(^+\) T cells. Importantly, HCV-specific CD8\(^+\) T-cells were most highly enriched for dual expression of Tim-3 and PD-1, compared to those of HIV- or CMV-specific CD8\(^+\) T-cells [median 46.10 ± SE 6.80% for HCV vs. 36.10 ± 7.52% for HIV, P=0.0403]; [median 46.10 ± SE 6.8% for HCV vs. 12.70 ± 3.7% for CMV, P=0.0001]. EBV-specific CD8\(^+\) T-cells tended to have lower PD-1 and Tim-3 expression when compared to T-cells directed against HIV and HCV. Moreover, analysis of the Mean Fluorescent Intensity (MFI) of PD-1 and Tim-3 on each category of antigen-specific CD8\(^+\) T-cells, demonstrated a significant increase among HCV-specific T-cells compared to those of HIV, CMV and also EBV (Fig 4.2b lower).

Among the co-infected participants, only one individual (C011) showed similarly high frequencies of Tim-3 and PD-1 expressing CD8\(^+\) T-cells, specific for both HIV and HCV, but significantly lower levels within CMV-specific CD8\(^+\) T-cells (Fig 4.2a, lower panel). In all other co-infected individuals HCV-specific CD8\(^+\) T-cells showed greater dual Tim-3/PD-1 expression compared to those of HIV specificity. The clinical status of this one individual revealed decades of co-infection with both viruses and a high APRI score (Aspartate Aminotransferase to Platelet Ratio Index-a non-invasive indicator of liver fibrosis in chronic HCV (216) ), indicating an advanced stage of liver damage.
4.1.4.4- Co-expression of Tim-3 and PD-1 on total and antigen-specific T-cells correlates with clinical parameter of liver injury in HCV/HIV co-infection

To investigate the clinical relevance of our findings, we analyzed the correlation of the frequencies of Tim-3/PD-1-co-expressing CD8$^+$ T-cells (total and antigen-specific) among co-infected individuals with their APRI score. Among all co-infected individuals, the frequencies of the dual Tim-3/PD-1 positive CD8$^+$ T-cells correlated with their corresponding APRI score [Spearman $r=0.70$, $P=0.0006$] (Figure 4.3a). When co-infected individuals were categorized based on being on or off HAART regimen, this correlation was observed among both groups [On HAART: Spearman $r=0.74$, $P=0.008$; Off HAART: Spearman $r=0.70$, $P=0.02$] (Fig. 4.3a-lower panel). No correlations were observed between the frequencies of single Tim-3$^+$ or PD-1$^+$ /CD8$^+$ T-cells and this fibrosis index among the co-infected subjects (data not shown). Also there was no correlation between APRI and CD4 count, neither between APRI and HIV viral load (data not shown). We did not find any correlation between PD-1/Tim-3 expression on antigen-specific CD8$^+$ T-cells and the viral load of the corresponding antigen (data not shown). Of note, among HCV mono-infected individuals no correlation was observed between the frequencies of dual Tim-3/PD-1 positive CD8$^+$ T-cells and the APRI score of each corresponding individual (data not shown). On the other hand, a positive correlation was also observed between the frequencies of CD4$^+$ T-cells co-expressing Tim-3/PD-1, and APRI of the co-infected subjects [$r=0.75$, $P=0.007$] (Fig 4.3a). More importantly, the frequencies of Tim-3/PD-1 dual positive CD8$^+$ T-cells, specific for both HIV and HCV, based on available tetramer stains, correlated positively with the corresponding APRI
score of each individual [Spearman r=0.66, P=0.013 for HCV-specific; Spearman r=0.84, P=0.0003 for HIV-specific] (Fig 4.3b).

4.1.4.5- Higher frequencies of dual Tim-3/PD-1-expressing HCV-specific CD8\(^+\) T-cells identified in HCV/HIV compared to HCV mono-infection

In order to determine if the presence of HIV infection is associated with dual up-regulation of Tim-3 and PD-1 on HCV-specific T-cells, we compared the frequencies of Tim-3/PD-1 co-expressing CD8\(^+\) T-cells specific for HCV tetramers, between the HCV/HIV and HCV mono-infected cohorts. Figure 4.4a depicts flow cytometry plots from representative individuals. Co-infected individuals showed significantly elevated frequencies of dual Tim-3/PD-1 positive HCV-specific CD8\(^+\) T-cells [median 31.80 ± SE 2.21\% for HCV vs. 46.10 ± 6.8\% for HCV/HIV; P=0.01] (Fig 4.4b).

4.1.4.6- Blocking of Tim-3 or PD-1 pathways augment the proliferation of peripheral virus-specific T-cells in HCV/HIV co-infection

We stimulated PBMCs from co-infected individuals with either HIV or HCV pools of peptides, and different compartments of CD8\(^+\) T-cells related to their Tim-3 and PD-1 status were analyzed for the production of IFN-\(\gamma\). Figure 4.5a represents the flow cytometry plot of IFN-\(\gamma\) production by PBMCs in a chronically co-infected individual. Similar to what we had previously shown in HIV mono-infection (128), the majority of functional HIV-specific CD8\(^+\) T-cells were within the Tim-3 negative compartment. Additionally, in the same line with previous reports (133), these functional cells were
highly single PD-1 positive. As previously shown, direct \textit{ex-vivo} T-cell responses to HCV antigens are generally weakly detectable, especially among HCV/HIV co-infected individuals (91, 199). In accordance, we encountered undetectable responses to direct \textit{ex-vivo} HCV peptide stimulation. To study the effect of blocking Tim-3 and PD-1 pathways on rescuing these responses in the context of co-infection with HIV, PBMCs from co-infected individuals were expanded in culture with pools of HIV or HCV peptides, in the presence or absence of anti-Tim-3 and/or anti-PDL-1 blocking antibodies. Figure 5b demonstrates the expansion of both CD8$^+$ and CD4$^+$ T-cells in response to HIV and HCV peptides, in different blocking settings. Compared to non-blocked control, compiled data from CD8$^+$ T-cells of co-infected individuals (figure 4.5d) demonstrated a significant increase in proliferation, in response to stimulation with HIV-Gag pool at the presence of anti-Tim-3 antibody [$\%$ CD8: 2.8 ± 1.20 vs. 1.24 ± 0.58, $p=0.0054$, $n=11$] and anti-PDL-1 antibody [$\%$ CD8: 3.10 ± 1.32 vs. 1.24 ± 0.58, $p=0.0179$, $n=11$]. Dual blocking also resulted in a better CD8$^+$ T-cell expansion compared to control [$\%$ CD8: 8.6 ± 1.56 vs. 1.89 ± 0.89, $p=0.103$, $n=6$], which was shown to be synergistic in two of the studied individuals. Similar results were found in response to stimulation with HCV-NS3 peptide pool [$\%$ CD8: 2.03 ± 0.80 vs. 0.91 ± 0.26, $p=0.0046$, $n=11$; for Tim-3 blocking], [$\%$ CD8: 2.35 ± 0.70 vs. 0.91 ± 0.26, $p=0.016$, $n=11$; for PD-1 blocking], [$\%$ CD8: 2.35 ± 1.11 vs. 1.56 ± 0.30, $p=0.026$, $n=6$; for dual Tim-3/PD-1 blocking]. We were also able to assess the effect of each type of blocking on CD4$^+$ T-cell expansion in a limited subset of co-infected individuals ($n=4$). Although a trend of increased proliferation was observed in all settings, only HIV peptide stimulation after dual blocking was significantly effective in CD4$^+$ T-cell expansion (figure 4.5d).
We were able to examine IFN-γ production by proliferated CD8+ T-cells among a subset of co-infected individuals (n=3 for single blocking, n=2 for dual blocking). Figure 4.5c represents FACS plots from one of these individuals, demonstrating that when PBMCs were co-cultured with anti-Tim-3 antibody, both HCV- and HIV-specific proliferated CD8+ T-cells expressed more IFN-γ, compared to negative control [% proliferated CD8+ T-cells: 64 vs. 0.8 for NS3 pool; 62 vs. 8.2 for Gag pool]. Similar results were observed during blocking with anti-PDL-1 [% proliferated CD8+ T-cells: 42% vs. 0.8 for NS3; 49% vs. 8.2% for Gag pool]. Pooled data on the frequencies of IFN-γ production by proliferated CD8+ T-cells, demonstrate a trend of increase with either anti-Tim-3 or anti-PD-L1 blocking (Figure 4.5d). When dually blocked with anti-Tim-3 and anti-PDL1 antibodies, proliferated CD8+ T-cells were shown to produce IFN-γ at frequencies more than double of those produced by the unblocked control [% IFN-γ on CFSE_{dim} CD8+ T-cells (median ± SE): 14.10 ± 1.55 vs. 6.4 ± 1.69 for Gag; 13.30 ± 6.08 vs. 4.4 ± 3.1 for NS3, n=2] (Figure 4.5d).
4.1.5- Discussion

The current study investigated whether the presence of HIV infection affects HCV-specific T-cell phenotype in HCV/HIV co-infection. Our findings integrate previous studies on the role of T-cell exhaustion in the pathogenesis of mono-infection with HIV or HCV, originally attributed to the up-regulation of PD-1 on T-cells (127, 130, 133). Tim-3 as another marker of more advanced stages of T-cell exhaustion was also shown to be associated with HIV disease progression (128) and up-regulated in HCV infection (158). Here we demonstrate a detailed analysis of T-cell expression of both Tim-3 and PD-1 in HCV/HIV co-infection. We report for the first time that in HCV/HIV co-infection, HCV-specific T-cells demonstrate a more exhausted phenotype compared to those being HIV-specific, with regard to Tim-3/PD-1 co-expression. In addition, HCV specific T-cells from HIV-infected individuals are more exhausted than those in HCV mono-infection. Within the same co-infected individuals, CMV-specific T-cells consistently showed lower frequencies of dual Tim-3/PD-1 expression. This may emphasize the essential role that these exhaustion markers play in the ineffective control of some persistent viruses. CMV is an example of a persistent pathogen that is efficiently controlled in immuno-competent individuals (217, 218). However, in individuals infected with other chronic viruses including HIV alone or HCV alone, a subset of CMV-specific T-cells were shown to express elevated levels of Tim-3 (128, 158) and PD-1 (219), but generally at lower frequencies compared to HIV- or HCV-specific T-cells. Surprisingly, EBV-specific T-cells showed an intermediate phenotype in terms of PD-1 and Tim-3 expression, when compared to HIV and CMV, thus reflecting possibly different degrees of the ability of T-cells to control certain persistent viruses. In contrast to CMV-specific
CD8$^+$ T-cells, we found that both HCV- and HIV-specific CD8$^+$ T-cells have greater frequencies of dual expression of PD-1 and Tim-3. In addition, the co-existence of HIV infection was associated with an added effect on the exhaustion phenotype of both total and HCV-specific CD8$^+$ T-cells as depicted by the frequencies of PD-1/Tim-3 co-expression. The question of what drives this elevated expression on HCV-specific cells needs to be addressed in future studies. The aggravated exhaustion phenotype of HCV-specific T-cells in co-infection may be driven by the lack of proper CD4 help found in HIV infection, as mouse LCMV models demonstrate that the absence of CD4 help enhances the exhaustion phenotype (220).

Within co-infected individuals, both HIV- and HCV-specific CD8$^+$ T-cells showed heterogeneity with regards to Tim-3 expression, similar to what was previously shown in HIV mono-infection (128). HIV-specific T-cells were more heterogeneous in expressing dual Tim-3/PD-1. This heterogeneity as mirrored by HIV viral loads may reflect the relative functionality of the T-cell response, with higher frequencies of Tim-3/PD-1 co–expressing CD8$^+$ T-cells being associated with more advanced exhaustion and dysfunction. Thus, an exhaustion gradient may explain the observed heterogeneity among the studied cohorts. HCV-specific T-cells on the other hand, were more consistently high in the frequency of dual Tim-3/PD-1 expression, which may reflect higher degree of exhaustion among CD8$^+$ T-cells targeting HCV antigens. Functionally, this phenotype was associated with poor cytokine production in samples and poor proliferative capacity, which could be reversed in part, by blocking antibodies. We generally found that HCV-specific T-cells were less functional than the HIV-specific cells, in a given individual, and these features correlated with dual expression of Tim3/PD-1. Tim-3 expression also
marked a poorer ability to produce INF-g, even if the cells co-expressed PD-1. It is unclear what the nature of dual expressing cells represents; however, we would favour that this population may represent a mid-stage of T-cell exhaustion, from PD-1 expression to Tim-3.

Our findings may also represent clinical significance related to HCV disease, as we found that in HCV/HIV co-infection the dual expression of PD-1 and Tim-3 either on total CD8\(^+\), CD4\(^+\) or HCV-specific CD8\(^+\) T-cells was associated with the degree of liver fibrosis as determined by APRI score, whereas single expression of PD-1 or Tim-3 was not. APRI scores have been used as a non-invasive test for estimating liver fibrosis, with reportedly high sensitivities and specificities, and a prognostic value similar to the established Model of End-Stage Liver Disease (MELD) in HCV infection, with or without HIV (216, 221-225). Our current data also demonstrate an association between HIV-specific T-cell frequencies of PD-1/Tim-3 with liver fibrosis progression. We have previously shown that HIV-specific T-cells accumulate in the liver during HCV/HIV co-infection (226), which may help explain our observations here. Thus, the presence of these specific T-cells may affect the whole cytokine milieu of the liver. It is recognized that exhausted T-cells are activated T-cells, and the effect of such cells in the hepatic milieu on the induction of hepatic fibrosis will require further study. We also found strong correlations between Tim-3/PD-1 co-expression on blood CD4\(^+\) T-cells and HCV disease progression, further highlighting the importance of CD4\(^+\) T-cell function in HCV control. Additional studies are required to elaborate on this exhaustion phenotype in hepatic T-cells and to clarify if the association of this phenotype with liver fibrosis in co-
infected individuals is related to the further impairment of T-cells to clear HCV in the liver and aggravate the hepatic damage.

Our analysis showed that HAART had no influence on our observed association, indicating that the exhaustion phenotypes induced in co-infection cannot be readily reversed. In terms of Tim-3 expression, we have previously shown that HIV-infected individuals demonstrate variable Tim-3 levels in response to HAART, with some individuals maintaining high levels of Tim-3 expression despite achieving undetectable HIV-1 viral loads (128). It should be noted however, that the time point of initiating HAART may also play an important role on the effect of HIV-related T-cell exhaustion on the progression of HCV disease. Larger co-infected cohorts, with different time points at which HAART therapy is initiated, are warranted to clarify the effect of HIV on HCV-related liver damage.

Our demonstration of the \textit{ex vivo} reversal of proliferation of both HIV- and HCV-specific T-cells from co-infected individuals, with the blockade of Tim-3 pathway, mirrored what has been found in HIV or HCV infection alone (128, 158). With regard to simultaneous blocking of PD-1 and Tim-3 pathways, although we observed a synergistic effect on T-cell proliferation in a subset of co-infected individuals, future studies are needed to examine why only some individuals appear to benefit from dual blocking. It should be mentioned however, that T-cell exhaustion is associated with several immunoregulatory pathways, which may vary between individuals, giving varying effects with blocking one or two pathways. This has been recently demonstrated by reversal of T-cell function during HCV infection by dual blocking of PD-1 and CTLA-4 pathways at the site of viral replication (129). Blocking of multiple pathways, although may serve to
enhance T-cell immune responses, may also result in the onset of an autoimmune process and should be approached with caution.

It is currently unclear if the T-cell phenotype that we observed in this study leads to loss of viral control, or if it is an indirect outcome of other underlying mechanisms including viral escape from CTL epitopes. Previous studies in HIV infection however, have demonstrated that epitope escape is associated with a reversion of the exhaustion phenotype (227). Our findings could be a prerequisite for designing further studies to elucidate the causality of this observed phenomenon. In addition, it is still unclear to us, and others, why phenotypically ‘exhausted’ T-cells would cause further liver damage leading to progression. As exhausted T-cells are also activated cells, further studies defining how such cells interact in the hepatic milieu would be informative. Together, our data may suggest a potential implication of the pharmacological effect of blocking Tim-3 signalling in managing HCV/HIV co-infection. These data complement and integrate previous studies that have identified important roles for Tim-3 and PD-1 in the pathogenesis of HIV or HCV mono-infection.
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<td>N</td>
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**F=Female, M=Male**

**HAART=Highly Active Antiretroviral Therapy**

**APRI=Aspartate Aminotransferase to Platelet Ratio Index**

**Y=Yes, N=No**

**VL=Viral Load**

**NA=Not Available**

* = HLA-A2+/ CMV+
Figure 4.1.

a)

b)
**Figure 4.1. Frequencies of dual Tim-3/PD-1-expressing T-cells are elevated in blood in HCV/HIV co-infection**

a) Representative flow cytometry plots show frequencies of total CD8<sup>+</sup> T-cells that express Tim-3, PD-1 and dual Tim-3/PD-1 in an HCV/HIV co-infected (OM 405) and an HCV mono-infected individual (OM 566). Initial gating on forward scatter area (FSC-A) versus height (FSC-H) was used to remove doublets. Lymphocytes were then gated on the remaining cells on a FSC versus SSC plot. Next, gates on CD3<sup>+</sup> cells were generated. From CD3<sup>+</sup> cells, both CD8 positive and negative populations were then presented against either Tim-3 or PD-1. For the dual Tim-3/PD-1 analysis, gates were first generated on CD8<sup>+</sup> and then presented against both Tim-3 and PD-1 stains. PD-1 and Tim-3 gating was confirmed by conducting a “Fluroscent minus one” (FMO) staining for each fluorochrome-conjugated antibody.

b) Compiled data of the frequencies of total CD8<sup>+</sup> T-cells expressing Tim-3, PD-1 and dual Tim-3/PD-1 are shown among HCV/HIV co-infected, HCV mono- and HIV mono-infected individuals.

c) Total CD4<sup>+</sup> T-cell frequencies of Tim3, PD-1 and dual Tim-3/PD-1 are shown in two representative flow cytometry plots, HCV/HIV co-infected (K009) and HCV mono-infected (OM 546). Same procedure of CD8<sup>+</sup> gating was applied in CD4<sup>+</sup> T-cell analysis.

d) Compiled data of the frequencies of total CD4<sup>+</sup> T-cells expressing Tim-3, PD-1 and dual Tim-3/PD-1 are shown among the three studied cohorts. Only a subset of total co-infected samples were available for the study of CD4<sup>+</sup> T-cells (n=15). Statistical comparative analyses were performed using the non-parametric Mann-Whitney test.
Figure 4.2.

a)
Figure 4.2. Tim-3 and PD-1 are dually expressed at elevated frequencies on HCV-specific CD8\(^+\) T-cells in HCV/HIV co-infection

PBMCs from chronically co-infected HLA-A2\(^+\) individuals were stained with the following tetramers: HCV-NS3:1073 (CINGVCWTV), HCV-NS3:1406 (KLVALGINAV), HIV-Gag p17:76 (SLYNTVATL), HIV-Pol:476 (ILKEPVHGV) and CMV-pp65:495 (NLVPMVATV). a) Shown are representative flow cytometry plots from three different co-infected subjects (upper panel: OM 539, middle panel: OM 403, lower panel: C011). Gates are set relatively to the appropriate fluorescent minus one (FMO) control to determine the percentage of T-cell subsets that express Tim-3 and PD-1. The last plot in each row is generated by gating on the tetramer positive population and demonstrates the frequencies of dual Tim-3/PD-1-expressing, antigen specific CD8\(^+\) T-cells. b) Compiled data of the expression frequencies (upper figure) and mean fluorescent Intensity (lower figure) of PD-1, Tim-3 and dual Tim-3/PD-1 are shown on HIV-, HCV-, CMV and EBV-specific CD8\(^+\) T-cells in co-infected subjects.
Figure 4.3.

a)  

On HAART  
\[ r = 0.74 \quad p = 0.008 \]  

Off HAART  
\[ r = 0.70 \quad p = 0.02 \]  

b)  

HCV-Specific CD8  
\[ r = 0.66 \quad p = 0.013 \]  

HIV-Specific CD8  
\[ r = 0.84 \quad p = 0.0003 \]
Figure 4.3. Dual expression of Tim-3 and PD-1 on total and antigen-specific T-cells is associated with clinical parameter of liver injury in HCV/HIV co-infection

a) Shown are the correlations between the percentages of dual Tim-3/PD-1-expressing CD8$^+$ and CD4$^+$ T-cells of co-infected individuals with their corresponding APRI score (an index of liver fibrosis) using the Spearman rank test. APRI was calculated according to the following formula (228):

$$\text{APRI} = \left( \frac{\text{AST Level/ULN}}{\text{Platelet count}} \right) \times 100$$

*ULN= AST upper level of normal or 56 IU/L

The upper figures demonstrates this correlation among all studied co-infected individuals, with the two lower figures demonstrating two sub-groups of on- or off –HAART co-infected subjects. b) Shown are the correlation analyses between APRI score and the percentages of Tim-3$^+$/PD-1$^+$ HCV-specific (left) and HIV-specific (right) CD8$^+$ T-cells among co-infected subjects.
Figure 4.4.

(a) Graphs showing the percentage of HCV and HCV/HIV co-infection in CD8+ cells, with gates for PD-1 and Tim-3 expression.

(b) Scatter plot comparing the percentage of HCV tetramer+/CD8+ cells in PD-1, Tim-3, and PD-1/Tim-3 conditions, with significance level p=0.01.
**Figure 4.4.** HCV-specific T-cells that express dual Tim-3/PD-1 are identified at higher frequencies in HCV/HIV co-infection compared to HCV mono-infection

PBMCs from all co-infected and HCV mono-infected individuals were stained with the following HLA-A*0201-restricted HCV class I tetramers: NS3:1073 (CINGVCWTV), NS3:1406 (KLVALGINAV) and analyzed for the frequencies of Tim-3 and PD-1 expression. a) Shown is a representative flow cytometry data from two HCV mono-infected individuals using HCV-NS3:1073 (CINGVCWTV) tetramer. Staining data from a co-infected individual are also presented for comparison. b) Compiled data analyses of the frequencies of PD-1, Tim-3 and dual Tim-PD-1 expression on HCV-specific CD8$^+$ T-cells, are compared between HCV/HIV co- and HCV mono-infected subjects, using the non-parametric Mann-Whitney test.
Figure 4.5.

a)
Figure 4.5. Blocking the Tim-3 pathway improves both HIV- and HCV-specific T-cell proliferation and cytokine expression in HCV/HIV co-infection

a) Shown are representative flow cytometry plots of Tim-3 and PD-1 expression status of functional CD8\(^+\) T-cells from a chronic co-infected individual (OM530), after direct ex-vivo stimulation with pools of HIV-Gag or HCV-NS3 peptides. SEB is used for stimulation as a positive control. Plots are originally gated on CD8\(^+\) T-cells and eventually gated on IFN-γ producing cells. b) Shown are representative flow cytometry plots of PBMCs from a co-infected individual (OM 539), proliferating in culture after 6 days of stimulation with HIV-Gag or HCV-NS3 pools of peptides at the presence of anti-Tim-3 and/or anti-PDL-1 antibodies. The percentages represent the proportion of antigen-specific CD8\(^+\) or CD4\(^+\) T-cells that have proliferated (CFSE\(^{\text{dim}}\) population). Blocking with IgG-1 isotype was used as a negative control. c) Shown are representative flow cytometry plots demonstrating IFN-γ production in proliferated CD8\(^+\) T-cells from one co-infected individual (OM530) after 6 days of stimulation with HIV-Gag or HCV-NS3 pools of peptides at the presence of anti-Tim-3 or anti-PDL-1 antibodies. For IFN-γ analysis, PBMCs were re-stimulated with the corresponding peptides for 5 hours. Plots are initially gated on CD8\(^+\) T-cells and then on the proliferated cells (CFSE\(^{\text{dim}}\) population). The percentages represent the proportion of proliferated antigen-specific CD8\(^+\) T-cells that have produced IFN-γ. d) Shown are summary data on the effect of blocking Tim-3, PD-1 or both pathways on the proliferation and IFN-γ production of CD8\(^+\) and CD4\(^+\) T-cells in response to HIV-Gag and HCV-NS3 pools of peptides, in
chronically HCV/HIV co-infected individuals. Data are analyzed by performing paired two-tailed student’s t-test.

**Supplement Figure 4.1.**

Shown are representatives of the frequencies of PD-1/Tim-3 expression on EBV-specific CD8+ T-cells in two co-infected individuals (C117 and OM 577). EBV-BMLF1: 259 (GLCTLVAML) tetramer was used for staining.
Chapter 5:

Role of T-cell Cross-reactivity During HCV/HIV Co-infection
Characterization of cross-reactive CD8+ T-cell recognition of HLA-A2-restricted HIV-Gag: SLYNTVATL and HCV-NS5b: ALYDVVS KL epitopes in individuals infected with Human Immunodeficiency and Hepatitis C Viruses

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5.1.1-Abstract

The immunologic mechanisms underlying the faster progression of HCV disease in the presence of HIV co-infection are not clearly understood. T-cell cross-reactivity between HCV and influenza-specific epitopes has been associated with rapid progression of HCV disease (189). We asked whether T-cell cross-reactivity between HCV and HIV could exist during HCV/HIV co-infection and affect pathogenesis. Our search for amino acid sequence homology between HCV and HIV proteomes revealed two similar HLA-A2-restricted epitopes, HIV-Gag (SLYNTVATL: SL9) and HCV-NS5b (ALYDVVSKL: AL9). We found that 4 out of 20 HLA-A2+ HIV infected individuals had CD8+ T-cells that recognized both HIV-SL9 and HCV-AL9 epitopes. However, the AL9 epitope was generally shown to be a weak agonist. Although HCV mono-infected individuals in our study did not show AL9-specific responses, we found that about half of HCV/HIV co-infected individuals had dual responses to both epitopes. High dual T-cell recognition among co-infected subjects was usually due to separate T-cell populations targeting each epitope, rather than true cross-reactivity. The one individual with truly cross-reactive T-cells to both epitopes showed the most advanced degree of liver disease. In co-infected individuals, we observed a positive correlation between the magnitudes of T-cell responses to either SL9 or AL9 epitope, which was also positively associated with a clinical parameter of liver damage. Thus, we find that HIV infection induces T-cells that can cross-react to heterologous viruses or prime for T-cells that are closely related in sequence. However, the induction of cross-reactive T-cells may not be associated with control of disease caused by the heterologous virus. This demonstrates that degeneracy of HIV-specific T-cells may play a role in the immuno-pathology of HCV/HIV co-infection.
5.1.2-Introduction

Hepatitis C virus (HCV)-related liver disease progresses faster in individuals co-infected with Human Immunodeficiency Virus-1 (HIV) (3); however the underlying immunologic mechanisms are not clear. Many studies indicate that exposure to an infectious agent may alter the immune responses of the host to other infections, a phenomenon referred to as heterologous immunity (168). Several lines of evidence signify the extent of T-cell degeneracy and the role of molecular mimicry on provoking an immune response. The stimulating target for CD8+ T-cells is the complex of a specific foreign peptide and a self MHC class I molecule (229). During molecular mimicry, a variant of an original peptide ligand interacts with the cognate T-cell receptor (TCR), inducing a total or partial T-cell activation. Only a few TCR contact residues on the peptide ligand are required for T-cell stimulation and certain amino acid substitutions in the peptide sequence can still result in the activation of T-cells (166). The ability of a TCR to cross-recognize multiple antigens is shown to have many implications for CD8+ T-cell functioning, including protective responses against pathogens (175), immuno-dominance (172), and maintenance of memory responses (230).

Activated memory T-cells that are specific for a virus are shown to further restrain the responses from naive T-cells specific for the same virus (231). Accordingly, in a primary infection even relatively weak T-cell responses to non-dominant epitopes may affect the immuno-dominance hierarchy of T-cell responses to a subsequent infection. This could result in an enhanced response to sub-dominant cross-reactive epitopes at the cost of responses to more immuno-dominant epitopes.
Cross-reactivity between HCV-and influenza-specific CD8+ T-cells and an association with the severity of the clinical course of HCV disease has been previously demonstrated (188, 189). Urbani et al identified individuals with severe course of HCV infection who had a narrowly focused response to the HCV-NS3: CVNGVCWTV epitope and these cells cross-reacted with the Influenza-A Neuraminidase (FLU-NA): CVNGSCFTV epitope (189). This suggested that pre-existing responses to the FLU-NA epitope may have adversely affected HCV responses by narrowing it to the NS3 epitope. In another recent study, although FLU-NA response was not prevalently seen in the studied cohort, HCV-NS3 specific CD8+ T-cells were identified from HCV-infected individuals that cross-reacted with the FLU-NA epitope (190). These authors showed that the cross-reactive T-cells only weakly responded to the FLU-NA epitope, indicating that the FLU-NA peptide was a weak agonist, and was mainly a consequence of the presence of a pre-existing response to the HCV NS3 epitope. These studies show that cross-reactivity to heterologous viruses may occur. However it is still unclear how such responses alter the subsequent responses to the heterologous viruses.

Individuals with HCV/HIV co-infection have high levels of circulating antigen to both viruses in their plasma, and given that HIV CD8+ T-cell responses are robust in most HIV-infected individuals, it is unclear what effect heterologous cross-reactivity may have on HCV-specific responses. In this study, we investigated if HIV-specific T-cells could recognize heterologous HCV epitopes and if this cross-recognition could alter the immuno-pathological profile of HCV infection in HCV/HIV co-infected individuals. We focused on two defined HLA-A2-restricted epitopes, HIV-Gag (SLYNTVATL: SL9) and HCV-NS5b (ALYDVVSKL: AL9). T-cell responses targeting HIV-SL9 are mainly
prevalent during the chronic phase of HIV infection (232). HCV-AL9 is also a defined epitope, associated with protection during acute infection (57). This study aimed to specifically address if the presence of HIV-SL9-specific T-cells influence the responses towards HCV-AL9, and hypothesized that in HCV/HIV co-infection, individuals with SL9-specific T-cell responses weakly mount AL9-targeted responses.
5.1.3- Materials and Methods

5.1.3.1- Study Participants

Blood samples were obtained from 20 HIV mono-infected and 17 HCV/HIV co-infected and 5 HCV mono-infected individuals and cryo-preserved until use. All studied individuals were HLA-A2\(^+\), as identified by HLA-A2 antibody staining (BD Pharmingen). Informed consent was obtained in accordance with the guidelines for conduction of clinical research at the University of Toronto and St. Michael’s hospital institutional ethics boards. Peripheral blood mononuclear cells (PBMCs) were isolated as previously described(215). Clinical data of the studied individuals are presented in Table1. None of the HCV infected individuals were treated with interferon/ribavirin prior to sampling. All studied co-infected individuals had HCV infection prior to being infected with HIV.

5.1.3.2- Identifying amino-acid sequence homology between HIV and HCV by BLAST proteomic search

A detailed search for amino acid sequence homology between HCV and HIV proteome was conducted using Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) database. BLAST search was conducted in two different ways. First, through a general search for short, nearly-exact amino-acid matches between HCV (H77 strain) poly-protein and different parts of HIV (HXB2 strain) proteome. We found 4 similar hits, as defined by relative similarities in short amino-acid sequences, within HIV Gag, 1 within HIV Pol, and 1 within HIV Vif
regions. In the second approach, we conducted a BLAST search within identified HCV CTL epitopes, to find amino-acid sequence matches within the whole HIV proteome. We found 7 hits, as defined by relative amino-acid sequence similarities within HCV-core epitopic region, none being identified HIV epitopes. However, our search within HCV-NS5b epitope map resulted in the identification of a relatively high degree of amino acid sequence similarity between HIV (HXB2) Gag p17: 76-84 (SLYNTVATL: SL9) and HCV (H77) NS5b:2594-2602 (ALYDVVSKL: AL9), with 4 out of 9 amino acids and HLA anchorage residues being similar, and both being defined HLA-A2-restricted epitopes.

5.1.3.3- IFN-γ ELISPOT screen

PBMCs from each individual (2x10^5/well) were incubated in 96-well, polyvinylidene plates, pre-coated with capture anti-human IFN-γ mAb and stimulated with 10 µg/ml of either HIV-Gag p17: 76-84: SLYNTVATL (SL9) or HCV NS5b: 2594-2602: ALYDVVSKL (AL9) peptide (Poimmune PEPscreen®: Custom Peptide Libraries) or DMSO control. An irrelevant HLA-A2-restricted MAGE peptide (Melanoma-Associated Antigen: 271-279: FLWGPRALV) was used as a control peptide for the mono-infected screen. HLA-A2-restricted peptide HCV NS3: 1073-1081: CINGVCWTV (CI9) with no amino-acid sequence similarity to HIV-SL9 was used as a random control for the co-infected screen. Staphylococcus enterotoxin-B (SEB, 3µg/ml) was used as a positive control stimulant. The frequency of responses were measured using an automated ELISPOT counter, and a positive response was defined as 2-fold higher than background (DMSO), with a minimum of 50 spots per 10^6 PBMCs and represented as spot-forming units (SFU/million PBMCs).

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5.1.3.4- CD8⁺ T-cell enrichment

CD8⁺ enriched T-cells were isolated from PBMCs using a StemSep magnetic bead negative selection system (StemCell Technologies). Briefly, PBMCs were re-suspended in separation medium (PBS with 2% FBS) and incubated with enrichment cocktail antibodies, directed against CD4, CD14, CD16, CD19 and CD56, and further incubated with magnetic colloids and passed through a gravity feed column. Unlabeled CD8⁺ T-cells were collected, washed and re-suspended in RPMI-1640 (Gibco Laboratories) for further analysis.

5.1.3.5- Generation of short-term T-cell lines

Antigen-specific T-cells were expanded from either the CD8⁺ T-cell enriched compartment or whole PBMCs in 24-well plates. Cultures of 10⁵ CD8⁺-enriched cells and 5x10⁵ autologous irradiated (3000 rads) feeder PBMCs were stimulated with synthetic SL9 or AL9 peptides (10 µg/ml) in RPMI supplemented with 10% heat-inactivated human AB serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml), in the presence of anti-CD28 and anti-CD49 antibodies. Recombinant interleukin-7 (rIL-7) (10ng/ml) and IL-15 (10ng/ml) (R&D Systems) were added. rIL-2 (50 IU/ml) (Invitrogen Inc.) was added on day 3 and the cells were harvested for experiments after 7-10 days.
5.1.3.6- Pentamer staining

Pentamer staining for SL9 or AL9 sequence was performed prior to peptide stimulation, at room temperature for 15 minutes (Fluorescent-labeled Pro5™ MHC class I pentamers, Proimmune). Pentamer-stained cells were then washed with 2% FBS in PBS and stimulated with each SL9 or AL9 peptide (10 µg/ml), followed by intra-cellular staining (ICS) and flow cytometry analysis.

5.1.3.7- Ex-vivo CD8+ T-cell stimulation and intracellular staining (ICS)

One million PBMCs from each individual were stimulated with 10 µg/ml of either SL9 or AL9 peptide or DMSO control for 6 hours in the presence of co-stimulatory antibodies (CD28 and CD49d, 1µg/ml BD Biosciences), as well as monensin (0.7 µL/mL; BD Biosciences) and brefeldin-A (10 µg/mL; Sigma-Aldrich). CD107a antibody (BD Pharmingen) was also added during the time of stimulation. Following the stimulation, the cells were washed and stained with cell surface antibodies against CD3 and CD8 (BD Biosciences) and then fixed, permeabilized and stained for intracellular cytokine IFN-γ (BD Biosciences). Percentage of responding antigen-specific CD8+ T-cells was measured by flow cytometry, using either a FACSArria flow cytometer, or a FACSCalibur instrument (BD Biosciences). Further analysis was performed using FlowJo version 7.5 (Tree Star Inc.).

5.1.3.8- Statistical Analysis

Data were analyzed by performing two-tailed student’s t-test using GraphPad Prism version 4.00 (GraphPad Software Inc.). Mann-Whitney test was used for non-
parametric data. Correlations were tested for significance by Spearman rank analysis. P-values of <0.05 were considered significant.

5.1.4- Results

5.1.4.1- Subject Characteristics

The two groups of studied individuals are depicted in Table 5.1; HIV mono-infected (n=20) (of whom 15 were untreated and 5 were receiving highly active antiretroviral therapy (HAART) for greater than one year at the time of evaluation) and HCV/HIV co-infected (n=17) (7 on HAART). HIV mono-infected individuals had negative HCV serology and undetectable HCV viral loads by bDNA assay. In addition, we examined 5 HCV mono-infected individuals. None of the co-infected individuals received prior pegylated-interferon/ribavirin treatment for HCV infection. All the co-infected individuals were infected with HCV first. HIV mono-infected individuals had a median HIV viral load of 1,560 copies/ml (± SE 28,143) and a median CD4 T-cell count of 670/mm$^3$ (± SE 52.7). HCV/HIV co-infected individuals had a median HIV viral load of 7,359 copies/ml (± SE 18,942), a median CD4 T-cell count of 352/mm$^3$ (± SE 59) and a median HCV viral load of 1,330,000 IU/ml (± SE 1,722,000). Between the two cohorts, HIV mono-infected subjects had higher CD4 counts (p=0.0008). There was no significant difference of HIV viral load between the two cohorts.
5.1.4.2- Identifying Individuals with T-cell responses to both HIV-SL9 and HCV-AL9 antigens

In order to examine if HIV mono-infected individuals with an SL9-specific T-cell response would also respond to HCV-AL9 antigen, 20 HLA-A2\(^+\), HIV mono-infected individuals were screened for responding to both SL9 and AL9 peptides, using IFN-\(\gamma\) ELISPOT assay. Of HLA-A2\(^+\) HIV mono-infected individuals, 15 subjects (75\%) showed response to SL9 peptide stimulation (more than 2-fold of background). Of these individuals, 4 patients (26\%) showed significant responses to AL9 peptide as well, despite not being HCV infected (HCV Ab negative, HCV VL negative). We refer to these individuals as HIV infected dual responders. No AL9 responses were detectable in the absence of SL9 in these individuals. One individual (OM 358), also responded to a MAGE self antigen peptide as well, and could not be studied further due to lack of additional samples (Figure 5.1a). These findings suggest that the presence of an HCV-specific AL9 response in HIV mono-infected individuals may be a result of cross-reaction of T-cells specific to the HIV-SL9 epitope.

We examined the prevalence and the magnitude of dual recognition of SL9 and AL9 antigens in HCV/HIV co-infection by screening 17 HLA-A2\(^+\) co-infected individuals by ELISPOT. Among the screened co-infected individuals, overall responses to SL9 peptide were weaker in magnitude, compared to HIV mono-infected cohort. A total of 10 out of 17 co-infected individuals (59\%) responded to SL9 antigen. Responses to AL9 peptide were observed in a total of 9 out of 17 co-infected individuals (53\%). Of co-infected individuals, 8 out 17 (47\%) had responses to both antigens (We refer to these individuals as HCV/HIV co-infected dual responders) (Figure 5.1b). Only one of the
screened HIV/HCV co-infected subjects (6%) showed an AL9-specific response in the absence of an SL9 response.

In a similar screening of a limited number of HLA-A2\(^+\) HCV mono-infected patients (n=5), we observed no response to either AL9 or SL9 peptides (data not shown). Our observation of lack of HCV-AL9 response among the HCV mono-infected studied subjects was consistent with a previous report demonstrating infrequent HCV-NS5b responses in HLA-A2\(^+\) individuals with chronic HCV infection (57). Lechner, F. et al. demonstrated that 0/10 studied individuals being chronically infected with HCV, had CD8\(^+\) T-cells detecting the HCV-NS5b: 2594 (AL9) epitope. We observed a greater frequency of responses to the HCV-AL9 epitope in HCV/HIV co-infection in comparison to HCV mono-infection. The AL9 response was predominantly associated with the presence of the HIV-SL9 response as shown by correlation analysis (see below).

5.1.4.3- Detection of Cross-reactive CD8\(^+\) T-cells in identified dual responders

In order to determine whether dual responses in the HIV mono-infected group were due to true cross reactivity by SL9-specific CD8\(^+\) T-cells, PBMCs from the identified dual responders were directly *ex-vivo* stained with the corresponding pentamers. All three HIV mono-infected dual responders showed CD8\(^+\) T-cells distinctly double-stained for both pentamers, indicating the presence of a cross-reactive T-cell population (Figure 5.2a). Similar pentamer staining was also conducted on PBMCs from HCV/HIV co-infected dual responders. In contrast, only one of the 8 co-infected dual responders (OM 539) was identified to have T-cells cross-recognizing both antigens. This individual had two
populations identifying AL9 or SL9 with a small population being dual stained with both pentamers, thus being cross-reactive to both epitopes (Figure 5.2b, upper panel). The majority of co-infected dual responders demonstrated separate, non cross-reactive recognition of T-cells, directed against either epitope (Figure 5.2b lower panel). Compiled data of the percentages of pentamer+/CD8+ T-cells from all co-infected dual responders indicate that cross-reactive T-cell population recognizing both HIV-SL9 and HCV-AL9 among HCV/HIV co-infected individuals is infrequent, although populations recognizing either epitope are common (figure 2c). This indicates that in HCV/HIV co-infection, the majority of SL9- and AL9-specific CD8+ T-cells are not cross-reactive to each other but mainly comprise of T-cells directed toward separate epitopes.

5.1.4.4- SL9-specific CD8+ T-cells weakly respond to stimulation with the heterologous AL9 antigen

To examine whether SL9–specific T-cells could be activated by AL9 antigen, SL9 pentamer-stained CD8+ T-cells from dual responders were assessed for IFN-γ and CD107-a production by flow cytometry. Direct ex-vivo stimulation of HIV mono-infected PBMCs with either SL9 or AL9 peptide demonstrated that SL9-pentamer positive CD8+ T-cells responded to stimulation with not only their cognate peptide, but also the heterologous HCV-AL9 peptide, as shown by IFN-γ and CD107-a production (Figure 5.3a). However, these cross-reactive responses to AL9 peptide were much weaker compared to those from SL9 peptide stimulation [% CD8+/pentamer+ in one representative (OM 125): IFN-γ: 40.8 for SL9 vs. 1.9 for AL9; CD107-a: 37.9 for SL9 vs. 11.1 for AL9]. Similar functional properties were observed in the only co-infected
individual with a dual pentamer-stained population (OM 539), showing that SL9-specific CD8\(^+\) T-cells are weakly responding to cross-reactive AL9 stimulation [\% CD8\(^+\)/pentamer\(^+\), IFN-\(\gamma\): 15.59 for SL9 vs. 7.14 for AL9] (figure 5.3b).

5.1.4.5- Substantial heterogeneity in expansion of the cross-reactive CD8\(^+\) T-cell population within SL9-generated T-cell lines from HIV mono-infected blood

In order to determine if HCV-AL9-specific CD8\(^+\) T-cells could be expanded among HIV mono-infected dual responders after \textit{in-vitro} peptide expansion, antigen-specific short-term T-cell lines were generated. ELISPOT analysis of responses demonstrated that in one individual (OM 54), both SL9- and AL9-generated T-cell lines produced IFN-\(\gamma\) in response to either peptide stimulation (Figure 5.4). In the other two individuals, we could not expand functional AL9-specific T-cells either with the cognate SL9 peptide or the cross-reactive AL9 antigen to a similar degree as with cells taken from OM54. The compiled data analysis of IFN-\(\gamma\) response from antigen-specific T-cell lines of the three HIV mono-infected dual responders demonstrated the presence of functional cross-reactive T-cells in only one of these individuals (Figure 5.4). SL9-generated line from OM 18, showed a borderline positive IFN-\(\gamma\) response to AL9 peptide stimulation. These individual variations depict the heterogeneity between individuals in the ability of T-cells to expand in response to a cross-reactive epitope.
5.1.4.6- SL9- and AL9-specific T-cell responses positively correlate with each other and with clinical parameter of liver injury in HCV/HIV co-infected responders

To understand whether the degree of T-cell responses to HIV-SL9 antigen associate with responses to HCV-AL9 peptide, correlation analysis was performed on the direct ex-vivo IFN-γ ELISPOT responses from all studied co-infected individuals. A positive correlation was observed between these two types of responses \[r=0.58, P=0.0004\]. No correlation was found between responses to HIV-SL9 antigen and to a less similar HLA-A2-restricted HCV epitope, the HCV-CI9 peptide (Figure 5.5a). Among dual responding co-infected individuals, no correlations were observed between either SL9- or AL9-specific responses and HIV or HCV viral load, although HCV viral loads were substantially high in all co-infected individuals.

To investigate the clinical relevance of our findings, we examined if there is a correlation between the magnitude of SL9- and AL9-specific T-cell responses in co-infected individuals with their corresponding APRI score (Aspartate Aminotransferase to Platelet Ratio Index), as a clinical indicator of the stage of liver fibrosis (216), in which a higher APRI score correlates with greater hepatic fibrosis. We first looked at this correlation among all the studied co-infected individuals. The magnitude of IFN-γ responses specific for HIV-SL9 correlated positively with the corresponding APRI score of each individual \[r=0.70, p=0.002\]. A trend towards positive correlation was observed for HCV-AL9-specific responses (Figure 5.5b, left). We then looked at this correlation among co-infected dual-responders only, and observed that responses specific for both HIV-SL9 and HCV-AL9 correlated positively with the corresponding APRI score of each individual \[r=0.68, p=0.01\] for SL9; \[r=0.55, p=0.03\] for AL9 (Figure 5.5b, right).
5.1.5- Discussion

The overall impact of T-cell cross-recognition of heterologous human infections is not clearly defined, but has been suggested to skew the immuno-pathogenesis outcome (189, 191). Several mechanisms are currently identified that largely explain the ability of T-cell receptors to cross-recognize different unrelated antigens (233). Molecular mimicry, where ligands share key structural features, is one of the first mechanisms proposed for T-cell cross-reactivity (234).

The present study started out with a focus on the role of molecular mimicry in T-cell cross-recognition. We elaborated on T-cell cross- recognition of two well-defined HIV and HCV epitopes during HCV/HIV co-infection. We provided evidence that in HIV infection and in the absence of HCV infection, CD8$^+$ T-cells that are specific for the immunogenic HIV epitope SL9 recognize the sequentially similar HCV-AL9 antigenic peptide. However, this interaction seems to be of low affinity, as shown by limited production of cytokine IFN-$\gamma$ and degranulation marker CD107a by these cells in response to the heterologous HCV-AL9 peptide. This was further supported by functional analysis of antigen-stimulated lines generated from PBMCs from HIV infected individuals, in which the AL9 peptide weakly expanded cross-reactive CD8$^+$ T-cells. Although pentamer staining could have interfered with the ability to stimulate stained T-cells with heterologous epitopes used in our assays, we had confirmed those findings by demonstrating weak induction of heterologous responses with independent peptide stimulations without pentamer co-staining, hence ruling out the effect of potential pentamer interference. Our findings are also in line with those recently described by Kasprowicz et al., in which HCV specific CD8$^+$ T-cells to an HCV-NS3 epitope weakly
cross-reacted with an influenza neuraminidase epitope, producing low affinity and weak responses to the latter epitope (190).

Contrary to our original hypothesis, our data suggest that co-infected individuals with higher HIV-SL9 T-cell response mount higher HCV-AL9 responses as well, which were more frequent than was observed in HCV mono-infection. This may suggest that the co-existence of the SL9 response, did not prevent the acquisition of an AL9 response, but rather helped its induction. Surprisingly, these apparently “cross-reactive” responses to AL9 in co-infected individuals were more often due to separate T-cell populations by pentamer staining, although truly cross-reactive T-cells could also be seen. It is possible that the separate T-cell population identified by the AL9 pentamer could represent a cross-reactive SL9 population with such poor avidity to the AL9 epitope as to abrogate binding to the AL9 pentamer. In this regard, Kasprowicz et. al., was able to identify such populations using of MHC Class-I tetramers with enhanced binding capacity (CD8 hi tetramers) (190) to certain cross-reactive epitopes. We however did not see any improvement in avidity of the SL9 staining with CD8hi tetramers directed to the AL9 epitope (data not shown). In addition, Kasprowicz et. al., also reported no difference in the staining and appearance of AL9-specific CD8+ T-cells between normal and CD8 hi tetramers, which supports the findings on T-cell responses to HCV-AL9 epitope being defined as high-affinity responses (57). Given, that we identified two separate populations in some individuals, it is possible that other HIV epitopes, rather than SL9 could induce AL9 responses through alternative recognition of different TCR determinants in this situation. Further exploration is warranted to examine this possibility. Nevertheless, our findings suggest that exposure to the SL9 epitope expands SL9-specific
T-cells with TCR that may cross-react with AL9 antigen, but may also expand T-cells with low to no affinity for SL9 but with affinity to AL9, hence generating two populations responding to separate pentamers. These findings are consistent with previous observations noted with EBV infection in humans, in which, acute EBV is shown to alter the T-cell receptor repertoire of the memory T-cell population (166, 191). Upon infection of a heterologous virus, these previously expanded cells would then be recruited to respond to the heterologous virus (166, 191). Further studies in our population would have to be performed to determine if similar V or J betas are used by SL9- and AL9-specific T-cells in our cohort.

We also observed heterogeneity in the functional nature of cross-reactive T-cells in co-infected individuals. For e.g., in only one of three individuals tested were we able to show successful in-vitro expansion of cross-reactive CD8+ T-cells. The expanded population in this individual showed similar functionality in response to stimulation with either HIV-SL9 or HCV-AL9 peptides. These findings suggest that depending on the private specificity of the TCR of an individual, cross-reactive T-cells can be induced with varying avidities to the heterologous epitope, and with varying functionalities.

The main question however, is whether the consequences of this T-cell cross recognition in HCV/HIV co-infection affect HCV disease outcome in the host. T-cell cross-reactivity could result in two scenarios:

1) The T-cell responses in co-infected individuals could become skewed, as described in ‘original antigenic sin’(180, 184), leading to T-cell responses mainly to the initial pathogen. Hypothetically, this would lead to a loss of responses to either HCV-AL9 or HIV-SL9 antigen.
2) HIV infection could prime for the induction of heterologous HCV responses that would not have occurred without exposure to HIV, by recruiting TCRs that could potentially cross-react with HCV. This may lead to the generation of functionally weaker T-cell responses towards the heterologous HCV-AL9 in those co-infected individuals with detectable HIV-SL9 response due to recruitment of TCRs of weaker specificities for AL9 compared to those who do not mount an SL9-specific T-cell response.

Original antigenic sin, as referred to in the first scenario, was first described in humans as a strong humoral response to an original influenza virus strain, with a modest response against a following flu infection with a heterologous strain (180). Later, Klenerman et al. demonstrated this phenomenon in cytotoxic T-lymphocytes in mouse LCMV infection (184). According to this phenomenon, the immune response to a current infection may be dominated by T-cells targeting epitopes of a previously encountered pathogen. This by virtue, as shown in other viral co-infections (189), could result in a loss of breath of responses towards HCV after an encounter with HIV, and lead to progression of HCV disease. However, for AL9 and SL9 epitopes we did not observe this, and in fact found a positive correlation between the magnitudes of responses against the two described antigens.

The second scenario could be potentially advantageous if the presence of HIV infection would have primed T-cells to respond to similar HCV epitopes, but could be disadvantageous if the resulting responses were of low avidity to the new virus. The generation of weak heterologous responses as mentioned in the second scenario, could hypothetically result in an opportunity for HCV to escape and also lead to more severe HCV disease.
To further address the above scenarios, we used APRI scoring for validation of the clinical significance of our findings. APRI have been used as a non-invasive test for estimating liver fibrosis, with reportedly high sensitivities and specificities, and a prognostic value similar to the established Model of End-Stage Liver Disease (MELD) in HCV infection, with or without HIV (216, 221, 223, 224). Our observation that among studied co-infected subjects, the highest APRI score of liver belong to the only individual with dual, cross-recognizing CD8+ T-cells, may suggest a negative role that the presence of these cross-reactive T-cells may play in advancing HCV-related liver disease in HCV/HIV-co-infection. Antigen-specific lines generated from PBMCs of the same individual were shown to mount the highest magnitude of IFN-γ response to both SL9 and AL9 antigens. We have previously shown that functional HIV-specific T-cells accumulate in the liver in HCV/HIV co-infection (226). Accordingly, the presence of these cross-reactive CD8+ T-cells in the blood may not be strong enough to facilitate HCV clearance, but the trafficking of these T-cells to the liver and their dual response to both HIV and HCV antigens may negatively affect the cytokine milieu of the liver and add to the initial tissue damage. How exactly the cross-talk between these two viral epitopes may affect HCV-related liver pathogenesis requires additional investigation with a much larger cohort of HLA-A2+ co-infected individuals.

In conclusion, our findings provide evidence for some degree of T-cell cross-reactivity between two immunogenic HIV and HCV epitopes. Although cross-recognition by the same T-cells seems to be an infrequent event in HCV/HIV-co-infection, it might be partially responsible for the clinical outcome of HCV disease in co-infection with HIV. It would be an underestimation to only consider structural similarities of epitopes
responsible for T-cell cross-reactivity between HIV and HCV. A thorough analysis is warranted to identify the extent of this phenomenon in these two viral infections and its impact on disease outcome.
### Table 5.1

#### a) Characteristics of HIV mono-infected Individuals:

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<th>CD4 Count (/mm3)</th>
<th>HAART</th>
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#### b) Characteristics of HCV/HIV co-infected Individuals:

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<th>CD4 Count (/mm3)</th>
<th>HAART</th>
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<th>HCV VL (IU/ml)</th>
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<td>1a</td>
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**OM#: Participant’s Code**  
**LTNP: Long-Term Non-Progressor**  
**VL: Viral Load (Plasma)**  
**HAART: Highly Active Retroviral Therapy**  
**APRI: AST to Platelet Count Ratio Index**  
**N/A: Not Available**
Figure 5.1. IFN-γ ELISPOT cross-reactivity screen.

PBMCs from HLA-A2⁺, HIV mono-infected (a) and HCV/HIV co-infected individuals (b), were screened for responding to either HIV-Gag: SLYNTVATL (SL9) and/or HCV NS5b: ALYDVVSKL (AL9) peptides by direct ex-vivo IFN-γ ELISPOT assay. An irrelevant HLA-A2-restricted MAGE peptide: FLWGPRALV (FV9) was used as a control peptide for the HIV mono-infected screen. HLA-A2-restricted peptide HCV-NS3: CINGVCWTV (CI9) was used as a random control for the co-infected screen. A positive response was defined as 2-fold higher than DMSO background (presented by the red horizontal line), with a minimum of 50 spots per $10^6$ PBMCs and represented as spot-forming units (SFU/million PBMCs).
Figure 5.2. Pentamer binding analysis of antigen-specific CD8+ T-cells from identified dual responders.

PBMCs from individuals identified from the ELISPOT screen were stained with HLA-A2-restricted class I pentamers HIV-Gag p17:76 (SLYNTVATL) and HCV NS5b: 2594-2602: ALYDVVSKL (AL9) and analyzed for the frequencies of dual-stained CD8+ T-cells. a) Shown are flow cytometry plots from the three HIV mono-infected dual responders. b) Shown are representative flow cytometry plots of two co-infected screened dual responders (OM 539: upper, OM 530: lower). The last plot on each row is gated on CD8+ T-cells. c) Shown are compiled data from the percentages of single and dual pentamer+/CD8+ T-cells from all co-infected dual responders.
a) DMSO

- HIV-SL9 Peptide: 0.7%
- SL9 Pentamer: 2%

b) DMSO

- HIV-SL9 Peptide: 3%
- SL9 Pentamer: 7%
Figure 5.3. Functional properties of the cross-reactive T-cells.

PBMCs stained with HIV-Gag p17: 76-84: SLYNTVATL (SL9) pentamer were analyzed for IFN-γ and CD107a production after a 6-hour stimulation with either AL9 or SL9 peptide. Shown are representative flow cytometry plots of functional analysis of SL9-specific CD8⁺ T-cells from blood of a) HIV mono-infected dual responder, OM 125 and b) HCV/HIV co-infected dual responder, OM 539. The last two columns were gated on SL9-pentamer⁺/CD8⁺ T-cells. DMSO treated PBMCs were used as negative control and responses greater than twice the DMSO response were considered positive.
**Figure 5.4.** Analysis of T-cell cross-recognition after *in-vitro* antigen-specific expansion.

Shown are the compiled ELISPOT data on the frequencies of T-cells producing INF-γ in response to either SL9 or AL9 peptide re-stimulation in short-term T-cell lines from the three HIV mono-infected responders. The horizontal red lines show the threshold of positive response to both antigens.
Figure 5.5. Analysis of correlation among different antigen-specific responses and their association to parameter of disease progression in HCV/HIV co-infected individuals.

a) Shown are correlation analyses between the magnitudes of INF-γ responses specific for HIV-SL9 and HCV-AL9 (left), and HCV-CI9 and HIV-SL9 (right) among all studied co-infected individuals. All data are presented after background subtraction. b) Shown are correlation analyses between antigen-specific INF-γ responses and APRI score among all studied co-infected individuals (left), and co-infected dual-responders (right). Correlations were analyzed using the Spearman rank test and APRI was calculated according to the following formula (228):

\[ \text{APRI} = \left( \frac{\text{AST Level/ULN}}{\text{Platelet count}} \right) \times 100 \]

*ULN= AST upper level of normal or 56 IU/L
Chapter 6:
General Discussion
6-General Discussion

HCV-related disease becomes more severe and progresses faster in the presence of HIV infection, leading to a great burden of advanced liver disease in HCV/HIV co-infected population. In addition, the current standard-of-care for HCV infection ( pegylated interferon-α and ribavirin) is associated with poor treatment results in co-infection with HIV (235). The liver disease associated with HCV infection is known to be induced by host immune responses, and it is hence paradoxical that HCV pathogenesis becomes aggravated in the immuno-incompetent setting of HIV infection. As a result, it is important to better understand the mechanisms underlying the negative effects of HIV infection on HCV disease. The focus of this doctoral work was to identify and elaborate on different aspects of host T-cell immune function in HCV/HIV co-infection.

6.1- Identification of functional HIV-specific T-cells in HCV/HIV co-infected liver

The first question in this investigation was to address whether HIV-specific T-cells would be identified in the liver of co-infected individuals, and if so how they would affect the immune milieu of the infected liver. Based on several lines of evidence, we indeed expected to find those T-cells in the liver of co-infected individuals. These evidence were based on data that liver functions as the final destination of all primed T-cells (204), as well as the actual detection of HIV RNA in the liver of HIV-infected patients (119). Based on these data, one would speculate that HIV-specific T-cells could
be found in the liver, including those T-cells previously primed at the draining lymph-nodes and eventually trafficked to the liver, as well as those which could have potentially been primed at the site of liver. Regardless of the source of these specific T-cells, we were more interested to examine if these HIV-specific liver T-cells would still be immunologically functional, and how differently would they function compared to HCV-specific liver T-cells or the circulating ones.

We found that in HCV/HIV co-infection, HIV-specific T-cells that produce cytokines are detected in the liver in frequencies comparable to those of HCV-specific T-cells. This observation may explain the added contribution of HIV-specific immune responses to the ongoing intra-hepatic damage induced by HCV-specific T-cell responses that are inefficient to clear the virus. With these data, we propose a novel mechanism for enhanced HCV-related liver disease progression in HIV co-infection. This proposed mechanism revolves around the innocent bystander role that intra-hepatic HIV-specific T-cells may play in HCV-initiated liver damage. Although our data were limited by the cross-sectional nature of our studied cohort and the low number of available liver biopsies, they still provide a foundation for future studies on the immuno-pathology of HCV/HIV co-infection. In addition, our data demonstrate that in co-infection, HAART was associated with reduction in both HIV and HCV-specific intra-hepatic cytokine responses. This finding could have potential implications in the management of this co-infection. If HIV-specific T-cells are immunologically contributing to advanced liver inflammation and fibrosis, then a possible proposal could be to treat dual infected co-individuals with HAART, regardless of their peripheral CD4+ T-cell count in order to reduce the inflammatory responses in the liver (current recommendations are to start
HAART for CD4 counts <350/ul). Such an intervention would expect to ameliorate progression of HCV disease. This model would favour always treating HIV infection first, prior to initiating the IFN-α/Ribavirin treatment for HCV infection. Further prospective studies are required to better understand the role of HIV-specific T-cells in aggravated liver damage during HCV/HIV co-infection and the role of HAART in this setting.

### 6.2- Identification of elevated frequencies of circulating Tim-3/PD-1-expressing T-cells in HCV/HIV co-infection

We expanded our investigation on the role of HIV-specific T-cells in HCV/HIV co-infection by studying whether the presence of HIV infection would affect the functional phenotype of HCV-specific T-cells in co-infection. This aspect of investigation was initiated based upon previous studies on the role of T-cell exhaustion in the pathogenesis of mono-infection with HIV or HCV. Our findings demonstrate that the co-existence of HIV infection is associated with an added effect on the exhaustion phenotype of both total and HCV-specific T-cells as depicted by the frequencies of co-expression of two defined exhaustion markers- PD-1 and Tim-3. It is currently unclear if the T-cell phenotype that we observed in this study leads to loss of viral control, or if it is an indirect outcome of other underlying mechanisms including viral escape from CTL epitopes. The question of what drives this elevated expression on HCV-specific T-cells still needs to be addressed in future studies. The aggravated exhaustion phenotype of HCV-specific T-cells in co-infection may be driven by the lack of proper CD4 help found in HIV infection, but also by a direct affect from HIV virus. Our initial finding of the
accumulation of HIV-specific T-cells in the liver during HCV/HIV co-infection may help explain the association that we observed between the frequencies of PD-1/Tim-3-expressing HIV-specific T-cells and the progression of liver fibrosis. Since exhausted T-cells are activated, the presence of these specific T-cells may affect the whole cytokine milieu of the liver and the induction of hepatic fibrosis. Additional studies are required to elaborate on this exhaustion phenotype in hepatic T-cells.

Functionally, the exhaustion phenotype that we observed was associated with poor cytokine production and poor proliferative capacity of circulating T-cells, which could be partly reversed by blocking antibodies. Since T-cell exhaustion is associated with several immuno-regulatory pathways, blocking of multiple pathways should be approached with extra caution. In vivo manipulation of these pathways may lead to enhanced T-cell immune responses, but may also result in the onset of an autoimmune process with dire consequences. Nevertheless, identification of novel pathways that describe T-cell exhaustion in HIV and HCV infections will have important implications in our understanding and treatment of these chronic persistent viral infections. Our findings could have a fundamental role in further investigations for developing novel immunotherapeutic strategies to reverse the immune defects associated with HIV and HCV infections.

6.3- Identification of T-cells cross-recognizing HIV and HCV epitopes

Reports on T-cell cross-reactivity between two HCV and Flu epitopes and the association between the existence of these T-cells and the clinical outcome of HCV
disease (189), encouraged me to explore the possibility that T-cell degeneracy could affect the immunopathogenesis of HCV disease at the presence of HIV infection. I started out on this project with a focus on molecular mimicry of T-cells and a general search for amino acid sequence homology between HCV and HIV whole proteomes in BLAST proteomic database. This search resulted in the identification of a number of similar hits, but mostly among undefined peptide sequences, and none as strikingly similar in amino acid sequence as the HIV-SL9 and HCV-AL9 epitopes that were chosen to be further studied. Examining T-cells targeting these two epitopes provided evidence that in HIV mono-infection, CD8^+ T-cells that are specific for the immunogenic HIV-SL9 epitope recognize the sequentially similar HCV-AL9 antigenic peptide, but with much lower affinity. This observation was further supported in the context of HCV/HIV co-infection. The heterogeneity that we observed in terms of the magnitude of this T-cell cross-reactivity among the studied individuals may suggest that depending on the private specificity of the TCR of an individual, cross-reactive T-cells can be induced with varying avidities to the heterologous epitope, and with varying functionalities. If this type of T-cell cross-reactivity could even partially affect the clinical outcome of HCV disease, then the variabilities of TCR repertoire among the infected individuals could potentially result in different degrees of disease severity. In addition, host’s history and the sequentiality of viral infections could affect the repertoire of T-cell memory pool. This in turn may affect whether T-cell cross-recognition should result in protective immunity or advanced immunopathology.

Alltogether, this body of reaserch demonstrates through different perspectives, how the presence of HIV infection may affect the adaptive immunity of a host towards
HCV; and how the alterations of the immune milieu induced by HIV infection could result in changes in the ultimate outcome of HCV-related disease. The causality of the observations and the analysis of the interplay between these two chronic viruses require further investigation.
Chapter 7:

Future Directions
7- Future Directions

The data generated through this doctoral project, like any other scientific research, built up not only answers but also more questions to be further addressed. Over the coming years, the development of more sophisticated techniques and a better understanding of the immune system will enable future studies to revisit old questions and to provide answers to the new questions with more powerful tools. This could integrate the data generated through this work into new projects. In this section of the thesis, I provide suggestions on potential future projects that can expand some of the findings achieved over the course of my doctoral work.

7.1- Prospective analysis of intrahepatic immune responses over the course of HCV/HIV co-infection

One of the shortcomings of my current findings on the accumulation of HIV-specific T-cells in co-infected liver is the lack of longitudinal data. The current data are indeed limited by the cross-sectional nature of the study; and although provide essential pieces of information, do not elaborate on what happens immunologically in the liver during a long period of co-infection. Prospective cohorts are warranted in order to better understand the role that HIV-specific T-cells play in contributing to fibrosis and in particular how HAART modulates these responses. These prospective cohorts should provide both blood and liver biopsy samples from co-infected individuals on a possible regular basis. This will enable a longitudinal analysis of an array of markers depicting the functional status of HIV-specific T-cells. The categorization of the cohorts based on the
reception of HAART could provide an opportunity to explore the long-term effect of HAART on modulating intrahepatic T-cell responses. In addition, my multiparameter cytokine/chemokine analysis of liver T-cells was limited by the small number of mononuclear cells isolated and the technical challenges faced regarding some antibody staining. In addition to liver biopsy analysis, the isolation of lymphocytes from extracted liver of transplant recipients would provide an opportunity for conducting larger multiparameter and proliferation analysis.

7.1.1- Analysis of the role of IL-21-expressing T-cells in HCV/HIV co-infection

Recently, the cytokine interleukin-21 (IL-21), which is responsible for viral control by the prevention of CD8\(^+\) T-cell exhaustion, was shown to be produced by CD4\(^+\) T-cells during LCMV infection (236). In addition, in HIV infection, the presence of virus-specific IL-21-producing CD4\(^+\) T-cells is shown to be correlated with better viral control (237). Meanwhile, in those infected with HIV who had high viral loads (>20,000 virus copies/mL), IL-21 producing CD4\(^+\) T-cells are observed to be infrequent or undetectable. Absence or reduced activity of IL-21-producing CD4\(^+\) T-cells may play a role in the more severe HCV disease observed in HCV/HIV infection. Further studies are required to examine the quantity and quality of these cells in HCV/HIV co-infection and a comparison with mono-infection, as well as the effect of IL-21 and other gamma-chain cytokines (IL-2, IL-4, IL-7, IL-15) on rescuing the function of CD8\(^+\) T-cells in blood and liver during HCV/HIV co-infection.
7.2- Identification and analysis of T-cell cross-reactivity in the liver during HCV/HIV co-infection

The identification of T-cell cross-recognition of two well-defined HIV and HCV epitopes in the blood of co-infected individuals puts emphasis on the role of heterologous immunity in HCV disease pathogenesis. However, it is currently unclear if the same phenomenon, as observed in the blood, takes place in the liver of co-infected individuals. This shall be addressed by intrahepatic analysis of antigen specific T-cells in the liver. As previously mentioned, one of the biggest limitations regarding liver analysis, is the number of isolated lymphocytes. Availability of cadaver liver or transplant extracted liver would be a valuable source for the analysis of this phenomenon within intrahepatic T-cells. Upon availability of more liver cells, the possibility of cross-recognition of less homologous HIV and HCV epitopes would also be feasible. It is indeed an underestimation to only consider the sequence similarities of epitopes for cross-reactivity search. A matrix analysis of cytokine production of overlapping peptides covering the whole proteomes of HIV and HCV would be a promising tool for identification of cross-rective T-cells in the liver, which of course requires larger numbers of isolated liver lymphocytes.
Chapter 8:

References


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