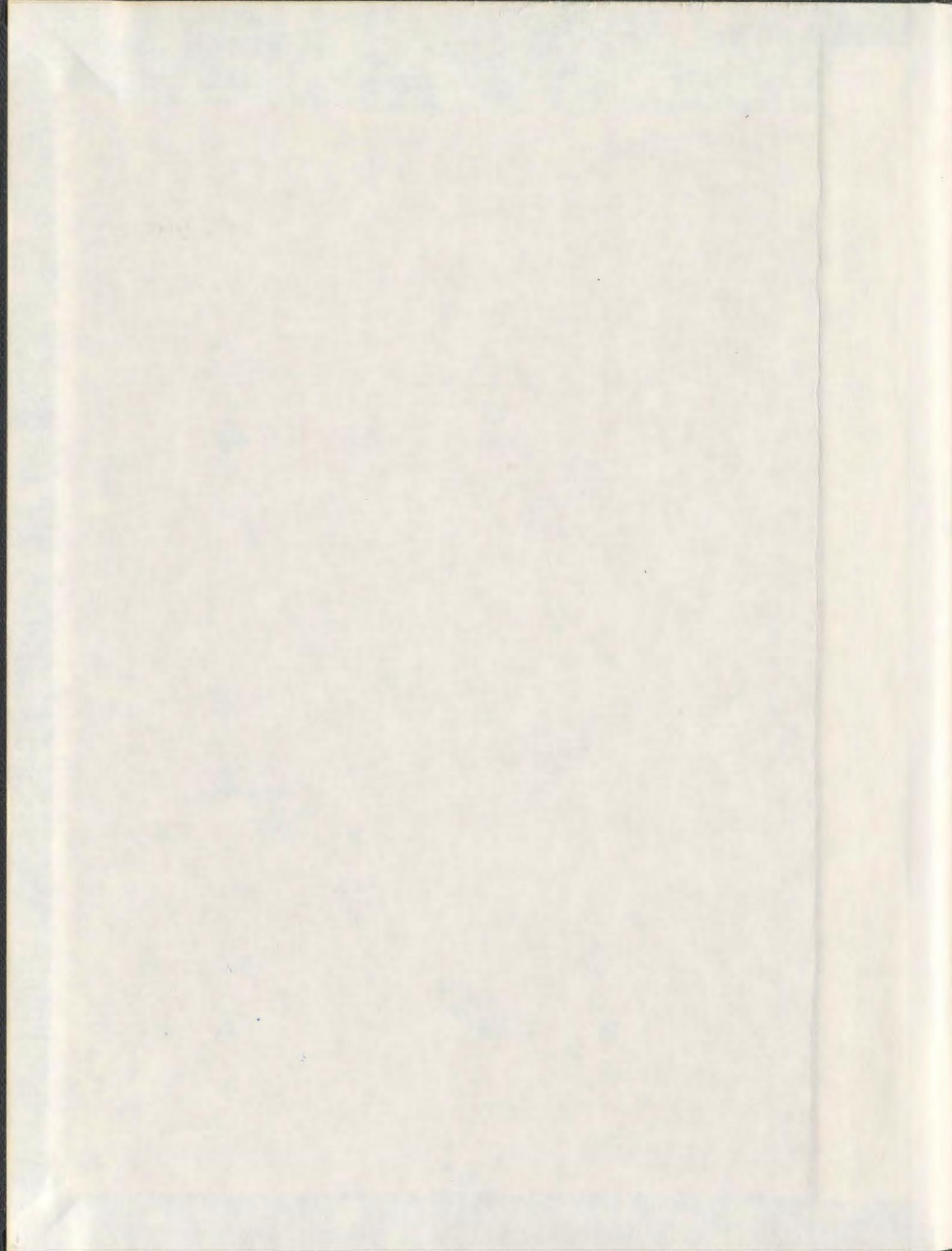
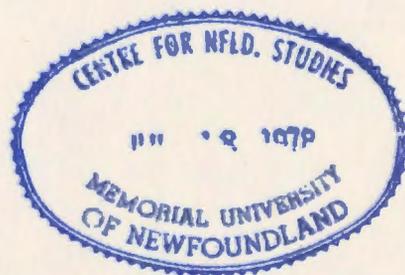


THE CHARACTER OF ANTI-HEPATITIS C VIRUS
T CELL RESPONSES IN HIV COINFECTION

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THE CHARACTER OF ANTI-HEPATITIS C VIRUS T CELL RESPONSES

IN HIV COINFECTION

by

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ABSTRACT

The human immune system has evolved to successfully eradicate most pathogens, however organisms such as hepatitis C virus (HCV) can circumvent the immune response and establish persistent infection in the majority of exposed individuals. While it is clear that broadly directed anti-HCV CD4⁺ and CD8⁺ T cell responses are important in spontaneous viral clearance, the immunologic correlates of persistent infection and treatment-associated viral clearance are markedly less well defined. There is conflicting data on how HCV modulates the immune system in mono-infection, and the data surrounding immunologic issues is even more limited in HIV coinfection.

The type of immune cells that permit or facilitate persistent infection, as well as the character of the anti-HCV T cell response in the context of immunodeficiency and differential infectious outcomes, is unclear. The objective of this study was to assess potential mechanisms of immune modulation by HCV, delineate the types of cells that may be involved, and define anti-HCV T cell responses in the context of coinfection.

In this work, we investigated whether HCV gene products induce interleukin-10 (IL-10), an immunomodulatory cytokine that facilitates chronic infection. We identified and phenotyped a novel cell population producing IL-10, not just in those infected with HCV, but in all healthy controls tested. IL-10 production by this new cell type may have a role in immune system homeostasis through immune regulation, but may also contribute to viral persistence. More of these previously undescribed cells produce IL-10 when exposed to HCV proteins, an effect that is accentuated when HCV-naïve cells from HIV-infected individuals are exposed to HCV proteins. We demonstrated more robust

anti-HCV T cell responses with viral clearance, and distinguished features of anti-HCV T cell responses associated with spontaneous versus treatment-induced clearance. We also described distinct HCV-specific T cell responses in HIV coinfection. Together, these findings emphasize the role of the immune system in HCV clearance, as well as in control of chronic infection, and illustrate the complex interactions between chronic pathogens in terms of their effects on the immune system.

ACKNOWLEDGEMENTS

The support and help of many individuals and groups on both the professional and personal sides have made this thesis possible, despite some rather unlikely circumstances. My supervisor, Dr. Michael Grant, has been both my role model and mentor since my undergraduate years. I am grateful for his support, pragmatism, and encouragement throughout my undergraduate and graduate studies. With completion of my PhD program, I will officially be off his student roster for the first time in eleven years, but I will always remain his student – he has much left to teach me. I am appreciative of the time and expertise Dr. Thomas Michalak and Dr. Deborah Kelly added to the project as members of my supervisory committee. Current and past members of the Grant lab have been instrumental in teaching me both science and life skills that were essential to my learning: Dr. Rodney Russell, Maureen Gallant, and Rosemarie Mason. The Immunology group at Memorial has been my home away from home since high school. Diane Codner, Dr. Jane Gamberg, Dr. Sheila Drover, Dr. Bodil Larsen, and Dr. Bill Marshall, have all helped make immunology and science accessible and exciting.

On the personal side, I have amazing support from my family and friends that makes anything possible. My parents, George and Juanita, have taught by example what it means to be learners and givers, and my sister Mandie has always had the courage to be supportive even when I was too shortsighted to see I needed it. Both sets of grandparents provided the unconditional (and delusional) belief that I could do anything. Dr. Sharon Oldford was always supportive and listened to the same issues over and over again – there has to be a reward for resilience! Dr. Ian Bradbury has been the ‘better half’ of this project all along, and I’m so glad he’s there both professionally and personally.

I gratefully acknowledge the salary support for my program from CIHR. The Office of Graduate Studies in the Faculty of Medicine, Memorial University, has a very supportive team. This was a first trial of an MD/PhD program, and while there are definitely some challenges that remain in training clinician investigators at Memorial, there are people there that can make it work. I appreciate the work and time that these people have contributed.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACD	Acid citrate dextrose
AEC	Alveolar epithelial cell
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ALT	Alanine aminotransferase
AM	Alveolar macrophages
AST	Aspartate aminotransferase
BCIP	5-Bromo-4-chloro-3-indolyl phosphate-p-toluidine
bp	Base pair
BSA	Bovine serum albumin
CCR5	Chemokine (C-C motif) receptor 5
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocytes
CXCR4	Chemokine (CXC motif) receptor 4
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing nonintegrin
df	Degrees of freedom
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E1	HCV envelope protein 1
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
ELISPOT	Enzyme-linked immunosorbent spot assay

EMC	Essential mixed cryoglobulinemia
FADD	Fas associated death domain
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GGT	Gamma-glutamyl-transpeptidase
HAART	Highly active antiretroviral therapy
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HVR	Hypervariable region
IBD	Inflammatory bowel disease
IFN- α	Interferon-alpha
IL	Interleukin
IRF-3	Interferon regulatory factor-3
ISDR	Interferon sensitivity determining region
IU	International units
IVDU	Intravenous drug use
Kb	Kilobase
KIR	Killer immunoglobulin-like receptor
KLRG1	Killer cell lectin-like receptor G1
LDL-R	Low density lipoprotein receptor
L-SIGN	Liver/lymph node-specific ICAM-3 grabbing nonintegrin
mAb	Monoclonal antibody
MCF	Mean channel fluorescence
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid

NBT	Nitro blue tetrazolium chloride
NCR	Natural cytotoxicity receptors
NNRTI	Non-nucleoside reverse transcriptase inhibitor (HIV antiretroviral drug)
NRTI	Nucleoside reverse transcriptase inhibitor (HIV antiretroviral drug)
NS3, NS4	Non-structural protein 3, 4 (HCV genome)
oxLDL	Oxidized low density lipoprotein
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PE	Phytoerythrin
PEG-IFN- α	Pegylated interferon-alpha
PerCP	Peridinin chlorophyll
PI	Protease inhibitor (HIV antiretroviral drug)
PKC	Protein kinase C
PKR	Double-stranded RNA activated protein kinase
PMB	Polymyxin B sulphate
PPAR- γ	Peroxisomal proliferator associated receptor- gamma
PVDF	Polyvinylidene difluoride
RIBA	Recombinant immunoblot assay
RIG-I	Retinoic acid inducible gene I
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SFC	Spot forming cells
SR-BI	Scavenger receptor class B type I

SVR	Sustained viral response
TCR	T cell receptor
Th1	T helper cell type 1
TGF- β	Transforming growth factor beta
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNP	Trinitrophenol
TRIF	Toll-IL-1 receptor domain-containing adaptor inducing IFN- β
ULN	Upper limit of normal

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PREFACE AND CO-AUTHORSHIP STATEMENT

In accordance with the thesis guidelines from the School of Graduate Studies, Memorial University of Newfoundland, this PhD thesis is written in manuscript format. Chapters 2 through 4 have been previously published, and the references are below.

Chapter 2:

Barrett L and M Grant. 2002. Brothers in harm: immunological and clinical implications of coinfection with hepatitis C virus and human immunodeficiency virus. *Clinical and Applied Immunology Reviews* 2: 93.

Chapter 3:

Barrett L, CM Dai, J Gamberg, M Gallant, M Grant. 2007. Circulating CD14⁻ CD36⁺ peripheral blood mononuclear cells constitutively produce interleukin-10. *Journal of Leukocyte Biology* 82: 152.

Chapter 4:

Barrett L, M Gallant, C Howley, MI Bowmer, G Hirsch, K Peltekian, M Grant. 2008. Enhanced IL-10 production in response to hepatitis C virus proteins by peripheral blood mononuclear cells from human immunodeficiency virus-monoinfected individuals. *BMC Immunology* 9: 28.

The candidate was responsible for the design of the experiments, the data analysis, the practical aspects of the research, and manuscript preparation with the exception of the following: Chunming Dai performed additional experiments with IL-10 RT-PCR. Chris Little and Jessica Rose provided cytokine positive control data.

CHAPTER 1

RATIONALE AND OVERVIEW

The human immune system has evolved to successfully combat viral, bacterial, fungal, and parasitic pathogens. Elements of innate and adaptive immunity come together to clear the invader in a vast majority of infections, however, there are a number of organisms that circumvent the immune response and establish persistent infection. *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Helicobacter pylori* are all capable of chronic infection, and many viruses in the herpes family (Epstein-Barr virus, Herpes zoster, Herpes simplex) are more likely than not to persist in the human host. Changes to the host immune system (e.g. manipulation of antigen presenting cells, alteration of T cell maturity, and production of immunosuppressive cytokines), as well as the pathogen itself (e.g. rapid generation time, infection of immunologically privileged sites, antigenic shift and drift), are all ways of establishing and maintaining chronic infection. The exact mechanisms of persistence at play in each infection are often not fully elucidated, indicative of complex host-pathogen interactions. Human immunodeficiency virus (HIV) and hepatitis C virus (HCV) both establish persistent infection, and in the case of HCV, little is known of how the virus manipulates the host immune system to establish and maintain infection.

HCV maintains chronic infection in the majority of exposed individuals, and while it is clear that strong and broadly directed CD4⁺ and CD8⁺ T cell responses are important in spontaneous viral clearance, the immunologic correlates of persistent infection and treatment-associated viral clearance are markedly less well defined. There

is conflicting data on how viral gene products modulate the immune system, and little information on the role of immune cells other than antigen presenting cells and T cells. The data surrounding these HCV-related immunologic issues is even more limited in HIV coinfection. From the clinical perspective, HCV-related disease is more aggressive and associated with higher mortality in HIV coinfection. There also exists an apparent dichotomy between the accelerated hepatic immunopathology of HCV-related liver disease despite increasing HIV-associated immunodeficiency.

The type of immune cells that permit or facilitate persistent infection, as well as the character of the anti-HCV T cell response in the context of immunodeficiency and differential infectious outcomes, is unclear. In this context, there were several objectives for this work:

1. Review the clinical spectrum of HCV and HIV related disease, as well as literature on the anti-HCV immune response in viral clearance and persistence;
2. Define cell types in circulation that secrete cytokines relevant to viral persistence;
3. Assess potential mechanisms of immune modulation by HCV;
4. Define the character of anti-HCV T cell responses in the context of HIV coinfection in general, as well as in subgroups with immunologically distinct outcomes; and
5. Compare the character of anti-HCV T cell responses in spontaneous and treatment-associated HCV resolution with responses in persistent HCV infection.

Chapter two reviews the current literature on HIV and HCV from the clinical and immunologic perspectives. Chapter three describes a novel type of circulating

mononuclear cell that produces an immunomodulatory cytokine known to be instrumental in the establishment and maintenance of other bacterial infections. Chapter four describes how this novel cell type responds to HCV-derived proteins in uninfected individuals, as well as those with HIV infection and chronic HCV infection. Chapter five describes the character of T cell responses in the context of HIV coinfection and the contribution of T cell responses to the natural history of disease. Chapter six delineates the components of the anti-HCV T cell response associated with HCV clearance, and differentiates between responses observed in spontaneous and treatment-induced HCV clearance that suggest more than one avenue to sterilizing responses. The impact and synthesis of these units, as well as the limitations and future research directions, is outlined in chapter seven.

CHAPTER 2

BROTHERS IN HARM:

IMMUNOLOGICAL AND CLINICAL IMPLICATIONS OF COINFECTION

WITH HEPATITIS C VIRUS AND HUMAN IMMUNODEFICIENCY VIRUS¹

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¹This is an updated and revised version of the manuscript published in *Clinical and Applied Immunology Reviews*, 2002.

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2.1. Abstract

Hepatitis C virus (HCV) infection has emerged as a huge global epidemic. Because HCV and the human immunodeficiency virus (HIV) share several routes of transmission, a significant proportion of HIV-infected individuals are coinfecting with HCV. Coinfection with these viruses complicates an already complex set of issues related to diagnosis, clinical disease progression, monitoring disease activity, treatment options and basic immunology. Compared to HIV infection, our understanding of the natural history and epidemiology of HCV infection remains preliminary, leaving many clinical and treatment issues surrounding HCV/HIV coinfection unclear. The potential impact of coinfection with HCV has become an important issue in HIV research and clinical care. This review addresses the virology and natural history of HIV and HCV infections, together with some of the relevant clinical and immunological aspects of HCV infection and how these may be modified in HIV coinfecting individuals.

2.2. HIV epidemiology and natural history

HIV infection is a pandemic with over 30 million persons infected worldwide. HIV spreads through parenteral, sexual and vertical transmission, therefore, risk factors for infection include high risk sexual encounters, needle sharing, intravenous drug use, exposure to unscreened blood and blood products, and breastfeeding from infected women. Before the advent of highly active antiretroviral therapy (HAART), the mean life expectancy for an HIV-infected individual was 9 years. Although the data from the HAART era is relatively new and followup is not yet sufficient to provide reliable estimates, it is clear that life expectancy in HIV infection has increased dramatically enough to justify reclassification of the disease as chronic rather than fatal.

HIV is an enveloped RNA virus belonging to the lentivirus family of retroviruses. The primary targets of HIV infection are CD4⁺ T cells, although the virus also infects macrophages and dendritic cells. The glycoproteins gp120 and gp41 in the viral envelope allow cell binding, fusion of the cell and viral lipid bilayers, and entry of the viral nucleocapsid into host cells. The CD4 molecule is the primary receptor for HIV on host cells, while C-C motif receptor 5 (CCR5), CXC motif receptor 4 (CXCR4) and other related chemokine receptors act as coreceptors (1, 2), as reviewed by Berger et al. (3). Individuals homozygous for deletion mutations in the CCR5 gene resist HIV infection, even after multiple high-risk exposures (4, 5).

Once HIV enters the host cell, an error-prone viral reverse transcriptase creates a DNA copy of the genome that translocates to the nucleus, splices into the host cell

genome and becomes an integrated provirus. Upon activation of the host cell, integrated proviral DNA is transcribed, viral proteins are synthesized, and new virions assembled and released. The antiretroviral drugs used to treat HIV infection interfere with viral replication at several stages of the viral life cycle. Protease inhibitors prevent the proteolytic maturation of precursor polypeptides into functional proteins, while nucleoside, nucleotide, and non-nucleoside analogue reverse transcriptase inhibitors decrease production of proviral DNA by inhibiting the viral reverse transcriptase. Combination therapy employing 2 or more drug classes is usually recommended due to the high rate of viral mutation and the associated rapid development of drug resistance. Several new groups of antivirals are now in use, including inhibitors of viral entry, integration and maturation. Entry inhibitors include maraviroc, a CCR5 antagonist (6, 7) with Federal Drug Administration approval, and CXCR4 antagonists (8). Other agents, such as enfuvirtide, limit viral entry through interaction with gp41 (9, 10). Integrase inhibitors of various types have been tested (11), and raltegravir is currently approved for use in heavily pretreated individuals (12, 13). Maturation inhibitors that act on the viral protease substrate are also in development. One compound, PA-457, prevents maturation of the capsid protein and is currently undergoing Phase 2 clinical trials (14).

Acute HIV infection is characterized by high viremia, transient loss of CD4⁺ T cells, and often a flu-like illness. This acute stage may last 2-6 weeks, throughout the early part of which the infected individual usually remains seronegative. HIV-specific CD4⁺ and CD8⁺ T cells effect the initial decline of viremia and maintain the diminished levels of virus throughout the prolonged clinically asymptomatic phase. The immune

response is insufficient to clear the virus, and eventually viremia rebounds, CD4⁺ T cell counts fall to dangerously low levels and opportunistic infections symptomatic of AIDS develop. Common opportunistic infections in late stage HIV disease, which often demand long-term prophylaxis, include *Toxoplasma*, *Mycobacterium avium intracellulare*, *Pneumocystis jiroveci*. Cytomegalovirus infection or reactivation may occur, however only those who are chronically immunosuppressed require secondary treatment. In addition to the many opportunistic infections, various virus-associated malignancies such as Kaposi's sarcoma (human herpes virus 8), non-Hodgkins lymphoma (Epstein-Barr virus), cervical cancer and anal cancer (human papilloma virus) increase in prevalence with the immunodeficiency of advanced HIV infection.

2.3. Hepatitis C virus

Since initial identification of the HCV genome in 1989 (15), an intense research effort has characterized HCV. HCV is a positive strand RNA flavivirus with a 9.6 kilobase (kb) genome translated as a single polyprotein and cleaved into ten products, four structural (core, E1, E2, p7) and six non-structural proteins (NS2, NS3, NS4A and B and NS5A and B (16) (Figure 2.1). Several alternate reading frame proteins (F protein, double frameshift protein, and short form of core +1) have also been described (17) and although they may not function in the HCV lifecycle directly, they are immunogenic (18, 19) and perhaps immunomodulatory (20).

The HCV lifecycle begins with viral entry into the host cell. A number of cellular receptors have been proposed, including CD81 (21), heparin sulphate (22), low density lipoprotein receptor (LDL-R) (23), scavenger receptor class B type I (SR-BI) (24),

liver/lymph node-specific ICAM-3 grabbing nonintegrin (L-SIGN) and dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) (25-27). These receptors are found on many cell types and suggest that HCV interacts with cells other than hepatocytes. Although thought primarily to be hepatotropic, HCV can also replicate in lymphoid tissue (28-33), and brain (34) and gut epithelial cells (35) are other possible viral reservoirs in chronic infection. However, conflicting reports suggest that the techniques used to demonstrate lymphotropism through detection of negative strand HCV RNA, purely a replicative intermediate, are unreliable (36-38). More recent studies using a modified poly-A tail technique (28) to reduce the impact of positive strand contamination detected negative strand intermediates in lymph nodes (33). It is generally accepted that lymphoid cells support HCV replication, while other tissues remain contentious.

One of the non-structural proteins, an error-prone RNA-dependent RNA polymerase (NS5B), drives the rapid development of viral quasispecies within a single infected individual during times of high viral replication (39). Quasispecies are viruses that are similar, but not identical, and do not have enough sequence diversity to be classified as distinct genotypes. There is compartmentalization of HCV quasispecies populations with clear hypervariable region 1 (HVR1) sequence differences between the liver, peripheral blood cells, and general circulation (40-42). Between individuals, HCV speciation is sufficient for delineation of multiple genotypes that differ by about 30% from each other at the amino acid level (43), with six major genotypes and up to three subtypes within each of these major types (44). There is differential distribution of these genotypes between countries, and subpopulations of HCV-infected individuals infected

through similar transmission routes often share predominant genotypes (Figure 2.2).

Studying the HCV life cycle and pathogenesis of HCV disease has been hindered both by lack of a cell culture system supporting significant viral replication and lack of suitable animal model other than the endangered and expensive chimpanzee. Development of infectious HCV cDNA clones (45-47) was key to demonstrating that HCV infection could cause liver disease. However, high levels of RNA replication are still only seen with one isolate (JFH-1), and in vitro replication is restricted to only 2 cell lines (Huh-7, HuH6) (reviewed by Bartenschlager (48)). Work with the genotype 1a RNA H77-S is promising (49), however this clone is only about 100-fold as infectious as the JFH-1 clone, limiting its use as a reliable in vitro model. A recently developed chimeric mouse model with a humanized liver (50) will hopefully facilitate elucidation of the mechanisms of HCV infection, replication and disease pathogenesis. This mouse model, as well another (51), are produced in immunodeficient mice and limit the ability to study HCV in the context of a competent immune system, an important restriction given that part of disease pathogenesis is immune mediated. In vivo infection of a tree shrew subspecies, *Tupaia belangeri chinensis*, has been described, but only after significant immunosuppression (52). Overall, the small animal models provide important but limited information on natural infection of immunocompetent hosts. Further information from chimpanzees and humans is needed to elucidate the mechanisms of pathogenesis related specifically to immune function.

2.3.1. HCV epidemiology and natural history

An estimated 170 million people are infected with HCV. Parenteral risk factors for infection include injection drug use, blood and blood product transfusions in the pre-HCV screening era, and HCV-infected liver transplants. Intravenous (IV) drug users, persons with inherited and acquired coagulopathies, and fetuses of infected mothers are at particularly high risk for infection. There are still a significant number of HCV infections without known risk factors, suggesting that other important routes of transmission remain to be identified. The risk of sexual (53) and vertical (54) transmission of HCV is considerably greater in HIV coinfecting individuals, probably reflecting the higher HCV loads in this group.

The mean incubation period of HCV is estimated between 7 and 20 weeks (55). Acute infection is generally characterized by moderate increases in serum transaminases and production of anti-HCV antibodies. Eighty percent of acutely infected individuals experience no clinical symptoms and, thus, are generally not investigated. The remaining 20% develop acute symptomatic hepatitis. Between 50% (56) and 85% (55) of acute infections become chronic, accompanied by an insipid disease course with approximately two thirds of those with chronic infection having elevated aminotransferases (55). Estimates of the proportion with chronic HCV infection that progress to cirrhosis vary between 20% and 30%. Approximately 25% of those with cirrhosis develop complications such as hepatocellular carcinoma (HCC) and decompensated liver disease requiring liver transplant (Figure 2.3).

The impact of HCV infection on HIV-related disease progression is not entirely clear. Certainly, survival analysis in populations with different HCV risk factors indicate an increased rate of mortality in HIV/HCV coinfecting individuals compared to HIV only patients, and this increased death rate cannot be attributed entirely to the increased incidence of liver disease (57, 58). However, not all HIV-infected individuals with HCV coinfection exhibit lower CD4⁺ T cell counts or higher HIV viremia than those with HIV infection alone (59, 60). Although there are conflicting reports, at least one study showed accelerated clinical progression of HIV disease in coinfecting individuals (58). This study included 119 HIV-infected subjects and 119 HIV/HCV-infected subjects followed for up to 108 months. It demonstrated an adjusted hazard ratio of 1.64 for clinical HIV disease progression in individuals with HCV coinfection versus those without HCV. The Swiss HIV Cohort Study also indicates a relative hazard of 1.7 for a new AIDS defining illness or death for HCV-seropositive compared to HCV-negative, HIV-infected individuals (57). A prospective Danish study of HIV individuals starting HAART therapy between 1995 and 2004 demonstrated increased overall mortality and mortality attributable to acquired immunodeficiency syndrome-related disease in the coinfecting population (61). One study of CD8⁺ T cell responses in coinfecting haemophiliacs found that HCV coinfection altered the phenotype of HIV-specific CD8⁺ T cells to a relatively low perforin state (62) that was associated with HIV disease progression. Conversely, an American longitudinal study followed 10,481 HIV infected individuals with 19% HCV coinfection for 1.9 years, and there was not an increased progression to an AIDS-defining illness or death (63). However, the followup was short considering the insidious disease

course of HCV infection. This study also followed individuals within one year of starting HAART therapy and found that HCV coinfection did not negatively impact HIV viral load reduction or CD4⁺ T cell count recovery. Clearly, there is still a lack of consensus on the effects of HCV on HIV related disease and response to treatment.

Earlier reports of little difference between HIV alone and HIV/HCV-infected groups could reflect a failure to identify all HCV coinfecting individuals. Second generation anti-HCV tests used by many groups to identify coinfecting individuals do not detect up to 20% of HCV-infected individuals coinfecting with HIV (64, 65). Consequently, a significant number of coinfecting individuals were probably not identified in early studies that did not use RNA based testing to determine HCV infection. These seronegative, HCV RNA-positive patients would mistakenly be grouped with the HIV group, perhaps falsely increasing the rate of clinical progression of the HIV group. Studies reporting no effect of HCV on HIV progression should be viewed with some scepticism if only HCV antibodies were used to diagnose HCV infection. Infection with different HCV genotypes also produces distinct clinical outcomes and early studies that did not stratify HCV infection by genotype may also be misleading when studying clinical progression. HCV genotype 1b is associated with a poor response to treatment, and with a poor prognosis once liver disease develops (66, 67). It may be that this is a more pathogenic HCV genotype not only on its own, but also in HIV coinfection.

HIV infection does impact clinical parameters of HCV infection. Coinfecting individuals have higher virus loads than those of individuals with only HCV (68-70). A study of 19 drug users showed that the mean number of HCV RNA copies per ml of

plasma was significantly higher in HIV coinfecting individuals (10^7 copies per ml) than in HCV only infected subjects ($<10^6$ copies per ml) (71). HCV-infected individuals infected with HIV during the study had significant increases in HCV RNA levels that were sustained throughout the followup time of up to 110 months. HIV infection also changes the rate of hepatic fibrosis progression (72, 73). An indirect measure of the fibrosis rate can be obtained by estimating the time from infection to cirrhosis. In one study, HIV/HCV coinfecting individuals had a median time of 26 years to cirrhosis while patients infected only with HCV had a median time of 38 years (74). Although some studies suggest otherwise, it appears that lower $CD4^+$ T cell counts are generally associated with accelerated progression to liver disease in HIV/HCV coinfecting patients (75). Not only does fibrosis occur more frequently and at earlier time points in coinfection, but the time between HCV exposure and development of hepatocellular carcinoma is shorter in coinfecting individuals than in subjects infected with HCV alone (76). The incidence of HCC in the coinfecting group may also be higher (77), in part because there is more rapid progression to all end-stage liver disease manifestations.

2.3.2. *Diagnosis of HCV infection*

Serological diagnosis of HCV infection utilizes both enzyme immunoassays (EIAs) and recombinant immunoblot assays (RIBAs). First generation assays only detect antibodies against an antigenic sequence primarily within the HCV NS4 region, the recombinant c100-3 antigen. The restricted scope of antigens in these tests resulted in many false negatives in some populations (78). It was also found that antibody production to antigens of c100-3 can be delayed for up to 6 months producing false

negative tests in early infection (79). Second generation tests include recombinant antigens from other regions of the hepatitis C genome, such as core (c22) and NS3 (c33c), in addition to c100-3. Antibodies to these antigens can be detected up to 8 weeks earlier than c100-3 reactive antibodies, resulting in earlier diagnosis of HCV infection, particularly in high risk populations (80). In addition to the core, c33c and c100-3 antigens, third generation tests include NS5 antigens. With their comprehensive array of HCV antigens, the third generation tests are the most sensitive for detecting acute HCV infection when seroconversion is still ongoing (81).

Detection of anti-HCV antibodies by third generation EIA is currently the primary screening test for HCV infection. Anti-HCV antibodies are found in most individuals that are chronically infected, and in many individuals with resolved HCV infection (82), however antibodies may decline to undetectable levels in the small minority of HCV infections that spontaneously resolve (83). Antibodies are not found early in acute infection and may not arise against a background of severe immunosuppression or immunodeficiency (84, 85). Therefore, antibody-based diagnosis of HCV infection is not always reliable. When clinical findings suggest possible HCV infection (high risk activity, elevated levels of liver enzymes in serum or HCV associated clinical phenomena in unusual populations, such as porphyria cutanea tarda in a very young individual), RNA based testing is necessary to accurately determine HCV infection status. Once diagnosed as HCV infected, genotyping is recommended in all viremic patients as it affects the likelihood of sustained virological response to treatment (86).

There are several groups of HCV-exposed individuals based on antibody and RNA testing: HCV antibody positive individuals with detectable RNA are considered to be chronically infected, and those with antibodies but undetectable RNA are said to have resolved infection. Another group, HCV antibody negative but detectable HCV RNA in serum, are referred to as seronegative chronic infections. Some individuals are HCV antibody and HCV RNA negative via serum testing but have elevated liver enzymes. Liver biopsy has demonstrated HCV RNA, as well as the negative strand replicative intermediate, in greater than 50% of those tested (87). In these cases, peripheral blood mononuclear cells (PBMC) have also been infected. Recent work has also demonstrated HCV RNA in the liver of individuals who are HCV exposed (antibody positive) but serum HCV RNA negative in the face of normal liver enzymes (88). This 'occult' infection has been described by other groups in both the liver and PBMC (89, 90). There is association of occult infection with milder but present liver disease and a weak association with HCC (91, 92), therefore in individuals with high clinical suspicion, excluding HCV infection by more invasive means may be an option worth considering.

Diagnosis of HCV infection in the coinfecting individual illustrates well the possible deficiencies of antibody-based testing for HCV infection. Antibody production in HIV infection may be impaired by a lack of CD4⁺ T cell help to B cells or by other types of immune dysregulation. Consequently, early generation ELISA testing in the HIV-infected population often reports HCV-infected individuals as either HCV antibody non-reactive or indeterminate (84). The percentage of false negatives for second generation EIAs in the general population has been estimated at approximately 5-10%

(93, 94). Our group and others found that upwards of 20% of coinfecting individuals may be HCV RNA positive, but seronegative using the second generation test (64, 65). While third generation assays have improved this situation during seroconversion by up to 25% (81) and reduced the number of indeterminate tests, it is still difficult to rely on seroreactivity in HIV-infected persons for HCV diagnosis. The situation may be compounded if an individual contracts HCV after becoming immunodeficient due to HIV infection. Even in individuals infected with HCV before HIV, there is some evidence that antibody titers fall and may become undetectable (95, 96), therefore, HCV RNA-based testing is definitely recommended for HIV-infected individuals if there is any underlying reason to suspect HCV infection.

In some situations, economics and infrastructure limit the use of PCR-based diagnosis. A recent strategy to increase early diagnosis in the context of blood donation and immunosuppression in HCV RNA positive, anti-HCV negative samples has been developed where a combination of HCV core antigen and antibody are detected (97). Another study looked specifically at HIV positive individuals and found the individuals are deemed HCV positive in 65% of cases earlier than by third generation anti-HCV assay alone (98). While not a replacement for RNA-based diagnosis, it may provide a useful adjunct in specific situations.

2.3.3. HCV monitoring

Defining reliable markers that allow comprehensive evaluation of HCV disease progression is challenging. Markers such as low serum albumin and increased International Normalized Ratio (INR), indicating reduced production of important

proteins made by the liver, can both be signs of serious liver damage or end-stage liver disease. Serum alanine transaminase (ALT) levels are commonly used to monitor liver damage in HCV-infected individuals that are otherwise asymptomatic. However, increases in serum ALT indicate hepatocellular damage that may have many causes other than HCV related disease, such as drug hepatotoxicity or acute alcohol ingestion. ALT levels may also be elevated late in liver disease, but do not allow sensitive detection of slow, progressive deterioration in the liver. While generally used as a marker of cholestatic disease, increases in serum gamma-glutamyl transpeptidase (GGT) may be a more sensitive marker for liver disease, especially in hemodialysed populations where the normal range for ALT levels are lower and relatively elevated ALT levels may be missed (99). Definitive estimation of the stage of liver disease relies on investigation of biopsy material. Pathologists examine liver biopsy specimens for evidence of lymphocytic infiltration, liver fibrosis and cirrhosis. Several scoring systems have been designed to quantitate the degree and extent of pathological changes occurring in the liver.

Various studies have addressed the need for non-invasive tests correlating with liver disease. Five biochemical markers (α 2-macroglobulin, apolipoprotein A1, haptoglobin, GGT and total bilirubin) have been combined to form the Fibrotest which can differentiate between non-clinically significant and clinically significant fibrosis, and may be useful in initial evaluation and follow-up of individuals undergoing therapy (100, 101). Other scoring indices include the AST to platelet ratio index (102), serum hyaluronic acid level (103), and the Forns index (age, GGT, cholesterol, platelet count,

and prothrombin time) (104). Although none have replaced liver biopsy, they are increasingly valued in clinical practice.

Monitoring disease activity and progression is difficult enough in HCV infection alone, but this challenge is compounded in the HIV coinfecting individual. Potent antiretroviral drugs used effectively to treat many HIV patients have a positive effect on both HIV viral load and CD4⁺ T cell counts (105, 106). Treatment is, however, a double edged sword as some of these drugs, in particular protease inhibitors and nonnucleoside reverse transcriptase inhibitors (NNRTIs) such as nevirapine, are highly hepatotoxic. Therefore, in individuals receiving HAART, ALT levels may rise more due to drug induced damage than to HCV-associated liver damage (107, 108). Moreover, maintenance of normal serum ALT levels in these individuals does not mean that insidious hepatic changes are not occurring. HIV/HCV coinfecting patients progress more quickly to liver-related morbidity, and have higher levels of HCV viremia than HCV-infected individuals (70, 72), but this is not always associated with abnormal ALT levels. In the face of accelerated liver deterioration in these patients, diligent monitoring of ALT levels and also periodic liver biopsies may be necessary.

2.3.4. HCV treatment

The ultimate goals of HCV treatment are eradication of HCV and regression of liver fibrosis. The current standard of treatment for HCV infection is pegylated interferon-alpha (PEG-IFN- α) injection weekly and oral ribavirin daily (66, 109). A sustained virologic response (SVR) is defined as undetectable serum HCV RNA 24 weeks after finishing therapy. Treatment regimes range from 6 months in non-genotype

1b infections to 12 months in genotype 1b infections. Most clinical trial results to date indicate low rates of SVR in genotype 1 (40%) compared with genotypes 2 and 3 (70-80%) (110, 111). Genotypes 4, 5, and 6 have intermediate levels of SVR (86). Sustained virologic response is associated with reduced fibrosis (112) however, treatment-associated improvement in individuals without significant change in HCV RNA viremia has also been reported (113). Response rates are affected by HCV RNA levels, sex, cirrhosis, age, weight, stage of fibrosis, steatosis and racial differences (67, 111, 114-117). A study of 475 patients showed a decreased response rate in African-Americans compared to White, Hispanic and Asian-Americans (118). These factors should be weighed, particularly if considering treatment in the HCV viremic individual with no clear evidence of liver disease.

Current guidelines recommend treatment in all HCV genotypes 2 and 3 without absolute contraindications, as the rates of SVR are relatively high (119, 120). For individuals who are genotype 1, 4, 5, or 6 with serum HCV RNA viremia, elevated ALT levels, and biopsy evidence of hepatitis, treatment should be considered (119, 121). Individuals with normal liver enzymes but HCV viremia may be treated but the decision is individualized based on level of patient concern, comorbidities, age, weight and genotype (86). There is some data that treatment of these patients is indicated from both a SVR (122) and cost effectiveness (123) perspective.

It is rare to identify acute (< 3 month) HCV infection as it does not often cause fulminant disease. Individuals with an exposure risk should be monitored for 12 weeks from the estimated or known time of infection. If they do not spontaneously clear the

virus, one report supports treatment with IFN- α . It demonstrated much higher response rates to IFN- α monotherapy when individuals were treated in the acute phase of HCV infection (124) compared with chronic infection. Forty-two of the 44 patients in this trial had undetectable serum HCV RNA up to 24 weeks after completing a 24 week course of IFN- α monotherapy. The majority of these individuals were genotype 1 and there was no significant difference in the response rate between genotypes.

As described above, a positive response in terms of HCV eradication is defined on the basis of serum HCV RNA. It is uncommon to look for HCV RNA in the liver in monitoring for these trials, but HCV RNA can sometimes be found in mononuclear cells and the liver, even in the absence of serum HCV RNA (125-128). Hence, undetectable serum HCV RNA does not confirm viral eradication. It is important in individuals who may undergo immunosuppressive regimens after anti-HCV treatment to consider that the virus is unlikely to be completely eradicated.

Toxic side effects of treatment are multiple and significant. They include malaise, depression, IFN-associated thyroiditis, bone marrow suppression, and hemolysis. Better treatments that would eradicate HCV more frequently or reduce replication in asymptomatic individuals are urgently needed. There has been a great deal of research into the basis of 'IFN-resistance' and the mechanisms by which IFN and ribavirin work in treatment of HCV (reviewed by Wohnsland (129)). Treatment at present is only a symptomatic therapy, with the goal of reversing or limiting damage mediated by a virus replicating unchecked. Other treatments have been directed at reducing symptoms in individuals failing IFN/ribavirin therapy. For example, treatment with interleukin-10 (IL-

10) reversed and reduced rates of fibrosis in a group of HCV-infected individuals (130). Ursodeoxycholic acid, nonsteroidal anti-inflammatory drugs and thymosin- α have been used as adjuncts to IFN- α with no clear benefit demonstrated (131). Newer studies of immunomodulators such as thymosin- α have suggested there may be a role in chronic hepatitis C patients who have previously failed interferon/ribavirin treatment (132).

As more information regarding the HCV lifecycle becomes available with the advent of in vitro infectious replication systems, specifically directed antivirals are being tested. Two NS3/4A protease inhibitors have reached Phase 2 trials. VX-950 and SCH503034, both peptidomimetic inhibitors, have been tested as monotherapy and in combination with IFN treatment and results are currently in press. Viremia recurs after cessation of therapy with these drugs and drug resistance accumulates rapidly (129). Early data in Phase 1 trials of polymerase inhibitors indicate a similar propensity for the development of resistance mutations, but less efficacy in terms of reducing HCV RNA viral load (129).

Making appropriate treatment decisions concerning HCV in HIV coinfecting patients requires consideration of more than just the response rate and side effect profile of treatment. Treatments for HIV are very effective in many individuals, restoring CD4⁺ T cell counts and delaying the development of AIDS-defining illnesses for increasingly long periods of time. When there are no contraindications, treatment of HIV infection is the first priority, unless there is evidence of decompensating liver disease (133). Many classes of antiretrovirals, including NRTIs, NNRTIs and PIs, can be hepatotoxic, with nevirapine and full dose ritonavir being some of the worst (108). Therefore, care should

be taken in coinfecting patients to optimise HIV treatment while minimizing use of the most toxic drugs (134, 135). With respect to HCV treatment, another potential drug interaction looms between ribavirin and zidovudine, a nucleoside analogue reverse transcriptase inhibitor (NRTI). In vitro studies have shown that ribavirin inhibits the phosphorylation of zidovudine and stavudine (d4T) by competing for thymidine kinases (136, 137). Although no adverse effects have been shown in vivo, close monitoring while individuals are receiving both drugs is essential.

While treatment of HIV is obviously necessary in patients meeting established criteria, the increase in CD4⁺ T cell counts and general increase in immune function can actually cause HCV-related liver disease to develop more rapidly. Such immune reconstitution has been related to increased rates of liver fibrosis (138, 139), suggesting that at least some of the hepatic damage in HCV infection is related to the host immune response and not a direct effect of the virus. Although there are conflicting reports (140-142), several studies found increases in HCV virus load in HIV-infected individuals given protease inhibitors (135, 143). These reports led to speculation that protease inhibitors could either induce HCV replication somehow or that competitive interference between HIV and HCV kept HCV levels lower in HIV-infected individuals with higher HIV loads. These hypotheses have not been confirmed and other studies reported no consistent effect of HIV treatment on HCV levels (140, 144).

Combination HCV treatment is recommended in stable, immunocompetent HIV/HCV coinfecting individuals without other absolute contraindications to either ribavirin or IFN- α (86, 145). Response rates to combination pegylated HCV treatments

in immunocompetent HCV/HIV coinfecting populations have been lower than those in the general population (146-152). As in HCV monoinfected individuals, baseline HCV RNA levels, and HCV genotype are involved in predicting response to treatment in the coinfecting patient, and the baseline CD4⁺ T cell count has also been associated with outcome in one study (153) as well as a recent meta-analysis (146), although the odds ratio confidence interval does cross unity in this pooled analysis. It may be that individuals on more effective HAART therapy with higher CD4⁺ T cell counts are more likely to clear the HCV virus, but this is still not clear. The abundance of data at this point suggests only treating individuals with HCV/HIV coinfection after control of HIV related disease.

2.3.5. Extrahepatic manifestations of HCV infection

Various extrahepatic manifestations occur in chronic HCV infection (reviewed by Zignego (154)), and some groups propose it be considered a systemic disease. Current classification divides the associated disease into 4 groups: association by high prevalence and pathogenesis (mixed cryoglobulinemia), association with higher prevalence than controls (B cell non-Hodgkins lymphoma, monoclonal gammopathies, porphyria cutanea tarda, and lichen planus), association to be confirmed (autoimmune thyroiditis, thyroid cancer, sicca syndrome, alveolitis-lung fibrosis, diabetes mellitus, non-cryoglobulinemic nephropathies, aortic atherosclerosis), and anecdotal observations (psoriasis, peripheral neuropathies, rheumatoid arthritis, polyarteritis nodosa, fibromyalgia, chronic urticaria, and cardiomyopathies).

Cryoglobulinemia, the most common of these clinical issues (155, 156), was actually treated with interferon- α prior to identification of HCV infection as the primary cause (157). Approximately 35-55% of people with chronic HCV infection have cryoglobulins (158) and 90% of patients with essential mixed cryoglobulinemia (EMC) are HCV-infected (159). HCV RNA, antigens and anti-HCV antibodies can be found in cryoprecipitates, suggesting a direct role for HCV in formation of these complexes (159). Up to 25% of HCV-infected individuals have clinical symptoms that occur independently of any overt liver disease (160), however a meta analysis of 19 studies between 1994 and 2001 has shown an increased odds ratio of 4.87 for cirrhosis in HCV patients positive for cryoglobulins (161). The most common clinical manifestations of EMC depend on the particular population studied, but include neuropathies (162), glomerulonephritis, and cutaneous vasculitis (163). Other syndromes and diseases suspected to be associated with HCV infection include porphyria cutanea tarda (164, 165), aplastic anaemia (166), non-Hodgkin's lymphoma (167), multiple myeloma (168), diabetes mellitus (169), arthritis (170, 171), idiopathic pulmonary fibrosis (172), and fibromyalgia syndrome (173).

The exact link between HCV and these diseases has not been firmly identified, but B cell dysfunction and other regulatory problems within the immune system are suspected. Studies have associated chromosomal translocation, t(14;18) with HCV infection (174) and mixed cryoglobulinemia (175), supporting a role for HCV in the development of clinical entities characterized by immunoglobulin dysregulation. Some of the diseases and syndromes associated with HCV are also associated with other

chronic viral infections such as HIV and hepatitis D virus (HDV) (176), raising the question of whether these symptoms are truly HCV-specific or epiphenomena related to the long-term immune stimulation accompanying chronic infection.

Whether HIV coinfection impacts the type and incidence of HCV-associated extrahepatic manifestations is not clear. Many of the syndromes have features of autoimmune disease and whether immunocompromised HIV-infected hosts are as likely to develop some of these symptoms remains unknown. Conversely, if these syndromes are not truly autoimmune and are more symptomatic of immune dysregulation, they may occur more frequently in severely immunocompromised individuals. Evidence strongly favoring or discounting either of these possibilities is lacking. A prospective cross sectional study of HCV/HIV coinfecting, HIV mono-infected and HCV infected individuals found similar rates of cryoglobulinemia in HCV mono-infected and HIV/HCV coinfecting individuals, however the prevalence of anti-thyroglobulin and anti-cardiolipin antibodies was increased in coinfecting individuals compared with HCV mono-infected individuals (177). This increased prevalence of autoantibodies was not associated with an increase in clinical autoimmune disease. While this study suggests that there is an interaction between HIV and HCV on immune regulation, the issue is far from resolved.

Treatment approaches to extrahepatic HCV manifestations vary, depending on other comorbid illness and the sensitivity of the individual to current HCV therapies. In general, specific antiviral treatment is useful in treating mixed cryoglobulinemia, glomerulonephritis, porphyria cutanea tarda, and panarteritis nodosa (160). Studies of combination PEG-IFN- α and ribavirin in mixed cryoglobulinemia have demonstrated

reasonable response rates in terms of HCV RNA clearance and improvement of cryoglobulinemia-related disease (178-180). Symptomatic therapy, in addition to specific anti-viral treatment, is indicated for autoimmune thyroid disease, lymphoma, aplastic anaemia, autoimmune thrombocytopenia, lichen planus, neuropathy, sicca syndrome, and diabetes mellitus (160). Autoimmune disorders such as thyroiditis, lichen planus, and sialadenitis are worsened by the immune activation instigated by interferon- α . Thus, treatment of patients whose disease is characterized by these symptoms should exclude interferon- α .

2.3.6. Pathogenesis of HCV disease

Controversy remains regarding the cytopathicity of HCV. Some evidence suggests that the virus itself is non-cytopathic (181), and that hepatic pathogenesis is directly linked to the pro-inflammatory immune response of chronic viral infection (182, 183). The pathologic picture of chronic HCV infection is typified by infiltration of CD4⁺ and CD8⁺ lymphocytes, B cells, NK cells and NK T cells. Similar to the case with hepatitis B virus (HBV) infection, there is an expansive hepatic lymphocytic infiltrate that consists largely of non-HCV-specific cells that produce IFN- γ (184), leading to hepatic fibrosis and portal inflammation (182). Other evidence indicates that HCV is cytopathic to host cells, causing changes similar to those seen in Dengue virus infection (185). HCV-infected individuals with cyclosporine-induced immunosuppression following transplantation (186) or with HIV-associated immune deficiency (75) may still have progressive liver disease, arguing against a purely inflammatory pathogenesis. In addition, there have been reports that HCV may preferentially infect B cells in the

peripheral blood (29, 187). Lower B cell counts have been reported in HCV-infected individuals (188), which may indicate destruction of infected B cells by the host immune response or a direct cytopathic effect of the virus. The true pathogenic origin of HCV-related disease both in the liver and extrahepatic compartment probably involves components of both virus cytopathicity and damage mediated by the host immune response.

2.4. Immunology of HCV Infection

2.4.1. Introduction

The immune response that arises against HCV during acute infection eliminates the virus only in about one quarter of cases. If viral clearance does not occur in the period directly following acute infection, it likely will not happen without antiviral treatment. Since the available treatments are effective in only a minority of cases, most HCV-infected individuals currently face lifelong chronic infection, irrespective of the anti-HCV immune response they ultimately develop. As in HIV infection, this immunological inadequacy remains a poorly understood aspect of the natural history of HCV infection. However, the roughly 25% of acutely infected individuals that do clear HCV infection provide examples of successful immune responses from which to discern the effective components of anti-HCV immunity. Even in cases of chronic HCV infection, the immune response is still meaningful, as illustrated by the higher plasma HCV virus loads and more rapid disease progression in settings of immunodeficiency mediated by anti-rejection drugs or HIV (69, 70).

The natural history of chronic HCV infection reflects the highly variable outcome of complex interactions between adaptive immunity and effective viral strategies for evading immunity. Since coinfection with HIV worsens the predilection towards inadequate immunity, it is not surprising that chronic HCV infection is a more frequent outcome in the HIV-infected population (189, 190). Even though it is uncommon for the immune system to eradicate HCV, some components of immunity provide long-term protection against symptomatic disease in most immunocompetent cases (55). Thus, there is a continuum of effective immunity against HCV ranging from sterilizing immunity and lifelong protection against disease to ever diminishing disease protection over the duration of infection. Evidence of what constitutes an effective immune response against HCV lies within qualitative and quantitative differences in anti-HCV immunity over this natural continuum. Aspects of anti-HCV immunity peculiar to individuals clearing the virus or suppressing replication to inconsequential levels offer rational targets to guide the design of prophylactic and therapeutic vaccines.

For the most part, people fully recovered from HCV infection have anti-HCV antibodies, but very little or no HCV RNA in their blood (126, 189-191). Viral clearance can also occur without development of anti-HCV antibodies, but this is difficult to document and probably quite rare. It is important to determine during acute infection how anti-HCV immunity differs between those who clear the virus and those who progress to chronic infection, as this is the key period for natural viral eradication. Immunological differences that persist between fully recovered individuals, healthy individuals with chronic infection and those with active liver disease can also be

informative, but to some extent will reflect the long term effects of chronic infection or clearance subsequent to the pivotal conditions that determined the outcome of acute infection.

The following sections review the evidence for anti-HCV immune responses in groups with HCV exposure but no infection, spontaneous clearance, persistent infection, successful treatment with sustained virologic response, and unsuccessful treatment with persistent viremia. We focus primarily on the anti-HCV CD4⁺ and CD8⁺ T cell response in monoinfected and HIV coinfecting groups, but also comment on relevant aspects of innate immunity.

2.4.2. Exposure to HCV without infection

HCV seronegative spouses and other family members of HCV-infected individuals exhibit a much higher incidence of anti-HCV helper and cytolytic T cell (CTL) responses than do unexposed controls (192-194). The T cell responses are broadly directed against different HCV proteins and there is speculation these responses may indicate previous HCV infection that was cleared as a result of these responses. Past infection in these cases was not confirmed by detection of HCV RNA in retrospective samples, however these results were obtained using a relatively insensitive HCV viral load test (approximately 1000 copies/mL). Clearance in these cases may be associated with exposure to levels of HCV too low to induce either anti-HCV antibodies or acute hepatitis. Lack of viremia was determined prospectively in a more recent study that looked at acutely infected HCV individuals and their sexual partners (195). Cellular immune responses, measured by IFN- γ production in enzyme-linked immunosorbant spot

(ELISPOT) assays, were detectable in eight of sixteen aviremic seronegative contacts. A relationship between lower HCV levels and lack of anti-HCV antibody induction is also suggested by descriptions of individuals with low level HCV infection who either lost or never developed anti-HCV antibodies (196). Conversely, antibody responses sometimes do become apparent as HCV levels rise in chronic HCV infection.

It would seem that exposure to low levels of HCV may induce antibodies and cellular immune responses in the absence of persistent infection. Whether these responses are protective in the context of challenge with higher viral loads remains to be determined, but these findings have important implications for prophylactic and therapeutic HCV vaccine development.

2.4.3. Acute self-limiting HCV infection

The presence of HCV-specific T cell responses without anti-HCV antibodies in some individuals who may have cleared HCV infection indicates a central protective role for cellular immunity (192, 193, 195), at least under some challenge conditions. This is true of most viral infections, however, the potential effector role of CTL in killing HCV-infected cells and the role of helper T cells, beyond that of helping CTL to proliferate and differentiate, has been studied by many groups without a clear consensus being reached. Many studies have demonstrated multispecific vigorous anti-HCV CD4⁺ and CD8⁺ T cells in acute infection (197-201). Not all individuals with strong CD4⁺ T cell responses cleared the virus (200) suggesting that CD4⁺ T cell responses alone are not sufficient for persistent control of virus. Despite expansion of CD4⁺ T cell responses, a dysfunctional phenotype in those who initially control the virus followed by relapse has been described

(202, 203). Other studies have underscored the importance of CD4⁺ T cell responses in individuals with acute infection. In 16 individuals with acute HCV infection, all had CD8⁺ T cell responses but only those with concurrent CD4⁺ T cell responses cleared the virus (204). These 'successful' CD4⁺ T cell responses were typically Th1 in nature (i.e. T cells produce IL-2, IFN- γ and TNF- α (205, 206)). No one particular antigenic region of the genome has been associated with particularly 'effective' CD4⁺ T cell responses, and major histocompatibility complex (MHC) class II epitopes have been defined from most non-structural regions (184). A recent study of 22 individuals with spontaneous HCV clearance identified several promiscuous human leukocyte antigen (HLA) class II epitopes from nonstructural regions that were found more frequently in spontaneous clearers than those with chronic infection (207). Estimates of the number of HCV-specific CD4⁺ T cells in acute infection differ widely based on the technique (MHC tetramer, IFN- γ production, or surface flow cytometry) used (201, 208).

Generally, CD8⁺ T cell responses in viral infection are essential for clearance (209). In the case of HCV, CD8⁺ T cell expansion in acute infection is associated with control of viremia (200, 210), and a lack of CD8⁺ T cell response resulted in persistent infection. While these cells expand reasonably well during acute infection, development of cytotoxic and IFN- γ effector function is delayed (199, 200, 211, 212), which may allow the establishment of persistent infection. Broad specificity, functional CD8⁺ T cell responses are associated with HCV clearance and HLA class I restricted epitopes have been described in all regions of the genome (210, 213-216), although there have been no clearly defined immunodominant regions. The exact breadth of the response, and

whether there is a threshold for clearance versus persistence, remains to be determined.

CD8⁺ T cells have been classified in various ways. In other persistent infections such as HIV and EBV, the differentiation of effector T cells in acute infection has been divided into early, intermediate, and late, based on expression of costimulatory molecules CD27 and CD28 (213, 217). Like HIV and EBV infections, HCV-specific CD8⁺ T cells during acute infection are CD28⁺ and CD27⁺ with low levels of effector molecules such as perforin, suggesting an early stage of differentiation (213) that does not include HCV-specific cytolytic 'late' effectors. Another model uses the lymph node homing receptor C-C motif chemokine receptor 7 (CCR7) to differentiate memory and central effectors (218). The large population of CCR7⁻ CD8⁺ T cells in acute infection were consistent with memory effector cells, but they did not proliferate *ex vivo* indicating a functional impairment beyond the differentiation explanation (219). Because interleukin 7 is involved in memory T cell differentiation, IL-7-R (CD127) distribution on T cells has been investigated. The memory T cell phenotype that develops in acute resolving HCV infection is characteristic of the phenotype found in other resolving infections such as respiratory syncytial virus (RSV) and influenza (220). CD127⁺ T cells secrete IL-2, are long lived, and are associated with the development of a central memory phenotype common in viral clearance. High levels of CD127 (206) expression are associated with HCV clearance, although this phenotype is also found in persistent HCV infection (221) in association with low killer cell lectin-like receptor G1 (KLRG1), questioning their functional role in clearance. Programmed death-1 (PD-1), a negative regulator of T cell activation and function, is increased in persistent infections such as HIV (222). In acute

HCV infection, PD-1⁺CD8⁺ T cells were present but decreased with resolving infection and remained at high levels in unresolving infection (223). These studies suggest further functional stunting of HCV-specific T cells, although the mechanism is unclear.

Since some chronically-infected individuals retain anti-HCV CTL and helper T cell responses (83, 224), or at least manifest these responses temporarily, there must be qualitative or quantitative differences within the response that differentiate those that clear the virus or remain healthy from those developing chronic hepatitis. Another indication of the central role of cellular immunity comes from the associations reported between expression of particular HLA alleles and viral clearance. These antigens present viral peptides to T cells and presumably, the alleles associated with viral clearance present a range of HCV peptides that allow for a more effective immune response. Several studies concluded that the HLA class II alleles DRβ1*1101 and DQβ1*0301 (which are strongly linked) are more common in persons who clear HCV infection compared to persons with chronic HCV infection (225, 226) and an Irish study associated DRβ1*01 with viral clearance as well (227). These findings have been supported by a recent meta-analysis, however it should be noted that the 11 study populations were European with the exception two (one from Thailand and another from North America) (226, 228). Since these HLA class II alleles present peptides to CD4⁺ helper T cells, this provides circumstantial evidence that strong helper T cell responses against HCV promote viral clearance. More multiethnic studies where the HLA diversity is increased would be informative.

Viral clearance is associated with higher frequencies of HCV-reactive CD8⁺ T

cells in the 6 month period following acute infection (229), indicating that these cells may be the ultimate effector arm of the immune response against HCV. As such, it is reasonable that there would be associations between HLA class I genotypes and viral persistence or clearance. However, there have been few reports associating alleles of the classical HLA class I molecules presenting viral peptides to CD8⁺ CTL with the outcome of acute HCV infection. One Egyptian study of genotype 4 infected individuals associated HLA B50 with viral clearance but this was in only 2 individuals (230), and similar findings in other genotypes have not been found. The lack of strong association may mean there are factors other than CD8⁺ T cell mediated immunity involved in clearance.

T cell, B cell and hepatocyte cell lines are permissive to HCV infection (185). However, the host cell receptor for HCV is not known with certainty and there are only recently developed in vitro culture systems. There is also a high degree of virus variability within the putative principal neutralizing determinant or hypervariable region 1 (HVR1) of the HCV envelope protein, making reliable measurement of HCV neutralizing antibody titers possible only in chimpanzee challenge experiments (231). These animal experiments have shown that pre-existing or co-administered antibodies specific for the challenge virus can prevent infection. Antibodies may also play a role in viral clearance in human infections, most likely through preventing new cycles of infection by viruses released from infected hepatocytes into the circulation. Surrogate measures of neutralizing antibodies have been derived in vitro from inhibition of recombinant HCV envelope protein binding to human cells (232) and from measurement

of serum antibody binding to HVR1-derived synthetic peptides from the HCV envelope 2 (E2) protein (233). These assays have shown associations between the development of high titers of antibodies against either HCV E2 HVR1, HCV itself or recombinant E2 and resolution of HCV infection (234). Several reports also suggest that anti-HCV antibodies from chronically infected individuals may lack the diversity normally associated with polyclonal B cell responses (235). Anti-HCV antibodies from chronically infected individuals often express a common idiotype, and show evidence of κ or λ immunoglobulin light chain dominance characteristic of oligoclonal B cell responses (236). In HIV infection, such responses led to speculation that B cell clonal dominance established early in infection inhibits adaptation of the humoral immune response to emerging viral mutants (237). A similar hypothesis for the effect of oligoclonal responses in HCV infection may explain the ineffectiveness of the anti-HCV response in adapting to HCV variants in vivo (236).

Innate immunity is a cornerstone of anti-viral immunity, and recent studies have increased our knowledge of the role of non-specific immunity to pathogens in general. Receptors for pathogen-associated molecular patterns, or PAMPs, have been identified for RNA (particularly toll-like receptor (TLR)-3), and these instigate the immune response mediated by type I interferons (238, 239). Indirect evidence for the importance of innate immune responses in controlling HCV infection stem from observations of IFN-signalling obstruction by various HCV proteins (240-242). Given the central role for NK cells in early antiviral immunity (243, 244), a model of HCV persistence with NK cells at the center has been proposed (245) although there are few studies in acute in vivo or in

vitro infection (246) as of yet to substantiate the hypothesis. NK cells interact with class I ligands on host cells through killer immunoglobulin-like receptors (KIRs) that either activate or inhibit natural killer activity (247). The Bw4 motif (common to multiple HLA-A and B alleles) and the dimorphic HLA-C motifs C1 and C2 are inhibitory. The natural ligands for HLA-C1 and C2 are the NK cell KIR2D receptors, while the KIR3D receptor family binds Bw4. One group found that the genotype KIR 2DL3/HLA C1C1 was associated with clearance of acute HCV infection in those who did not acquire the virus by transfusion (248), while another study of HIV/HCV coinfecting individuals did not support this finding (249). Overall, the role for NK cells and genotype in clearance of acute HCV infection remains unclear, however the innate immune system likely has the most impact in infections with limited viral load.

HCV is relatively hepatotropic, and it is interesting to wonder whether circulating anti-HCV T cell responses are the same as intrahepatic lymphocyte populations in frequency, specificity and function. Acute infection in humans is generally a benign disease, and as such, liver biopsy is not often performed, and information on intrahepatic lymphocyte populations comes from chimpanzee studies that have demonstrated multispecific CD4⁺ T cell responses in the liver of resolving acute infection occur more frequently than in persistent infection (200). Further human based data is lacking.

Cellular immune responses in seronegative HCV-exposed individuals, combined with the associations between HLA class II molecules and HCV clearance, highlight the role of helper T cells in protection from HCV infection. However, natural history studies suggest that humoral immunity and CTL can also play important roles in resolution of

HCV infection. Coinfection with HIV lowers the natural rate of HCV clearance to below 15% (189, 190) and increases the likelihood of recurrent infection after initial HCV control (190, 250). This effect could also largely be attributed to primary helper T cell dysfunction and depletion associated with HIV infection, supported by improved viral clearance rates in coinfecting individuals treated with HAART, CD4⁺ T cell counts greater than 250 cells/mm³, and HIV viral loads <5000 copies/mL (251). At least one study has found that the magnitude and breadth of the circulating anti-HCV CD8⁺ T cell response is related to the CD4⁺ T cell count in HIV coinfection (252). Seronegative HCV infection is clearly more common in the HIV coinfecting population than the general population and may reflect a reduced capacity to make new antibody responses (64, 95). This could also favor establishment of chronic infection. Cytotoxic T cell responses against other viruses tend to be preserved through advanced HIV infection (253, 254), but reduced HCV-specific CD8⁺ T cell activation secondary to helper T cell dysfunction could also contribute to the establishment of chronic HCV infection in HIV coinfecting individuals. There have been few studies of HCV-specific CTL activity in HIV coinfecting individuals addressing this possibility. The key variable in the coinfecting population, in terms of the nature of HCV-specific immune responses, is probably the time of exposure to HCV in relation to progression of HIV-related immunodeficiency.

2.4.4. Chronic HCV infection

Except in clear cases of pre-existing immunodeficiency, establishment of chronic HCV infection reflects both the failure to generate sterilizing immunity and the success of viral strategies to evade the resultant immune response. A key component of the

immune evasion strategies of both HIV and HCV is the infidelity of genomic replication mediated by error prone RNA-dependent polymerases that lack proof-reading ability. Both viruses evolve into a swarm of quasispecies during acute infection and variability within sequences targeted by humoral and cellular immunity favors persistence of poorly recognized variants. A study of 12 individuals with acute HCV infection indicated that variation within the HVR1 of HCV E2 protein was greater in those who developed chronic infection than in those who resolved infection (255). The appearance of new variants was also associated with HCV seroconversion, indicating an important role for potentially neutralizing antibodies in mediating immune selective pressure on HCV. Although these results appear to stress the role of humoral immunity in viral clearance, it is possible that the selective pressure of the antibodies only impacts HCV once it has already escaped the cellular immune response. If the cellular immune response is mediating the primary selective pressure, there would be no immediate selective advantage for HCV HVR1 variants. Whether escaping the cellular or humoral response, generation of diversity by HCV plays a critical role in persistence.

In chronically infected individuals, T cell-mediated recognition of endogenous HCV sequences is compromised relative to recognition of the index peptide sequences identified as CTL epitopes (256), although some studies have not found evidence of particularly strong CTL escape variants (257). Similar evolution takes place within helper T cell epitopes indicating that the virus evolves away from T cell recognition in chronic infection (258). In acute HCV infection, CTL escape variants also emerge in chimpanzees that progress to chronic HCV infection (259). These studies indicate that

mutational escape from T cell recognition is an important aspect of HCV's ability to evade the immune system during acute infection and to persist in chronic infection. HCV-reactive CD8⁺ and CD4⁺ T cells expand in peripheral blood during acute HCV infection (224, 260) and the strength and breadth of this anti-HCV T cell response correlates with resolution of infection (198, 210, 229, 261). The high virus loads associated with acute infection may produce what has been referred to as the "stunned" phenotype of HCV-reactive cells in which the expanded HCV-specific cells fail to release interferon- γ in response to stimulation (199). This phenotype disappeared as virus load fell, and although HCV-specific T cell frequencies also decreased compared to levels during acute infection, they remained higher in individuals resolving infection than in those developing chronic infection. Thus, just as in HIV infection, it appears that viral persistence reflects the ability of HCV to diversify and expand at a greater rate than the responding immune effector cells.

As in most chronic infections, HCV-specific peripheral blood T cell responses in chronically infected individuals are skewed towards Th2 type cytokine production (262). T cells isolated from the liver of chronically-infected individuals, however, continue to produce pro-inflammatory cytokines when stimulated (263, 264), which may be linked to the immunopathogenesis of HCV disease. PBMC from HCV-infected individuals appear to produce immunologically significant amounts of transforming growth factor- β (TGF- β), as antibody neutralization of TGF- β increased levels of anti-HCV CTL generated by *in vitro* stimulation (265). Certain peptides from HCV non-structural proteins also selectively stimulate T cells to produce IL-10 (266, 267), an immunosuppressive cytokine

with anti-inflammatory properties. We found that HCV proteins stimulate IL-10 production by CD36⁺ mononuclear cells and depletion of T cells does not potentiate this effect (268). Thus, a propensity for HCV proteins to stimulate production of immunosuppressive cytokines and inhibit production of pro-inflammatory cytokines may help tilt the balance against immune system mediated clearance of HCV.

Regulatory T cells with a CD4⁺CD25⁺ phenotype have been found in relative abundance in chronic HCV infection (269), and were associated with high levels of TGF- β production (270) in HCV infected individuals with normal ALT levels (271). Those with elevated ALT levels had lower TGF- β levels, supporting a role for the ongoing immune response in the development of hepatitis, as well as a potential mechanism of immune evasion. Depletion of CD25⁺FoxP3⁺ regulatory T cells increased IFN- γ production from CD8⁺ T cells in vitro (272, 273), and in persistently infected or cleared chimpanzees, CD8⁺ T cells have an altered phenotype in comparison to uninfected animals that suggests ongoing regulatory roles in chronic and cleared infection (274).

The immunophenotype of peripheral (223, 275) and hepatic (276) T cells in chronic HCV infection is similar to that of other persistent infections, with high PD-1 and low CD127 levels. Loss of CD127 has been associated with progression to viral persistence in acute infection (277), presumably by promoting a 'dysfunctional' CD8⁺ T cell phenotype that is unable to respond to the IL-7 signals necessary for memory cell survival and proliferation. Other studies have found intrahepatic CD8⁺ T cells in HCV infected persons are not as responsive to TCR stimulation as those from uninfected individuals, despite a terminally differentiated effector phenotype (278). Of note, the loss

of mature phenotype in chronically infected individuals extended to cytomegalovirus (CMV)-specific CD8⁺ T cells in one study and may suggest more generalized immunomodulation by HCV than previously thought (279).

In terms of innate immunity, various studies have indicated dysfunction at the level of NK cell receptor expression as well as cytotoxic function. Reduced expression of NKp46 and NKp30 and natural cytotoxicity receptors (NCR) were found in chronic HCV patients compared to healthy donors, and were associated with a decrease in target cell killing (280). Other groups have found that NK cells have conserved cytotoxic activity against most targets *in vitro* but expressed significant IL-10 when triggered (281).

There are few studies on the breadth and frequency of anti-HCV CTL responses in HIV coinfecting individuals with either chronic or resolved HCV infection. In a study of 26 coinfecting and 47 HCV mono-infected individuals, the frequency of CD8⁺ T cell responses measured by IFN- γ ELISPOT was similar between the groups but the breadth was reduced in coinfecting individuals (282). However, another group reported decreased frequency and breadth of CD4⁺ and CD8⁺ T cell responses in coinfecting individuals (283, 284). The frequency of peripheral anti-HCV CD8⁺ T cell responses was associated with the CD4⁺ T cell count in HIV coinfecting individuals (252), however, this is not consistent between studies. Intrahepatic CD8⁺ and CD4⁺ responses based on expanded T cells derived from liver biopsies of 10 coinfecting and 8 HCV mono-infected individuals found comparable CD8⁺ and CD4⁺ T cell frequencies in both groups, and CD4⁺ T cell responses correlated with the presence of CD8⁺ T cell responses (285). Another study of liver

biopsies based on pathology and not in vitro expanded cells found a depletion of intrahepatic CD4⁺ T cells in coinfecting individuals compared with HCV monoinfected that was not affected by peripheral CD4⁺ T cell count (286).

The propensity to stimulate immunosuppressive cytokines may be exacerbated by HIV coinfection, as HIV infection is also associated with a skewing of T cell responses towards production of Th2 type anti-inflammatory cytokines and peripheral blood T cell counts are often lower in HIV infection. Compared to monocytes from uninfected individuals, more monocytes from HIV-infected individuals appear primed to produce IL-10 when exposed to recombinant HCV proteins (268). CD8⁺ T cells from coinfecting individuals were reported to secrete TGF- β and attenuate T cell responses (287). Surprisingly, against this backdrop of Th2 skewed cytokine production, the results of one study indicated that HIV coinfection increased production of Th1 type cytokines by T cells stimulated with HCV proteins, relative to the cytokine production profile of T cells from individuals chronically infected with HCV alone (288). Although this result seems contradictory in terms of the higher HCV plasma virus loads and increased incidence of chronic HCV infection in HIV coinfecting individuals, it would fit with the accelerated rate of immune-mediated liver disease.

In summary, anti-HCV T cell responses are present but weaker in peripheral circulation compared with liver in those with chronic HCV infection. These responses are further attenuated by HIV coinfection. A stunned or exhausted CD8⁺ T cell profile, in the context of diminished CD4⁺ T cell responses over time, may partially explain the failure of both peripheral and intrahepatic T cell responses. No particularly

immunodominant epitopes have been consistently described, and augmentation of existing T cell responses may be important to viral clearance.

2.4.5. IFN/Ribavirin treatment with or without viral response

The innate immune system plays a role in priming adaptive responses, and therefore, it might be expected that treatment of HCV infection with interferon- α would result in the augmentation of detectable T cell responses. A study of T helper CD4⁺ T cell responses in 30 individuals treated with IFN- α monotherapy demonstrated enhanced NS3 and NS4 responses in patients with viral clearance, but increased CD4⁺ responses to NS5 and core in those with viral persistence (289). This group also described the association of viral clearance with increased frequency CTL, although the association is complicated by differing pre-treatment precursor frequencies and HCV viral load (290). While CD4⁺ and CD8⁺ T cell responses are important, there is also evidence that decreased dendritic cell frequency and function (by measurement of allogeneic CD4⁺ T cell stimulation) is associated with reduced response to PEG-IFN/ribavirin treatment (291). Few studies have compared T cell responses in the same individuals before and after treatment. One group who studied early therapy in acute HCV infection did not find a correlation between more frequent CD4⁺ or CD8⁺ T cell responses and viral response to combination therapy (216), suggesting that while T cell responses may contribute to viral clearance, they are not sufficient without other factors. There have been no studies of the effects of treatment on cell mediated or innate immunity in coinfecting populations to date.

Thus, although it seems intuitive that IFN-based therapy would improve cell mediated responses, the lack of agreement among a small number of studies suggests the mechanism of action of treatment is not just related to cell mediated immunity and may play a larger role at the level of innate immunity induction and regulation. This has implications for the development of more efficacious treatments and vaccine development.

2.4.6. Immune evasion strategies

Several strategies directed towards potentiating the immune response may contribute to HCV persistence. Both structural and non-structural HCV proteins have potentially immunoregulatory functions. Recombinant HCV core protein mediates several activities that can subvert an effective immune response, including in vitro inhibition of lymphocyte proliferation to core peptides (292), modulation of dendritic cell function (293-295), induction of tumor necrosis factor (TNF)-mediated apoptosis in T cell lines (296), enhancement of Fas-mediated death domain (FADD)-mediated apoptosis (297) and suppression of CTL responses, IFN- γ and IL-2 production in mice (298). Core protein can also regulate transcription of the interleukin-12 (IL-12) p70 subunit, which could disrupt expression of this pro-inflammatory cytokine critical for predisposing responding T cells towards a pro-inflammatory Th1-type cytokine production profile (299). The envelope protein E2 is thought to act in several ways with host cells to subvert or limit immune responses. Inhibition of NK cell activity through CD81 interaction (300, 301) and DC-SIGN (25) are two possible mechanisms.

Non-structural proteins have been associated with altered IFN signalling. A region of the HCV non-structural protein NS5A has potential immunoregulatory activity through inhibition of the antiviral cytokine IFN- α (302). The IFN-sensitivity determining region (ISDR) in NS5A is more likely to be mutated in those persons responding to IFN- α treatment (303). Wild type ISDR's are proposed to inhibit IFN function via both IFN-inducible double stranded RNA dependent kinase (PKR)-dependent and PKR-independent mechanisms (304). NS3/4A acts through interferon regulatory factor-3 (IRF-3), retinoic acid inducible gene I (RIG-I) and the toll-like receptor 3 (TLR-3) adaptor protein TRIF to limit IFN-related signalling (240-242). Most recently, NS4B has been shown to inhibit IFN signalling when overexpressed in HCV replicon supporting cell lines (305).

In summary, subversion of both intracellular and extracellular mechanisms of innate immunity may contribute directly and indirectly to viral persistence. Whether other mechanisms of immune subversion, including sequestration of effector cells in secondary lymphoid tissue, clonotypic restriction of CD8⁺ T cell receptors (TCRs), and T cell deletion, play a role remains to be determined (306).

2.5. Summary

In most cases, acute HCV infection progresses to chronic infection despite humoral and cellular antiviral immune responses. Viral diversification through rapid mutation and replication is probably a key viral strategy for persistence, but the high virus loads of acute infection and direct effects of viral proteins on cytokine production can also compromise the generation of pro-inflammatory Th1 type responses associated

with viral clearance. Individuals who clear HCV infection tend to have stronger T cell responses and higher levels of antibodies against the putative principal neutralizing determinant of HCV following acute infection. The T cell responses that arise in acute infection rapidly decline and often become undetectable in those clearing the virus and fall to much lower levels in those with chronic infection. In chronic infection, HCV-specific T cells in peripheral blood are more likely to produce Th2-type (IL-10, IL-2) cytokines, while T cells infiltrating the liver maintain a pro-inflammatory, Th1-like phenotype. HIV coinfection increases the prevalence of chronic HCV infection, increases plasma virus loads, increases the frequency of serologically silent HCV infection, and accelerates the rate of progressive liver disease. It is unclear whether the increased frequency of chronic and serologically silent infection simply reflects HCV infection in the setting of previous immunodeficiency or if a natural propensity of HCV to stimulate immunosuppressive cytokines is exacerbated by HIV infection. It also remains unclear whether the accelerated rate of disease progression is due primarily to increased HCV replication or if the increased immune activation, especially CD8⁺ T cell activation, in HIV infection hastens immunopathological liver damage. HIV coinfection complicates treatment of HCV infection as both ribavirin and interferon- α can produce side effects especially troublesome in HIV infected individuals. However, preliminary studies suggest that healthy HIV-infected individuals tolerate and respond to HCV treatment equally as well as non HIV-infected individuals. The liver damage associated with chronic HCV infection also reduces tolerance for antiretroviral drug regimes, creating a worsening spiral in which neither infection can be effectively treated and rapid

clinical disease progression follows. As a result, liver disease has become one of the leading causes of death for HIV-infected individuals in North America.

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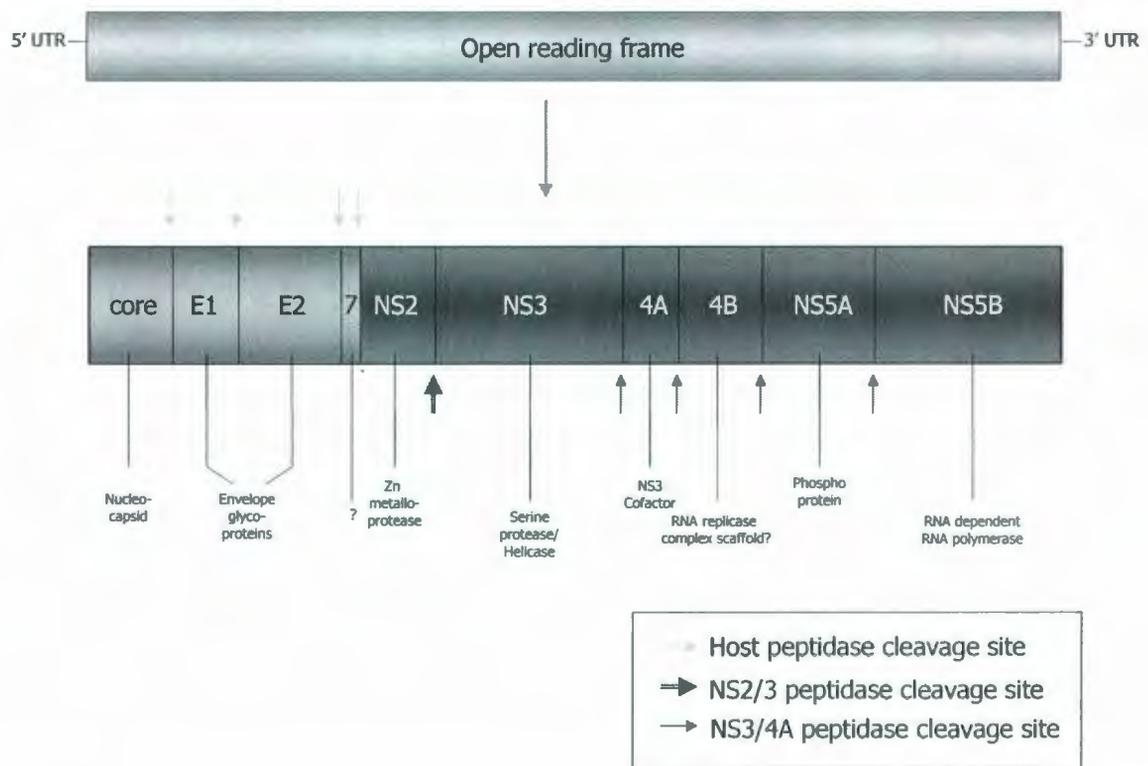
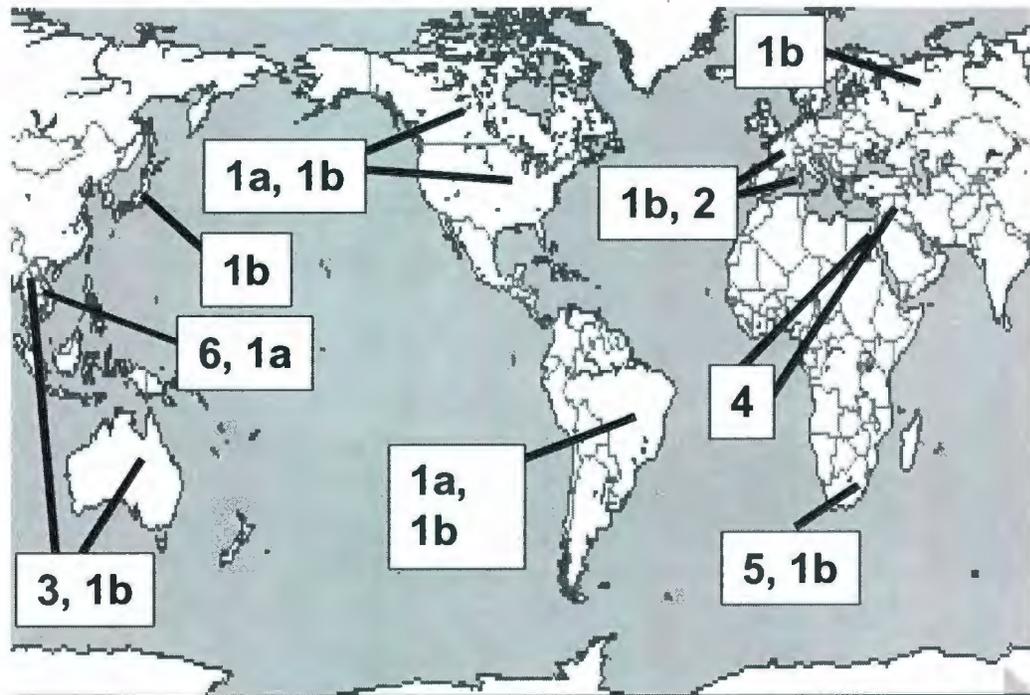
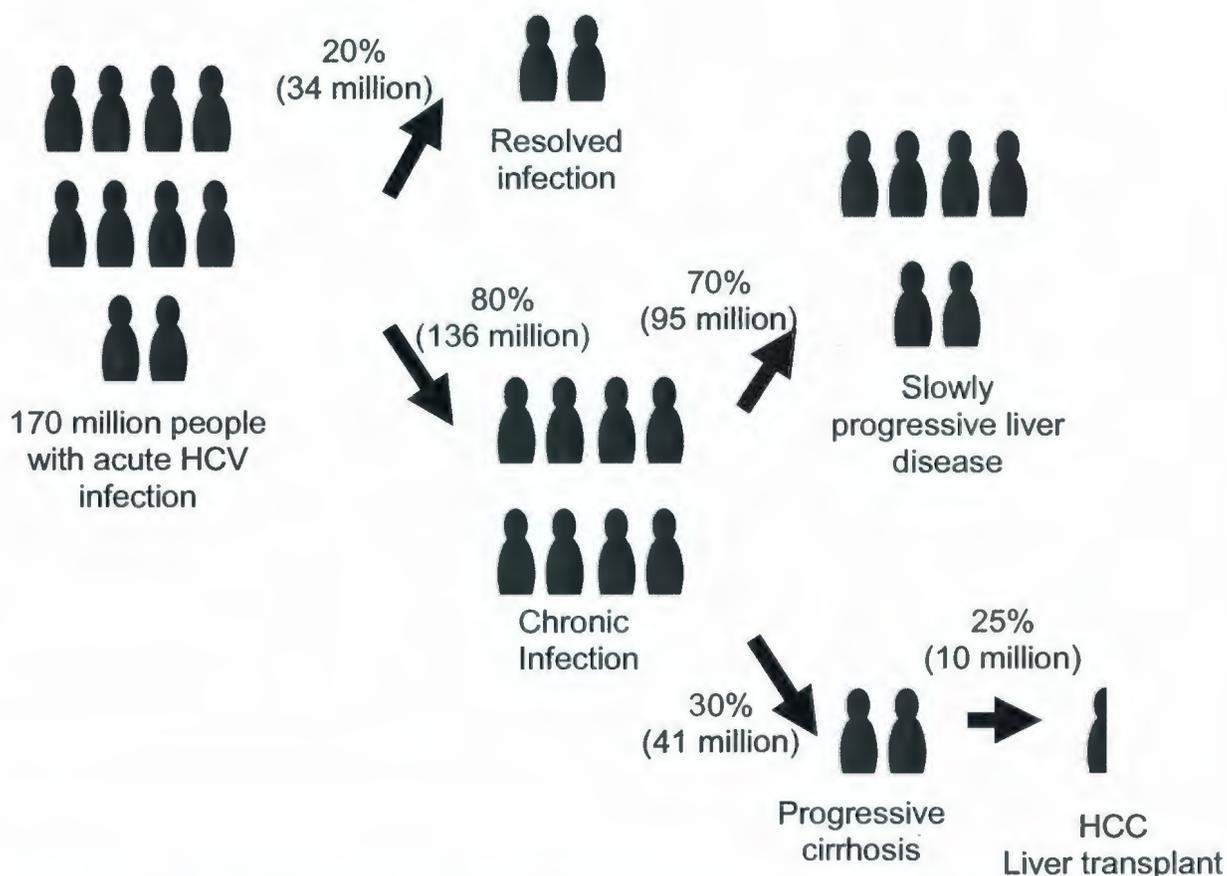


Figure 2.1. HCV genome and polyprotein processing. HCV has a 9600 base pair open reading frame genome that is translated as a polyprotein. The 4 structural proteins are cleaved by host peptidases (light grey arrows), and the remainder of the nonstructural proteins are cleaved by HCV peptidases (darker grey arrows). Most of the proteins have known functions in viral replication (indicated in text below each protein), but also functions related to immune regulation that are described in the text. Core, core protein; E1, envelope 1 protein; E2, envelope 2 protein; 7, protein 7; NS2, nonstructural protein 2; NS3, nonstructural protein 3; NS4A/B, nonstructural proteins A and B; NS5A/B, nonstructural proteins A and B.



<u>Genotype</u>	<u>High risk populations</u>	<u>Clinical Correlates</u>
1b	Blood transfusion recipients	Poor response to IFN/Ribavirin treatment Increased fibrosis, cirrhosis
1a, 3a	Intravenous drug users	
2, 4, 5, 6	Unclear	Unclear

Figure 2.2. Worldwide distribution of HCV genotypes. While it has been difficult to establish the clinical significance of various genotypes, it is clear that infection with genotype 1b carries a worse prognosis than infection with other genotypes, as well as a poor response to the best available HCV treatments. Certain genotypes are more common in blood transfusion recipients and IV drug users, and may be associated with different routes of transmission.



An estimated 170 million people are infected with HCV - approximately 10 million of these individuals could develop HCC or liver disease requiring a transplant.

Figure 2.3. Natural history of HCV infection. Estimates for the proportion of acutely infected individuals that become chronically infected range from 50%-85%. Many factors affect the time course of disease progression for those with chronic HCV infection, including HIV coinfection, concurrent alcohol abuse, age at time of infection, and gender.

CHAPTER 3**CIRCULATING CD14⁻CD36⁺ PERIPHERAL BLOOD MONONUCLEAR CELLS
CONSTITUTIVELY PRODUCE INTERLEUKIN-10**

Running Title: Constitutive IL-10 production

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3.1. Abstract

The impact of immune regulatory imbalance covers surprising physiological breadth. While dominance of anti-inflammatory cytokines such as interleukin-10 (IL-10) is associated with reduced immune responsiveness and susceptibility to persistent infection, conditions such as cardiovascular disease and diabetes are linked to chronic inflammation and lower IL-10 levels. An appropriate threshold for immune activation is critical for optimal protection from infection and conversely, from short and long-term side effects of immune effector mechanisms. To assess the possibility that IL-10 plays a role in setting this threshold and that healthy maintenance of immune silence may involve low level immune suppression, we sought out and characterized human peripheral blood cells constitutively producing the immunosuppressive cytokine IL-10. We determined the surface phenotype of circulating peripheral blood mononuclear cells (PBMC) constitutively producing IL-10 by surface and intracellular flow cytometry and visualized their ultrastructure by electron microscopy. The frequency of IL-10 producing and secreting cells was estimated by enzyme-linked immunospot (ELISPOT) and flow cytometry. Up to 1 percent of PBMC constitutively produce IL-10. These CD14⁻CD36⁺CD61⁺ non-adherent cells expressed general markers of hematopoietic and progenitor cells (CD45 and CD7) but no stem cell, T cell, B cell, NK cell, monocyte, or dendritic cell specific markers. Inflammation-associated toll-like receptors were also absent. The IL-10 producing cells had prominent nuclei, multiple mitochondria, and abundant rough endoplasmic reticulum. Healthy individuals have PBMC constitutively

producing IL-10. While the lineage of these cells remains unclear, their properties and frequency suggest a potential role in homeostatic or innate immune suppression.

3.2. Introduction

Activation of innate and adaptive immunity is essential for combating the constant challenge of invasive viral, bacterial, parasitic and fungal pathogens. Conversely, preventing inappropriate activation of immune responses and downregulating appropriate immune responses, once pathogens are cleared or other antigenic stimuli dissipate, is equally essential for avoiding immunopathology. Pathogen clearance by multiple arms of the immune system passively downregulates immune responses by decreasing antigenic stimuli, but when antigen persists, unresolved immune responses may be more damaging than the infection itself. In such situations, or when immune responses arise and propagate in the absence of infection, active immunoregulation by cytokines, regulatory cells or pharmacological intervention may be appropriate to prevent or limit pathology.

The immunoregulatory cytokine interleukin-10 (IL-10) plays an essential role in down-modulating both adaptive and innate immune responses (307). A variety of hematopoietic cells including monocytes, mast cells, T helper 2 (Th2) type T cells, regulatory T cells (Tr), dendritic cells (DC) and B cells produce IL-10, usually in response to particular stimuli (308). Interleukin-10 indirectly inhibits Th1 responses by blocking IL-12 production and major histocompatibility complex (MHC) class II upregulation by antigen presenting cells (APC) (309-311). Interleukin-10 also suppresses inflammation by inhibiting production of IL-1, tumor necrosis factor (TNF) and a variety of chemokines by APC, T cells and neutrophils (309, 312, 313). The physiological relevance of the immunoregulatory effect of IL-10 has been clearly demonstrated in

animal models of disease. Too little IL-10 renders mice more susceptible to chronic enterocolitis (314), but more resistant to intracellular pathogens such as *Listeria* and *Chlamydia* (315, 316), while IL-10 overexpression reduces intravascular inflammation and delays development of atherosclerosis in low density lipoprotein (LDL)-receptor deficient mice (317). In human studies, lower levels of IL-10 production are similarly associated with diseases thought to have an inflammatory component, such as atherosclerosis and diabetes (318, 319). Administration of exogenous IL-10 reduces symptoms in colitis and inflammatory bowel disease (IBD) and can moderate disease severity (320, 321). However, elevated IL-10 levels are associated with chronic bacterial infection (322-324), decreased dendritic cell maturation (325-328), and less effective immune surveillance against tumors (329). Thus, the broad range of IL-10-mediated immunoregulation constitutes a double edged sword with the beneficial effect of limiting immunopathology counterbalanced by increased susceptibility to infectious disease and cancer. While the regulatory role of IL-10 in various pathological states has been well described, there has been little investigation of IL-10 as a potential homeostatic regulator of the immune response. In this light, we report on the frequency, phenotype and ultrastructure of PBMC that constitutively produce and secrete IL-10 in healthy individuals. Constitutive production of IL-10 by a relatively large number of circulating cells in healthy individuals suggests that these cells could play a role in homeostatic immune regulation.

3.3. Materials and Methods

3.3.1. Subjects

Acid citrate dextrose (ACD)-preserved whole blood was collected by venipuncture from 12 apparently healthy volunteers (6 male, 6 female; ages 26 to 55 years) recruited from Memorial University Faculty of Medicine personnel. Informed consent was obtained from individuals for drawing blood, and ethical approval was obtained from the Memorial University Faculty of Medicine human investigation committee. PBMC were isolated by Ficoll-HyPaque Plus (Amersham Biosciences, Baie d'Urfé, Quebec, Canada) density gradient centrifugation. Cells were washed and suspended at 1×10^6 /mL in lymphocyte medium (RPMI supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 2 mM L-glutamine, 1% penicillin/streptomycin, and 20 μ M 2-mercaptoethanol; all from Invitrogen, Burlington, Ont).

3.3.2. Flow cytometry

Surface staining. The phenotype of fresh PBMC subpopulations were determined by extracellular flow cytometry using antibodies against human myeloid and lymphoid lineage-specific surface markers, as listed in Table 3.1. All steps were performed at 4°C, and incubations were performed in the dark to prevent fluorochrome photobleaching. Freshly isolated PBMC (5×10^5) were washed with cold PBS supplemented with 0.1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Missouri, USA), 5 mM EDTA (Sigma) and 0.02% sodium azide (Sigma), resuspended in a total volume of 600 μ L and incubated for 20 minutes with 0.5 μ g specific antibody against surface antigens or 0.5 μ g of appropriate isotype controls (Table 3.1). When the primary antibodies were

unlabelled, fluorochrome labelled secondary antibodies were added for 20 minutes and cells were washed before fixation. Cells were fixed with 0.5 mL 1% paraformaldehyde (Sigma) in PBS for 20 minutes, washed again, and resuspended in 250 μ L of 1% paraformaldehyde. Fixed, stained cells were stored at 4°C until analysis of at least 100,000 events on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Intracellular staining. Cells were labelled as above for detection of surface antigens with an additional permeabilization step after fixation. Cells were incubated with 0.5 mL DAKOCytomation (Mississauga, Ontario) permeabilization reagent together with antibodies against either various cytokines or appropriate isotype control antibodies (Table 3.1) for 20 minutes. After washing, cells were resuspended in 1% paraformaldehyde in PBS until analysis as above. CellQuest Pro was used for data analysis.

3.3.3. *Detection of IL-10 mRNA*

The presence of IL-10 mRNA in freshly isolated PBMC subsets was demonstrated by reverse transcriptase polymerase chain reaction (RT-PCR). Aliquots of 1×10^7 PBMC were washed with separation buffer (PBS supplemented with 5 mM EDTA and 0.5% BSA) and incubated for 30 min at 4°C with 0.5 μ g of isotype control, anti-CD14 or anti-CD36 antibodies (Table 3.1). The cells were washed again and incubated at 3×10^6 /ml for 45 min at 4°C in separation buffer with sufficient goat anti-mouse IgG or rat-anti-mouse IgM conjugated magnetic beads (Dynal ASA, Oslo, Norway) for a 10:1 bead-to-target cell ratio. A magnet was used to separate bead-bound and unbound cells and flow cytometry indicated depletion of >90% of the targeted subset. Total RNA from bead-

bound and unbound cells was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the Amersham cDNA synthesis kit (Amersham Biosciences, Baie d'Urfé, Québec). Specific primers for PCR were previously described; antisense and sense IL-10 primer sequences were 5'-ACCTGCTCCACGGCCTTGCTCT-3' and 5'-CACCCAGTCTGAACAGCTGC-3', respectively (330). Anti-sense and sense primers for the β -actin housekeeping gene were 5'-CAACCGTGAGAAGATGACC-3' and 5'-ATCTCCTGCTCGAAGTCC-3' respectively. PCR conditions for each 50 μ L reaction were: 1X PCR buffer, 2.5 mM $MgCl_2$, 200 μ M dNTPs, 2.5 U Taq DNA polymerase, and 20 pmol each of the sense and antisense primer (all from Invitrogen). Reactions ran for 35 cycles for IL-10 and 30 cycles for β -actin with the following cycle times and temperatures: 40 seconds at 95°C, 30 seconds at 60°C (IL-10) or 55°C (β -actin), 1 minute at 72°C, with a final extension of 7 minutes at 72°C. The expected product sizes were approximately 360 and 339 base pairs (bp) for IL-10 and β -actin respectively. Products were separated by electrophoresis on 2% agarose gels with 0.5 μ g/mL ethidium bromide (Sigma Chemical Co.) and visualized by ultraviolet light. Gels were analysed using a CCD camera and ChemiImager (AlphaInnotech, San Leandro, CA) software.

3.3.4. Depletion of $CD61^+$ PBMC

Ten million fresh PBMC were incubated for 20 minutes with 5 μ g purified anti-CD61 antibody (Table 3.1) in 0.5 mL flow cytometry buffer at 4°C. Cells were washed, and suspended in separation buffer with a 10:1 bead-to-target cell ratio of goat anti-

mouse IgG coated magnetic beads (Dyna) for 45 minutes at 4°C. A magnet was used to remove bead-bound CD61⁺ cells from the remaining PBMC. Depletion efficiency (98%) was determined by flow cytometric CD61 staining of the remaining PBMC.

3.3.5. *Depletion of adherent cells*

In some experiments, PBMC were depleted of adherent monocytes cells by plastic adherence. Freshly-isolated PBMC at 1×10^6 /well were incubated in 24 well tissue culture treated flat bottom plates (Corning Costar, Corning NY) at 37°C, 5% CO₂ for 60 minutes. Non-adherent cells were removed by gently resuspending settled PBMC in 1 mL of medium. Efficiency of monocyte removal (97%) was determined by flow cytometry.

3.3.6. *ELISPOT for IL-10 production*

The number of IL-10 producing cells within intact or CD61-depleted PBMC was determined by ELISPOT. Flat bottom polyvinylidene difluoride (PVDF)-coated 96-well plates (Millipore, Bedford, MA) were prewet with 100 µL 70% ethanol/well (Sigma Chemical Co.), washed with PBS, and coated either overnight at 4°C or for one hour at 37°C with 1.5 µg/well anti-IL-10 antibody (clone 9D7, Mabtech, Stockholm, Sweden) in PBS. Plates were washed five times with PBS and 2×10^5 intact PBMC or approximately 1.9×10^5 CD61-depleted PBMC were added to each well in triplicate. Plates were incubated at 37°C, 5% CO₂ for 18 hours, washed with PBS, and incubated for 2 hours with 0.1 µg/well of biotinylated anti-IL-10 antibody (clone 12G8, Mabtech). Plates were washed, and 100 µL of a 1:1000 dilution of streptavidin-alkaline phosphatase (Mabtech) was added for 1 hour. After washing, 100 µL freshly prepared nitro blue tetrazolium chloride (NBT) 3 mg/mL /5-bromo-4-chloro-3-indolyl phosphate-p-toluidine (BCIP) 1.5

mg/mL substrate (BioRad, Hercules, CA) in Tris buffer (pH 9.5) was added to each well for approximately 20 minutes. Plates were washed with distilled water to stop the color reaction and air-dried overnight before spot enumeration using the high resolution Zeiss reader system and associated KS software (Carl Zeiss Canada, Ontario, Canada). The number of IL-10 producing cells per million PBMC was determined by multiplying the average number of spots/duplicate well x 5.

3.3.7. *Electron microscopy*

The IL-10 producing PBMC population was enriched for visualization by electron microscopy through depletion of T cells, B cells, monocytes, and NK cells. 1.25×10^8 PBMC were obtained from a healthy donor, divided into 1.0×10^7 aliquots and incubated with the following antibodies for 25 minutes at 4°C: 7 µg anti-CD2; 6 µg anti-CD3; 6 µg anti-CD14; and 7 µg anti-CD19 (Table 3.1). Cells were washed and goat anti-mouse IgG coated magnetic beads (Dynal) added at a bead to PBMC ratio of 10:1 for 45 minutes at 4°C. Bead-bound cells were magnetically removed, and red cells lysed from the remaining unlabelled PBMC by 3 minute room incubation at room temperature with 0.5 mL erythrocyte lysis buffer (0.15 M ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) in distilled water), followed by 2 PBS washes. The remaining cells ($\sim 3.5 \times 10^6$ /aliquot) were incubated with 3.5 µg IgM anti-CD36 mAb (Table 3.1) for 25 minutes at 4°C, washed in flow buffer, and labelled with 12 nm gold particle conjugated goat anti-mouse IgM beads (Jackson Immunosciences Laboratories, West Grove, PA) for 40 minutes at 4°C. Cells were washed twice, and fixed in 1 mL of Karnovsky's fixative (4 g paraformaldehyde, 5% glutaraldehyde in

0.2M sodium cacodylate). After 6 hours, cells were placed in 1% osmium tetroxide for 20 minutes and washed. Cells were dehydrated with increasing alcohol concentrations from 75%-100% followed by an acetone wash and embedded in epoxide resin overnight at 70°C. 90 nm sections were cut, stained with uranyl acetate, and examined with a Jeol 1220X electron microscope.

3.3.8. *Statistical analyses*

All statistical analyses were performed using SPSS version 9 (SPSS Inc., Chicago, IL). Means were compared using either the non-parametric Mann-Whitney U test or the parametric Student's *t*-test. Nonparametric tests were used when either the populations were not normal or the number of samples each group was less than 12. Parametric tests were used if the population was normal and the group contained more than 12 individuals.

3.4. Results

3.4.1. *A PBMC subset in healthy individuals constitutively produces and secretes IL-10*

To investigate background levels of pro- and anti-inflammatory cytokine production by circulating PBMC, we used intracellular flow cytometry to evaluate constitutive IL-2, IL-10, IL-12, and IFN- γ production by freshly isolated PBMC from healthy individuals. None of the pro-immune or pro-inflammatory cytokines IL-2, IL-12, or IFN- γ were constitutively produced by freshly isolated PBMC from any individual, but CD3-negative PBMC with lymphoid light scatter characteristics constitutively producing IL-10 were detected *ex vivo* in every individual tested (Figure 3.1a). A significant fraction of CD36⁺ PBMC with lymphoid light scatter characteristics (moderate forward scatter and low side scatter) expressed varying levels of IL-10, while a much smaller fraction of CD36⁺ monocytoid cells (CD14⁺, high forward and side scatter) produced low levels of IL-10 (Figure 3.1b). Intracellular flow cytometry indicated approximately 1% (range 0.37%-1.14%) of PBMC from healthy individuals (n=12) constitutively producing IL-10 (Figure 3.2). Following PBMC isolation, IL-10 production decreased such that after 18 hours of cell culture, intracellular IL-10 was detectable in <0.1% of total PBMC (data not shown).

We used an IL-10 ELISPOT assay to confirm constitutive IL-10 production and to demonstrate IL-10 secretion by freshly isolated PBMC. For the 6 individuals tested, 318-1171 cells/10⁶ PBMC secreted IL-10 detectable by ELISPOT (Figure 3.2). Negligible numbers of spots developed in the control wells. While the ELISPOT data confirms spontaneous production of IL-10 by PBMC from healthy individuals, the

frequency of IL-10 producing cells detected is around one-tenth of that observed by intracellular flow cytometry, reflecting either greater sensitivity of flow cytometry or fairly rapid cessation of IL-10 secretion in vitro. There was a weak but significant correlation between the frequency of IL-10 producing PBMC detected by intracellular flow cytometry and ELISPOT ($r = 0.88$, $p < 0.05$). Constitutive production of IL-10 protein was also reflected by detection of IL-10 mRNA in freshly isolated PBMC subsets by RT-PCR (Figure 3.3). The CD14⁺ monocyte population and moreso, the CD36⁺ monocyte and lymphoid cell population accounted for IL-10 mRNA in freshly-isolated PBMC.

3.4.2. *Detailed phenotype of PBMC constitutively producing IL-10*

Initial flow cytometry studies identified the majority of IL-10 producing PBMC as non-T cells with lymphoid light scatter characteristics. We used the panel of antibodies listed in Table 3.1 to more extensively phenotype these IL-10 producing PBMC. None of the early precursor and stem cell markers (CD34, CD117), lymphoid lineage-specific markers (CD2, CD3 and CD19) or the myeloid lineage marker CD33 were present on the IL-10 producing cells (Figure 3.4). The general leukocyte marker CD45 was present, together with the non-lineage-specific adhesion molecules CD54 and CD62L. In the absence of other T cell subset makers such as CD4 or CD8, the thymocyte and T cell subset antigen CD7 was expressed on the IL-10 producing cells. None of human leukocyte antigen (HLA) class II, CD11c, CD40, CD68, CD80, CD83 or CD86 (molecules found primarily on professional APCs) were present on the IL-10 producing cells. Of several activation markers examined (CD25, CD38 and CD69), only

CD38, an activation marker increased on T cells and APC after antigen specific stimulation, was found on the IL-10 producing cells. These data indicate that the lymphoid PBMC constitutively producing IL-10 do not belong to any commonly recognized NK cell, B lymphocyte, T lymphocyte, or dendritic cell subset and they are not professional APC.

Two cell surface markers best defined the constitutive IL-10 producing cells and clearly distinguished them from other PBMC with lymphoid scatter characteristics. All the IL-10 producing cells expressed CD36 (Figure 3.5a) and low levels of CD61 (Figure 3.5b). Cells with high level CD36 expression (mean channel fluorescence $\geq 10^2$) were uniformly positive for intracellular IL-10 and 50%-80% of the CD36⁺CD61⁺ cells expressed IL-10 (Figure 3.5b). To demonstrate constitutive IL-10 production by circulating CD36⁺CD61⁺ cells, CD61⁺ cells were magnetically depleted from freshly isolated PBMC. This removed greater than 98% of the CD36⁺IL-10⁺ population in fresh PBMC (Figure 3.6a) as measured by flow cytometry. When PBMC depleted of CD61⁺ cells were incubated for 18 hours in an IL-10 ELISPOT assay, significantly fewer spots were formed (318 IL-10 producing spots/ 10^6 cells) than with intact PBMC (733 spots/ 10^6 PBMC) (Figure 3.6b; $t = 2.67$, $p < 0.03$). This degree of reduction in the ELISPOT assay relative to the 98% reduction in the immediate ex vivo flow cytometry assay indicates partial, but incomplete overlap between the PBMC producing IL-10 ex vivo as detectable by flow cytometry and the PBMC producing IL-10 in vitro as detected by overnight ELISPOT assay.

3.4.3. Relationship between phenotype and function of IL-10 producing PBMC

Since monocytes are the major PBMC population previously reported to express the CD36 and CD61 molecules distinguishing our IL-10 producing PBMC, we examined phenotypic and physical characteristics of the CD36⁺ IL-10 producing lymphoid cells that might further differentiate them from monocytes. Monocytes and lymphocytes have very different forward (size) and side (granularity) light scatter characteristics when analyzed by flow cytometry. Most of the PBMC constitutively producing IL-10 have light scatter characteristics similar to lymphocytes (Figure 3.1b), indicating they are smaller and less granular than monocytes. They also do not express the monocyte marker CD14 (Figure 3.7). Non-adherence to plastic further distinguishes the CD14⁻ CD36⁺CD61⁺ IL-10 producing cells from CD14⁺CD36⁺CD61⁺ monocytes, as there was no depletion of IL-10 producing cells following 2 hour PBMC incubation on plastic dishes (data not shown).

As previously shown, CD36 is present on the IL-10 producing cells and high CD36 expression appears to be associated with IL-10 production. CD36 is a scavenger receptor for apoptotic bodies and may play an evolutionary role in the innate immune response. Absence of CD14, the lipopolysaccharide (LPS) receptor that stimulates a monocyte pro-inflammatory response, phenotypically distinguishes the IL-10 producing cells from CD36⁺ monocytes. Since CD14 is functionally related to the family of toll-like receptors (TLR) that recognize pathogen-associated molecular patterns, we investigated expression of several common TLRs on the CD36⁺ IL-10 producing lymphoid cells. In contrast to the CD14⁺CD36⁺CD61⁺ monocytes,

CD14⁻CD36⁺CD61⁺IL10⁺ cells did not express TLR-2, TLR-4 or TLR-9 (Figure 3.7). The absence of these primarily pro-inflammatory receptors on the surface of the IL-10 producing cells is consistent with their production of the anti-inflammatory cytokine IL-10 and with a potential immunoregulatory role for these cells.

3.4.4. *Ultrastructural characteristics of the IL-10 producing cells*

To examine the ultrastructure of the IL-10 producing cells, we enriched for this population by depleting PBMC of T cells, B cells, monocytes and NK cells with magnetic beads and antibodies against CD2, CD3, CD14 and CD19. Remaining cells were incubated with gold bead-conjugated anti-CD36 antibodies and the CD36⁺ cells were visualized by electron microscopy. The CD36⁺ cells were approximately 6 μm in diameter, similar to resting lymphocytes, with a high nucleus:cytoplasm ratio and large amount of heterochromatin (Figure 3.8). Mitochondria were plentiful and rough endoplasmic reticulum was very prominent in the cytoplasm, suggesting these were highly synthetic, metabolically active cells.

3.5. Discussion

Induction of inflammatory and immune responses must be carefully regulated to allow for effective protective immunity while avoiding unnecessary inflammation and immunopathology. Stimuli must individually and collectively breach a certain threshold to initiate or sustain inflammation and immunity. Whether an appropriate threshold for immune activation is maintained simply through the requirement for accumulation of positive signals or whether low level basal immune suppression may be involved is an important issue in relation to susceptibility to chronic infection or chronic inflammation. Consistent with the general view that immune responses do not arise in the absence of stimuli, we found little or no constitutive production of pro-inflammatory cytokines IL-2, IL-12 or IFN- γ by PBMC from healthy individuals. However, up to 1% of freshly isolated PBMC constitutively produced and secreted IL-10, an important negative regulator of inflammatory and cellular immune responses. Phenotypic and ultrastructural characterization of the cells spontaneously producing IL-10 indicated a previously undescribed PBMC subset resisting inclusion in any common recognized lineage

Constitutive production of IL-10 by freshly isolated, non-stimulated PBMC was readily apparent by *ex vivo* intracellular flow cytometry or by ELISPOT, following overnight incubation in unsupplemented lymphocyte medium. Surprisingly, despite the widespread application of sensitive ELISPOT assays, only one group has previously reported constitutive IL-10 production by PBMC, and in this case only at the mRNA level (331). Counterstaining the IL-10⁺ PBMC by flow cytometry demonstrated consistent expression of the common leukocyte antigen, CD45, suggesting hematopoietic

origin. While Th2 cells are a common source of IL-10 in response to specific antigens, none of the IL-10⁺ cells expressed CD2, CD3, CD4, CD8 or CD19, ruling out their assignment within NK, T or B cell subsets. Immature DC and monocytes also produce IL-10, but the characteristics of the lymphoid IL-10⁺ cells were inconsistent with either of these PBMC subsets. Unlike immature DC, the IL-10⁺ cells expressed no HLA-DR or CD1a, a MHC-related protein expressed on antigen presenting cells that presents self proteins to T cells. In contrast to monocytes, the IL-10⁺ cells had lymphoid cell light scatter characteristics and didn't express CD14, CD33, CD68 or myeloperoxidase. The IL-10⁺ cells are easily distinguished from virtually all other PBMC by their lymphoid light scatter (smaller and less complex than monocytes) and expression of CD36 in the absence of CD14. Expression of other surface markers such as CD5, CD7, CD38, CD54, CD59 and CD61 is interesting and useful for depletion or isolation, but offers little obvious insight into the origin or role of these cells. The constitutive IL-10 production is consistent with a role for these cells in low level basal immunosuppression and expression of CD62L indicates an ability exit the blood stream through high endothelial venules and modulate immune responsiveness within the lymph nodes, where primary immune responses generally originate.

As noted in the results section, a small proportion of circulating monocytoid cells were also IL-10⁺ positive. These cells were very easily differentiated from the CD36⁺CD14⁻ population of lymphoid cells, and removal of CD14⁺ monocytes through plastic adherence or magnetic beads did not deplete IL-10 protein or mRNA bearing cells respectively. Absence of CD14, a primarily pro-inflammatory molecule related to the

Toll-like receptors (TLR), phenotypically distinguishes the CD36⁺IL-10⁺ cells from monocytes and also supports their characterization as potentially immunosuppressive, rather than pro-inflammatory cells. While signalling through CD14 also can result in late production of IL-10, the phenotypic polarization of these CD36⁺IL-10⁺ cells away from immune activation is further reflected by the absence of TLR 2, 4 and 9, additional pro-inflammatory receptors that are consistently expressed on CD36⁺ monocytes and are generally associated with immune activation. In the CD14⁻CD36⁺ PBMC subset, we observed a general relationship between IL-10 production and CD36 expression in that all CD36^{bright} cells were producing IL-10 and that the CD36^{bright} cells produced more IL-10 than CD36^{dim} cells. This relationship raises the possibility of a mechanistic link between CD36 expression and the function of these cells. One function of CD36 is in the uptake of apoptotic bodies by macrophages and dendritic cells (332, 333) and subsequent modulation of immune responses (334). The relationship between high CD36 expression and high IL-10 production could preferentially deliver apoptotic bodies to IL-10 producing cells, favouring immune suppression over inflammation. Delivery of early apoptotic cells to a macrophage subset producing IL-10 was recently demonstrated and proposed as a mechanism to prevent autoimmunity under steady-state conditions (335). Another CD36 ligand is oxidized LDL (oxLDL), a factor associated with the pathogenesis of atherosclerosis (336, 337). Selective delivery of oxLDL to IL-10 producing cells through this interaction with CD36 could play a protective role against the putative inflammatory component of atherosclerosis. The malaria-causing parasite, *Plasmodium falciparum*, also interacts with CD36 and through this interaction could

exploit localized immunosuppression to evade immunity and establish chronic infection (334).

While circulating monocytes express relatively low levels of CD36, few were constitutively producing IL-10, therefore, cell-specific factors other than just expression of CD36 are involved in constitutive IL-10 production. Monocytes exposed in vitro to thiazolidinediones, peroxisomal proliferator associated receptor- γ (PPAR- γ) agonists commonly used to treat type II diabetes associated insulin resistance, express higher levels of CD36 and develop an anti-inflammatory cytokine release profile (338). A rat model of type II diabetes and hypertension has a dominant CD36 mutation that precludes expression of CD36 at the transcriptional level (339), supporting a immunomodulatory role for CD36 in inflammatory diseases such as diabetes. In humans, the null mutation of a human blood group polymorphism, Nak^a results in loss of CD36 expression (340). This phenotype is prevalent in African and Japanese populations, and it would be interesting to compare circulating IL-10⁺ PBMC and the rates of various inflammatory diseases between Nak^{a null} (with impaired CD36 expression) and appropriate control groups.

Local delivery or production of IL-10 may be the mechanism by which CD36 engagement, in some cases, dampens inflammatory responses (334, 341). We speculate that the IL-10⁺CD36⁺ cells provide a low level innate barrier to immune responses and inflammation, suggesting that lower circulating levels of these cells could predispose to development of autoimmune or pro-inflammatory diseases. Homeostatic regulation of the immune system similar to what we are suggesting by these IL-10⁺ cells is mimicked in the interaction between lung alveolar epithelial cells (AEC) and alveolar macrophages

(AM) (342). Transforming growth factor beta (TGF- β produced by AEC maintains a homeostatic suppressive environment in normal lung by inhibiting AM. However, AM are still able to respond to and clear microbial pathogens when necessary by using protein conformational change to disengage from the AEC inhibition. AM inhibition is restored as the pathogen is cleared, resulting in minimal damage to nearby lung tissue. We suggest that constitutive production of IL-10 by the cells described in this study provides a similar tonic checkpoint on the immune system to downregulate unnecessary responses in the steady state.

The concept of immune regulatory cells has evolved through various incarnations of suppressor T cells to the relatively well-defined CD4⁺CD25⁺FoxP3⁺ regulatory T cells of today. These cells develop spontaneously and in some cases mediate their effector functions through non-specific cytokines such as IL-10 and TGF- β . However, they are activated through antigen-specific receptors and, therefore, constitute an adaptive immunosuppressive response that can appropriately protect against autoimmunity and inappropriately increase susceptibility to infection and cancer. The existence of circulating antigen-non-specific cells spontaneously producing the immunosuppressive cytokine IL-10 suggests that like immune reactivity, immune suppression may be organized in innate and adaptive layers with a similar series of interconnections still to be elucidated.

3.6. Acknowledgements

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Table 3.1. Antibodies for fresh PBMC phenotyping

Antigen¹	Fluorochrome²	Isotype³	Clone	Company⁴
CD1a	FITC	mIgG ₁	NA1/34	DakoCytomation
CD2	Purified	mIgG _{2b}	RPA-2.10	BD Pharmingen
CD3	Purified, FITC	mIgG ₁	UCHT1	DakoCytomation
CD4	FITC	mIgG ₁	RPA-T4	BD Pharmingen
CD5	APC	mIgG ₁	VI-PL2	BD Pharmingen
CD7	FITC	mIgG ₁	M-T701	BD Pharmingen
CD8	PerCP	mIgG ₁	HIT3a	BD Pharmingen
CD10	PE	mIgG ₁	HI10a	BD Pharmingen
CD11b	PE	mIgG ₁	ICRF44	BD Pharmingen
CD11c	PE	mIgG ₁	B-ly6	BD Pharmingen
CD13	PE	mIgG ₁	WM15	BD Pharmingen
CD14	Purified, FITC	mIgG _{2a}	M5E2	BD Pharmingen
CD19	Purified	mIgG ₁	HIB19	BD Pharmingen
CD20	FITC	mIgG _{2b}	2H7	BD Pharmingen
CD25	PE	mIgG ₁	M-A251	BD Pharmingen
CD33	PE	mIgG ₁	WM53	BD Pharmingen
CD34	FITC	mIgG ₁	581	BD Pharmingen
CD36	Purified, PE	mIgM	CB38	BD Pharmingen
CD38	APC	mIgG ₁	HIT2	BD Pharmingen
CD40	Purified	mIgG ₁	E5-A	Calbiochem
CD45	APC	mIgG ₁	HI30	BD Pharmingen
CD54	Purified	mIgG ₁	HA58	BD Pharmingen
CD56	FITC	mIgG ₁	B159	BD Pharmingen
CD57	FITC	mIgM	NK-1	BD Pharmingen
CD59	FITC	mIgG _{2a}	p282	BD Pharmingen
CD61	Purified, PerCP	mIgG ₁	VI-PL2	BD Pharmingen
CD64	FITC	mIgG ₁	10.1	Caltag
CD62L	FITC	mIgG ₁	DREG56	BD Pharmingen
CD66abce	FITC	mIgG ₁	Kat4C	DakoCytomation
CD68	FITC	mIgG ₁	KP1	DakoCytomation
CD69	FITC	mIgG ₁	FN50	BD Pharmingen
CD71	FITC	mIgG ₁	YDJ1.2.2	Immunotech
CD80	Purified	mIgG ₁	DAL-1	Caltag
CD83	FITC	mIgG ₁	PNIM2410	Immunotech
CD86	Purified	mIgG ₁	BU63	Caltag
CD117	PE	mIgG ₁	YB5.B8	BD Pharmingen
TLR-2	FITC	mIgG _{2a}	TLR.1	eBioscience
TLR-4	PE	mIgG _{2a}	HTA 125	eBioscience
TLR-9	Purified	mIgG ₁	26C593	Bio/Can Scientific
HLA Class I	Supernatant	mIgG ₁	PA2.6	ATCC
HLA Class II	Supernatant	mIgG ₁	L243	ATCC

Table 3.1. (con't)

Antigen ¹	Fluorochrome ²	Isotype ³	Clone	Company ⁴
Myeloperoxidase Isotype Controls	PE	mIgG ₁	1B10	BD Pharmingen
Mouse IgG₁	FITC			BD Pharmingen
Mouse IgM	PE			BD Pharmingen
Mouse IgG ₂	FITC			BD Pharmingen
Rat IgG ₂	FITC			Caltag
Secondary Antibodies				
Goat anti-mouse IgG	FITC, PE			Jackson ImmunoResearch
Goat anti-mouse IgM	FITC			Jackson ImmunoResearch
Goat anti-mouse IgA	FITC			Sigma
Cytokines				
Interferon-gamma (IFN- γ)	FITC	mIgG ₁	B27	Caltag
Interleukin-2 (IL- 2)	FITC	rIgG _{2a}	MQ1-17H12	Caltag
Interleukin-10 (IL-10)	FITC	rIgG ₁	JES9D7	Caltag
Interleukin-12 (IL-12)	FITC	rIgG ₁	C8.6	Caltag

¹Antibodies are against human antigens unless otherwise indicated.

²FITC-fluorescein isothiocyanate; PE-phycoerythrin; PerCP – peridinin chlorophyll protein; APC – allophycocyanin

³m-mouse; r-rat; g-goat

⁴Becton Dickinson Biosciences Pharmingen, San Diego, California; Bio/Can Scientific, Montreal, Quebec; Caltag, Burlingame, CA; DakoCytomation, Mississauga, Ontario; eBioscience, San Diego, CA; Immunotech, Mississauga, Ontario; Jackson ImmunoResearch, West Grove, Pennsylvania; Sigma, St. Louis, Missouri, USA

(a)

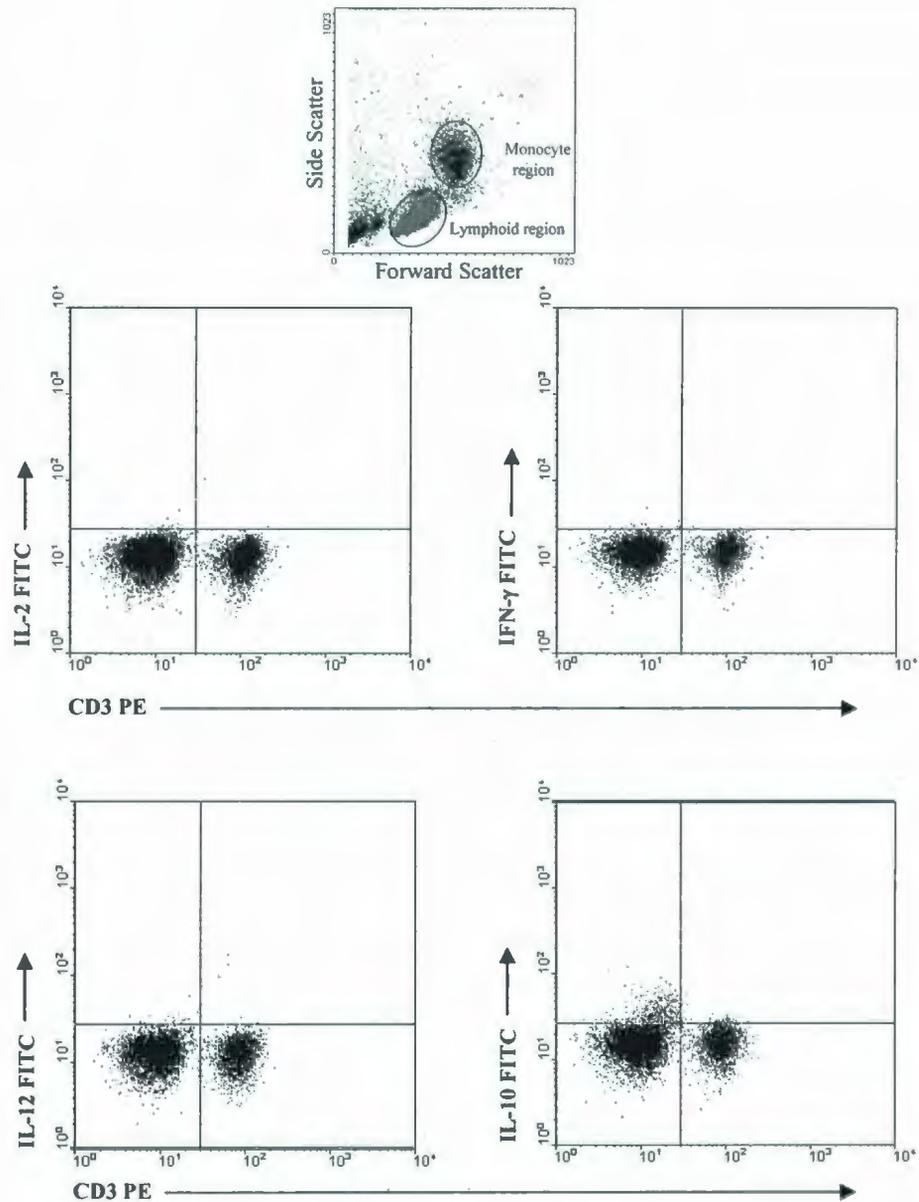


Figure 3.1. Ex vivo cytokine production by CD3⁺ PBMC from a healthy individual.

(a) Constitutive production of IL-2, IFN- γ , IL-12 and IL-10 by CD3⁺ and CD3⁻ PBMC with lymphoid scatter characteristics was assessed by intracellular flow cytometry. Results are representative of 12 individuals.

(b)

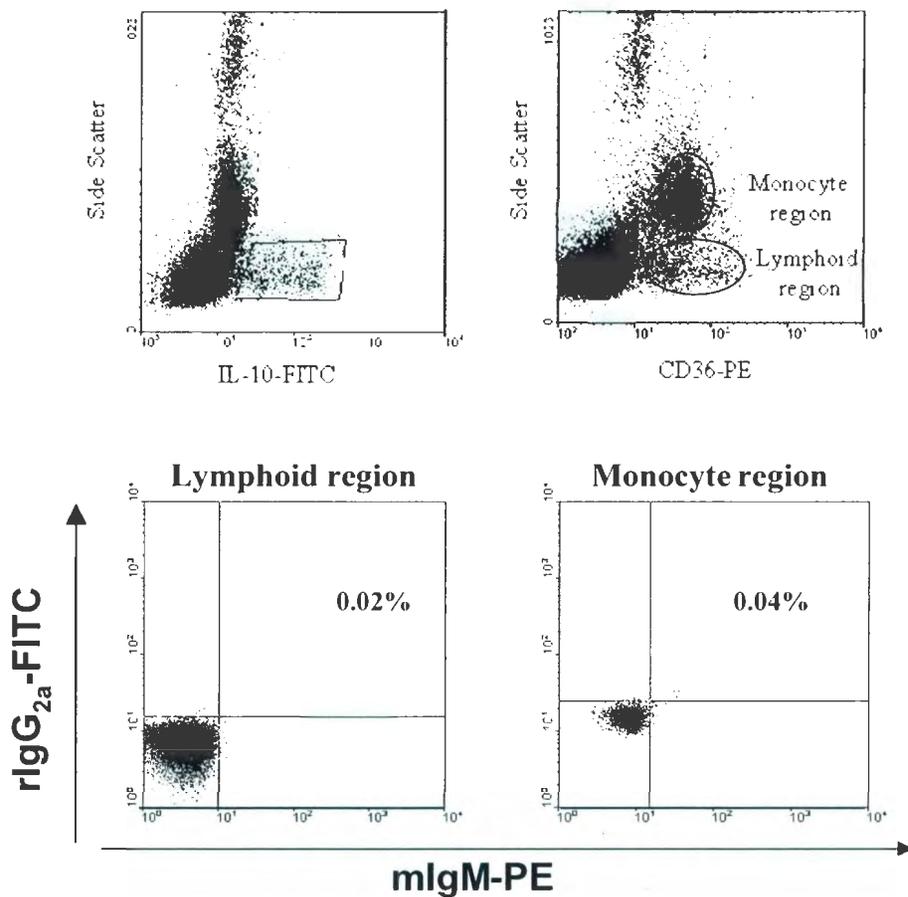


Figure 3.1. (con't) (b) Production of IL-10 by lymphoid and monocyte cells was distinguished by side scatter. Isotype controls are shown in the lower panels.

Individual	% PBMC Expressing IL-10	
	Flow Cytometry	ELISPOT
1	0.69	0.03
2	0.37	0.03
3	0.82	0.07
4	0.92	0.09
5	1.14	0.12
6	0.83	0.05

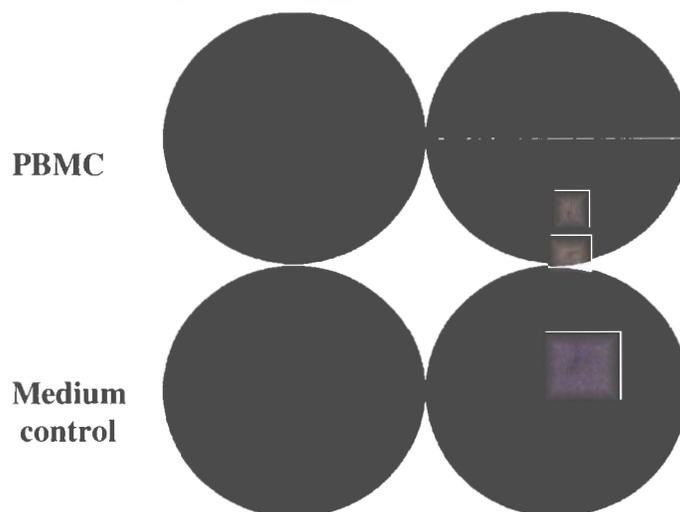


Figure 3.2. Comparison of IL-10⁺ cell frequency in PBMC determined by flow cytometry or ELISPOT in 6 representative individuals. The picture depicts test wells with PBMC from a representative individual with control wells underneath.

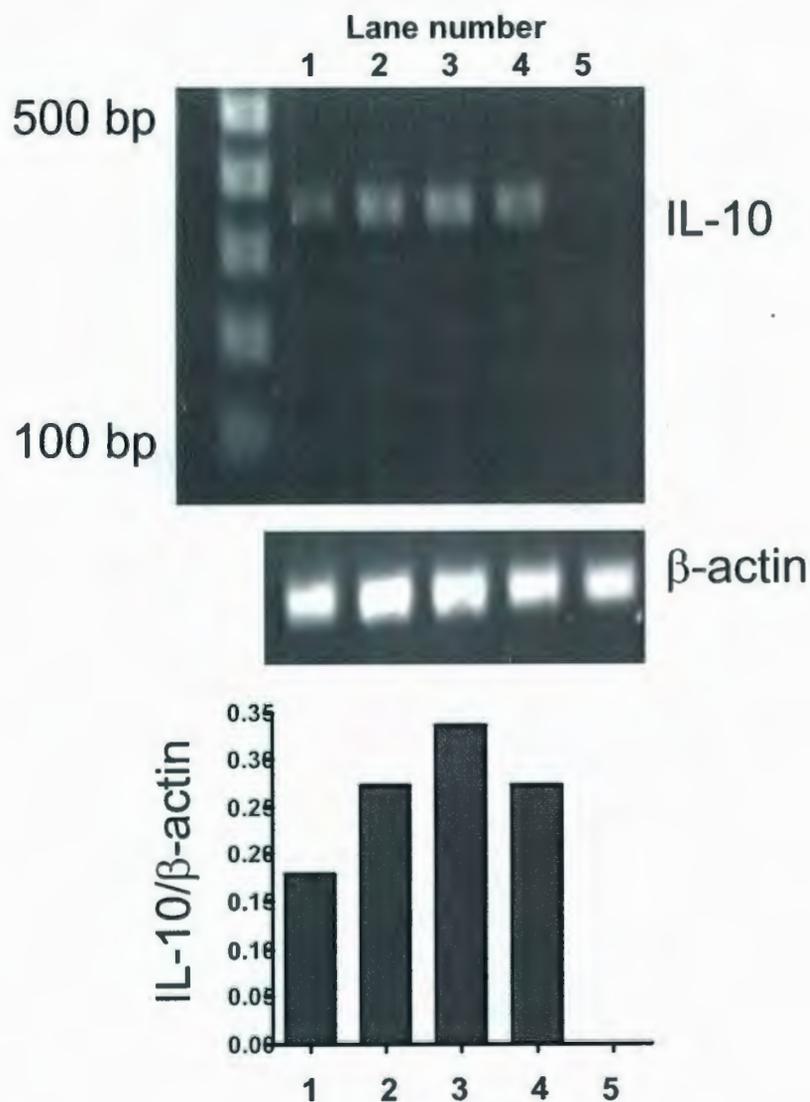


Figure 3.3. Relative levels of IL-10 mRNA in PBMC subsets. An equal amount of RNA isolated from PBMC (lane 1), CD14⁺ PBMC (lane 2), CD36⁺ PBMC (lane 3), PBMC depleted of CD14⁺ cells (lane 4) and PBMC depleted of CD36⁺ cells (lane 5) was converted to cDNA, subjected to IL-10 and β-actin specific PCR and separated on an agarose gel. The relative intensity of the IL-10 band compared to the β-actin band is shown in the bar graph below the gel.

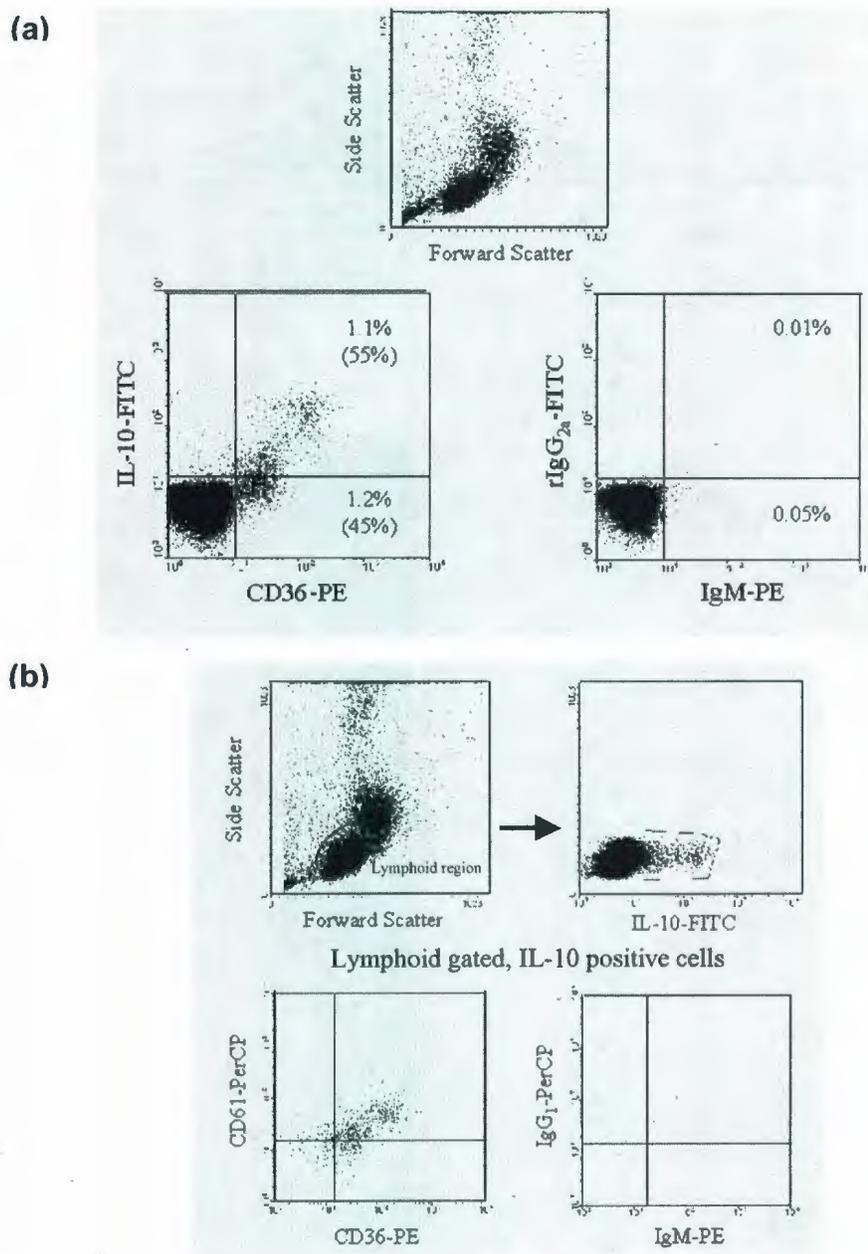


Figure 3.5. Relationship between IL-10 production, CD36 expression and CD61 expression on PBMC. (a) Cells within the lymphoid scatter region were analyzed for extracellular CD36 and intracellular IL-10. The lower right panel shows isotype controls. (b) Cells with lymphoid scatter expressing IL-10 were analyzed for CD36 and CD61 expression. The lower right panel shows isotype controls.

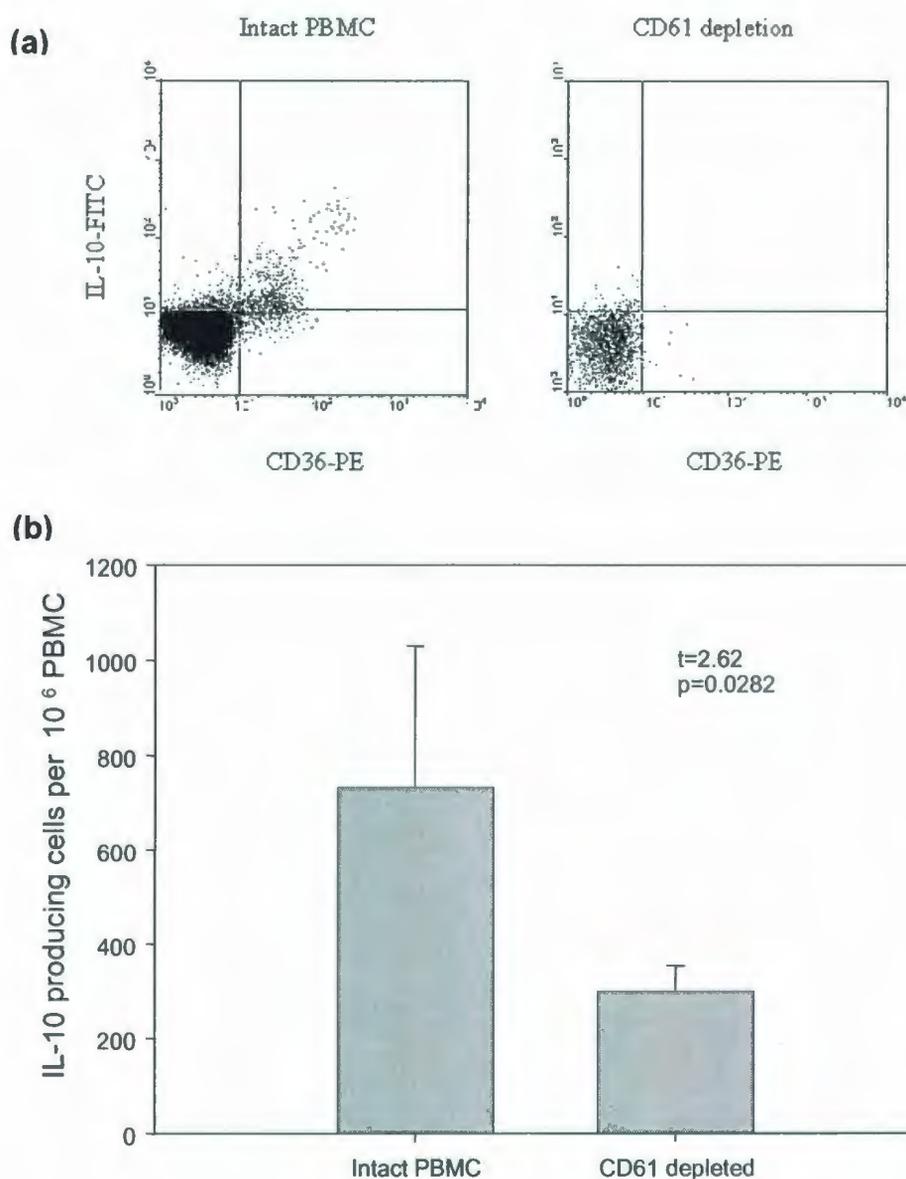


Figure 3.6. Comparison of IL-10 producing cells in PBMC before and after depletion of CD61⁺ cells. (a) Cells within the lymphoid scatter region were analyzed for extracellular CD36 and intracellular IL-10 with and without depletion of CD61⁺ cells. (b) The number of IL-10 secreting cells per 10^6 PBMC (n=6) was enumerated by ELISPOT before and after depletion of CD61⁺ cells.

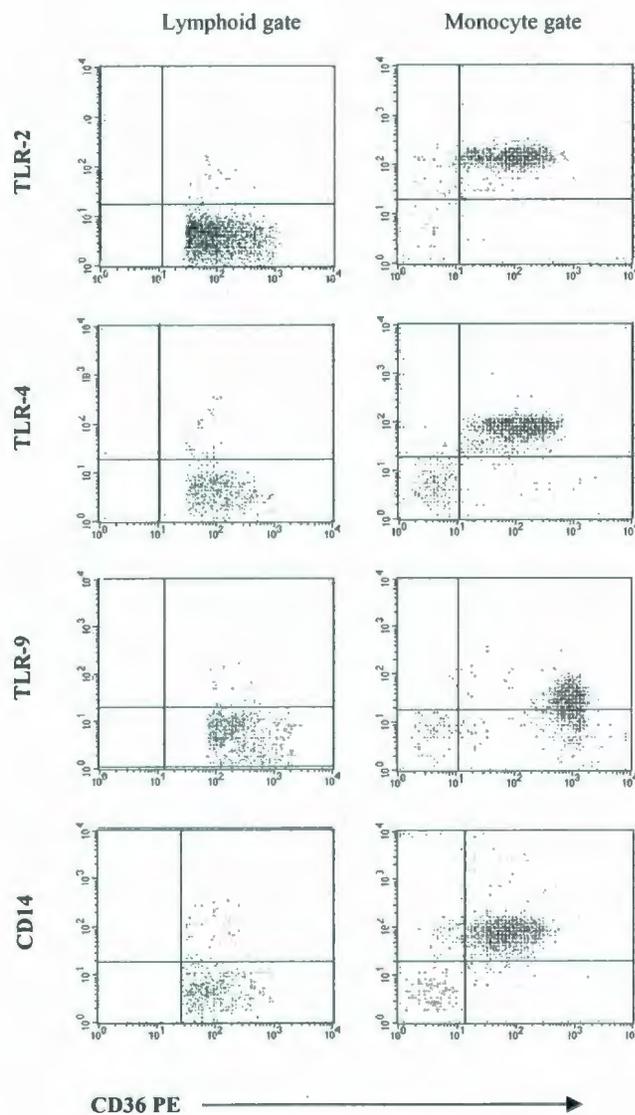


Figure 3.7. Comparison of expression of TLR 2, 4, 9 and CD14 on CD36⁺ monocytes and CD36⁺ cells with lymphoid scatter characteristics. Results shown are representative of those obtained with 12 individuals.

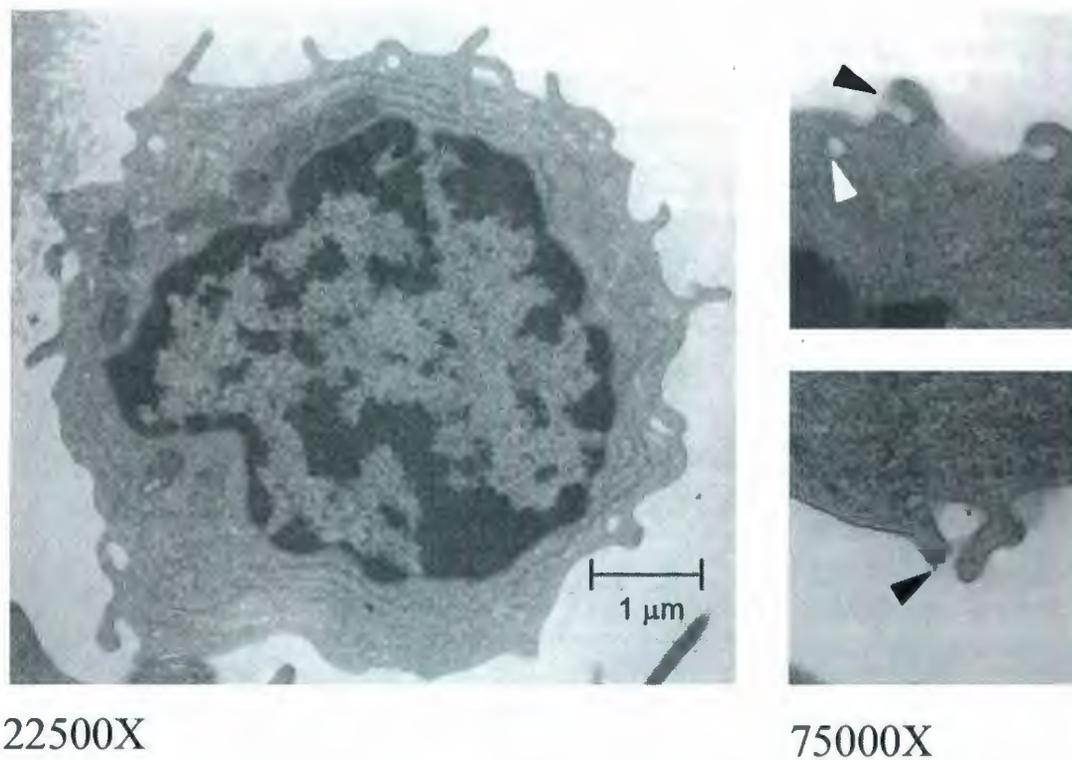


Figure 3.8. Electron micrograph of $CD36^+CD14^-$ cells. Freshly isolated PBMC were depleted of T cells, B cells, NK cells and monocytes, labelled with anti-CD36 followed by 12 nm gold particle conjugated goat anti-mouse IgM beads and then visualized by electron microscopy. Arrows on the right panels indicate gold beads associated with CD36.

CHAPTER 4**ENHANCED IL-10 PRODUCTION IN RESPONSE TO HEPATITIS C VIRUS
PROTEINS BY PERIPHERAL BLOOD MONONUCLEAR CELLS FROM
HUMAN IMMUNODEFICIENCY VIRUS-MONOINFECTED INDIVIDUALS**

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4.1. Abstract

Multiple immune evasion strategies by which HCV establishes chronic infection have been proposed, including manipulation of cytokine responses. Prior infection with HIV increases the likelihood of chronic HCV infection and accelerates development of HCV-related morbidity. Therefore, we investigated in vitro cytokine responses to HCV structural and non-structural proteins in peripheral blood mononuclear cells (PBMC) from uninfected, HIV-infected, HCV-infected and HIV/HCV-coinfected individuals. Intracellular flow cytometry was used to assess IL-2, IL-10, IL-12, and IFN- γ production by freshly isolated PBMC incubated for 16 hours with recombinant HCV core, non-structural protein 3 (NS3), and NS4 proteins. Anti-HCV cellular responses were assessed in HIV/HCV-coinfected individuals by ^3H -thymidine proliferation assay. Exposure to HCV antigens increased IL-10 production by PBMC, especially in uninfected and HIV-monoinfected individuals. This IL-10 response was attenuated in chronic HCV infection even with HCV/HIV-coinfection. The cells producing IL-10 in response to HCV proteins in vitro matched a PBMC subset recently shown to constitutively produce IL-10 in vivo. This subset was found at similar frequencies in uninfected, HIV-infected, HCV-infected and HIV/HCV-coinfected individuals before exposure to HCV proteins. HCV-specific T cell proliferation was detectable in only one HIV/HCV-coinfected individual who demonstrated no HCV-induced IL-10 response. This pattern suggests that selective induction of IL-10 in uninfected individuals and especially in HIV-monoinfected individuals plays a role in establishing chronic HCV infection and conversely, that

attenuation of this response, once chronic infection is established, favours development of hepatic immunopathology.

4.2. Introduction

Most viral infections induce cellular and humoral immune responses that act in concert to limit viral spread, clear infection and provide protective immunity against reinfection with the same virus. However, a number of viruses have evolved varied and sophisticated mechanisms to establish persistent infection, even in immunocompetent hosts (343-346). Epstein-Barr virus (EBV), for example, produces an interleukin-10-like virokinase that functionally mimics IL-10 in downregulating cellular immune responses (346).

Hepatitis C virus (HCV) causes significant morbidity and mortality. Approximately 80% of HCV-exposed individuals develop life-long infection and in many cases, progressive liver disease (55). Immune escape through mutations introduced by the error prone HCV RNA-dependent RNA polymerase is believed to play a major role in the establishment of persistent HCV infection as strong, broadly directed cellular responses during acute infection are associated with HCV clearance, while narrow, qualitatively impaired anti-HCV responses occur in chronic infection (199, 200, 202, 229, 347-349). Clearance of HCV after interferon-alpha (IFN- α) and ribavirin therapy is also associated with greater breadth and amplitude of anti-HCV cellular immunity (350, 351). These associations suggest that development of appropriate adaptive immune responses relates closely to HCV clearance.

The relatively small RNA genome of HCV encodes few proteins, all of which are involved in some way with virion structure or virus replication. Hence, any immunosuppressive activity must reflect secondary functions of the structural or non-

structural HCV proteins. Core, non-structural protein 3 (NS3), NS4 and NS5 have been investigated for a variety of secondary functions. The 20 kDa HCV core protein is associated with cellular transformation in vitro and modulation of cytokine signalling and cellular immune responses (294, 352, 353). The NS3 serine protease downregulates immunoproteasome activity in vitro and may impair immune recognition (354). NS4 is comprised of NS4A and NS4B, two proteins involved in viral replication. NS4A acts as a cofactor in forming the active protease NS4A/NS3 that participates in polyprotein processing during HCV replication (39), while NS4B is essential for replication and seems to affect the intracellular membranous web where viral replication occurs (355). NS5A inhibits antiviral PKR activity through its interferon sensitivity determining region (ISDR) (356). Thus, multiple HCV proteins appear to have evolved secondary functions affecting both innate and adaptive immune responses.

Viral infection generally triggers production of pro-inflammatory cytokines, but as described above, viruses have evolved gene products that promote persistent infection by tipping the cytokine balance towards immunosuppression. The setting in which viral exposure initially occurs is also relevant, therefore, we compared induction of cytokines by HCV proteins in PBMC from uninfected, HCV-infected, HIV-infected and HIV/HCV-coinfected individuals. Production of the immunosuppressive cytokine IL-10 was selectively triggered by HCV proteins, and the response was greater in HIV-monoinfected and uninfected individuals than in those exposed to HCV. This pattern suggests that selective induction of IL-10 in uninfected individuals and especially in HIV-monoinfected individuals plays a role in establishing chronic HCV infection and

conversely, that attenuation of this response, once chronic infection is established, favours development of hepatic immunopathology.

4.3. Materials and Methods

4.3.1. Study participants

HIV-infected and HIV/HCV-coinfected individuals were recruited from the St. John's General Hospital Infectious Disease Clinic, St. John's, NL, Canada. HCV-infected individuals were recruited from the Capital Health Queen Elizabeth Hospital Hepatitis Clinic in Halifax, NS, Canada. Ethics approval for this project was obtained from the Human Investigation Committee at each institution and all subjects provided informed written consent for blood collection and immunological studies. Uninfected individuals were recruited from Memorial University of Newfoundland Faculty of Medicine personnel.

4.3.2. Identification of HCV infection

HCV exposure was ascertained by testing for serum anti-HCV antibodies using second or third generation EIA assays from Ortho Diagnostics (Mississauga, ON). HIV status was determined by commercially available PCR testing by Abbott Diagnostics (Mississauga, ON).

To demonstrate the presence of HCV RNA, total nucleic acids were extracted from plasma samples, using NucliSens[®] Lysis Buffer and the NucliSens[®] Isolation Kit (Organon Teknika, Durham, NC). Briefly, 200 μ L of plasma was added to 1 mL of lysis buffer. Silica beads were added to bind free nucleic acids, followed by successive washes with 70% ethanol, 95% ethanol, and acetone. Nucleic acids were removed from the silica at 56°C with the elution buffer provided. Thirty μ L of eluant was frozen at -20°C if not used immediately for cDNA synthesis.

Complementary DNA (cDNA) was produced from isolated RNA using a first-strand cDNA synthesis kit (Amersham Biosciences, Baie d'Urfé, Québec). Eight μL of extracted nucleic acid was denatured at 65°C for 5 minutes and added to a reaction mix containing random hexamer primer, deoxyribonucleotides, and Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT). Reactions were placed at 37°C for 1 hour followed by MMLV-RT inactivation at 95°C for 5 minutes. cDNA was stored at -80°C until needed.

cDNA was amplified by polymerase chain reaction (PCR) with primers specific for the highly conserved HCV 5'-untranslated region. Primers and PCR conditions were modified from Shindo et al. (357) as follows: forward primer 5'-GGCGACACTCCACCATAGATC-3' and reverse primer 5'-GGTGCACGGTCTACGAGACCT-3'. The expected amplicon size was 324 bp. Final reactions included 20 mM TRIS-HCl buffer (pH 8.4), 50 mM KCl, 0.5 mM MgCl_2 , 0.025 U/ μL DNA polymerase (Invitrogen, Burlington, Ontario), and 0.5 μM MG18 and MG321 primers (Invitrogen, Burlington, Ontario) in 50 μL . Samples were initially denatured at 95°C for 2 minutes, followed by thirty cycles of amplification (95°C for one minute, 60°C for one minute, and 72°C for one minute). PCR products were analyzed on 2% agarose gels with ethidium bromide visualization and stored at -20°C .

4.3.3. PBMC isolation and *in vitro* stimulation

Acid citrate dextrose (ACD)-treated whole blood was obtained by venipuncture from each individual and PBMC were isolated by Ficoll-HyPaque Plus (GE Health Care, Baie d'Urfé, PQ, CA) density gradient centrifugation. Cells were washed, counted and

suspended at 1×10^6 /mL in lymphocyte medium (RPMI supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1% penicillin/streptomycin, and 2×10^{-5} M 2-mercaptoethanol; all from Invitrogen, Burlington, ON, CA).

To determine the effect of HCV proteins on PBMC from healthy and infected individuals, 1.5×10^6 freshly isolated PBMC were incubated in lymphocyte medium at 37°C for 16 hours with 10 µg/mL Brefeldin A (Sigma Chemical Co., St. Louis, MO, USA) and 2.5 µg/mL recombinant HCV core, NS3, and NS4 proteins (Virogen, Watertown, MA), 2.5 µg/mL recombinant HIV p24 from the same source (Virogen) or 2.5 µg/mL various control proteins. Initially, various concentrations (1, 2.5, 5, and 7.5 µg/mL) were tested in 3 individuals to determine if there was a dose response. All concentrations tested demonstrated results within 0.01% of each other in each individual (data not shown), and 2.5 µg/mL was used in subsequent studies as it was comparable to other studies of HCV proteins in human PBMC. The HCV antigens were expressed in *E. coli* as β-galactosidase fusion proteins, and supplied in a urea-based buffer, therefore, negative controls included β-galactosidase (Calbiochem, La Jolla, CA) in the same buffer as the viral antigens. Polymyxin B sulfate (PMB; Sigma) was added at 100 µg/mL to block any endotoxin-induced cytokine production as described previously (358). For some experiments, the lysosomal antigen processing pathway inhibitor chloroquine (Sigma) was added at 50 µM to PBMC 30 minutes before addition of HCV antigens to investigate whether antigen processing was involved in IL-10 induction. Cytokine production was assessed by intracellular flow cytometry.

4.3.4. *T cell depletion*

For some experiments, CD3⁺ T cells were removed from fresh PBMC by depletion with magnetic beads. Ten million fresh PBMC were washed and resuspended in cold depletion buffer (PBS supplemented with 5 mM EDTA and 0.5% BSA) at 3×10^6 /mL, and a 10:1 bead-to-target cell ratio with mouse anti-human CD3-coated magnetic beads (Dynal ASA, Lake Success, NY). Cells and beads were rotated together for 45 minutes at 4°C, and bead bound CD3⁺ cells were removed using a magnet. Depletion efficiency was >97% as determined by flow cytometry. The remaining cells were resuspended in lymphocyte medium and exposed to HCV and HIV proteins as described above.

4.3.5. *Flow cytometry*

Surface staining

PBMC subpopulations were phenotyped by surface and intracellular flow cytometry using the following antibodies: CD3-phycoerythrin (PE; Clone UCHT1, DakoCytomation, Mississauga, ON), CD4-fluorescein isothiocyanate (FITC; Clone RPA-T4, BD Pharmingen, San Diego, CA), CD8-peridinin chlorophyll (PerCP; Clone RPA-T8, BD Pharmingen), CD14-FITC or CD14-PE (Clone M5E2, BD Pharmingen), CD36-PE (Clone CB38, BD Pharmingen), CD19-FITC (Clone HIB19, BD Pharmingen), CD56-FITC (Clone HA58, BD Pharmingen) and CD61-PerCP (clone VI-PL2, BD Pharmingen). To each tube, IL-10 and CD36 were added, along with one other surface marker for each major cell type (e.g. CD36, IL-10, and CD3). Through this strategy, 3 color flow cytometry was used to identify the cell population of interest. All steps were performed

at 4°C, and incubations were carried out in the dark to prevent fluorochrome photobleaching. Approximately 5×10^5 PBMC were washed with flow cytometry buffer (PBS supplemented with 0.1% bovine serum albumin (BSA), 5 mM EDTA and 0.02% sodium azide, all from Sigma), resuspended in 600 μ L and incubated for 20 minutes with 0.5 μ g antibody against surface antigens or appropriate isotype controls. Cells were washed, fixed with 0.5 mL 1% paraformaldehyde (Sigma) for 20 minutes, resuspended in 250 μ L of 1% paraformaldehyde, and stored at 4°C until collection of at least 100,000 events on a FACScan flow cytometer (Becton Dickinson). CellQuest Pro™ and WinMDI™ were used for data analysis.

Intracellular staining

Cells were stained as above for surface antigens with an additional permeabilization step after fixation. Cells were incubated with either 0.5 mL 0.2% saponin (Sigma) in PBS or DAKO permeabilization reagent and anti-IL-10 FITC (clone JES9D7 Caltag, Burlingame, CA), anti-IFN- γ FITC, (clone B27, Caltag), anti-IL-2 FITC, (clone MQ1-17H12 Caltag) anti-IL-12 FITC (clone C8.6 Caltag) antibodies or appropriate isotype controls for 20 minutes at 4°C in the dark. After washing with 3 mL flow buffer, cells were resuspended in 1% paraformaldehyde until analysis.

Total PBMC were incubated for 16 hours with either 5 μ g/mL phytohemagglutinin, 10 nM phorbol 12-myristate 13-acetate (PMA)/100 nM ionomycin, or 200 ng/mL lipopolysaccharide to provide positive controls for IFN- γ , IL-2, IL-10, and IL-12 production, respectively. Brefeldin A was added one hour after the various

stimulants. Lymphoid cells were gated and stained for CD8 and IFN- γ production, total PBMC for IL-2 production, and monocytes were gated for IL-12 and IL-10 production.

4.3.6. Proliferation assays

Cellular immune responses were measured by standard 5 day thymidine incorporation assay. PBMC were washed twice with proliferation medium (lymphocyte medium with 10% human AB serum (Atlanta Biologicals, lot number M0102) substituted for FBS) and 1×10^5 cells/well were incubated in triplicate with either plain medium, 5 $\mu\text{g}/\text{mL}$ phytohemagglutinin (ICN Biomedicals Inc., Costa Mesa, CA), 2.5 $\mu\text{g}/\text{mL}$ *Candida albicans* (Greer Laboratories Inc., Lenoir, NC), or 2 $\mu\text{g}/\text{mL}$ HCV core, NS3, NS4 or HIV p24 (all Virogen) for 5 days. One μCi of tritiated (^3H)-thymidine (Perkin Elmer Life Sciences, Boston, MA) was added to each well on day 4, and the assay was harvested onto glass fiber mats 18 hours later using a semi-automated harvester (Tomtec Harvester 96 Mach M III, Hamden, CT). Incorporated ^3H -thymidine was measured by a 96 well scintillation counter (TopCount, Packard, Meriden, CT). Stimulation indices were calculated as follows:

Stimulation Index = $\frac{\text{cpm}_{\text{antigen}}}{\text{cpm}_{\text{background}}}$

Background counts were defined as the number of cpm in the wells containing only medium.

4.3.7. Statistical analyses

All statistical analyses were performed using SPSS version 9 (SPSS Inc., Chicago, IL). In Figure 4.2, the group sizes were small and unequal. Therefore, nonparametric tests were used to compare means. Within a group, the Mann-Whitney U test was used, while the Kruskal-Wallis test was used to test for differences between groups, as there were greater than 3 groups. Neither test depends on the data distribution for calculation, and the Kruskal-Wallis for multiple comparisons adjusts the alpha value to avoid inflation of type I error. The Fisher's exact test was used to compare the categorical data. All graphs indicate mean values and error bars represent standard error of the mean.

4.4. Results

4.4.1. Study participants

Eight healthy volunteers from St. John's NL, Canada provided blood samples for the study. Twenty-four HIV-infected, 10 HIV/HCV-coinfected, and 3 HCV-infected individuals were also recruited, and Table 4.1 describes the baseline characteristics of the infected groups. The age and sex of participants was similar across groups. Most individuals exposed to HCV developed chronic infection, although 2 of the HIV/HCV-coinfected individuals had cleared the virus and were anti-HCV antibody positive but HCV RNA negative. HIV related clinical parameters were similar between groups, although there was a statistically nonsignificant trend toward higher CD4⁺ T cell counts in the coinfected group. Most HIV-infected individuals were on antiviral therapy and had achieved viral suppression at the time of participation. Only one of the HIV/HCV-coinfected individuals had been treated (unsuccessfully) with HCV antiviral therapy, and the remainder of HCV exposed patients were untreated.

4.4.2. Exposure to HCV proteins induces IL-10 production in PBMC

To investigate the cytokine response of PBMC from different groups of individuals to HCV proteins, we incubated PBMC from uninfected, HIV-infected, HCV-infected and HIV/HCV-coinfected individuals for 16 hours with HCV core, NS3 and NS4 proteins. Production of IL-2, IL-12, IFN- γ and IL-10 was then assessed by intracellular flow cytometry. In no case did any of the HCV antigens, individually or together, induce IL-2, IL-10, IFN- γ , or IL-12 production (Figure 4.1a). Positive controls for each of these four cytokines demonstrated the efficacy of the antibodies (Figure 4.1b). However, IL-

$10^+CD3^-CD19^-CD14^-CD36^+CD61^+$ cells with lymphoid light scatter characteristics increased in number following 16 hour exposure to HCV proteins (Figure 4.2a, dark bars). We previously described these IL-10⁺ cells in freshly-isolated PBMC from uninfected individuals, but found that their number fell to almost undetectable levels following 16 hour incubation in unsupplemented lymphocyte medium (359). All of the individuals in this study (uninfected n=6, HIV-infected n=24, HIV/HCV-coinfected n=10, HCV monoinfected n=3) had circulating CD36⁺CD61⁺ PBMC constitutively producing IL-10 (Figure 4.2a, black bars). The average percentage of PBMC producing IL-10 ex vivo ranged from 0.37% (range 0.22-0.6%) in the HCV-infected group to 0.75% (range 0.2-1.2%) in the uninfected group. Although there was a trend towards a lower frequency of these cells in the HIV-infected, HCV-infected and HIV/HCV-coinfected groups, there was no statistically significant difference (Figure 4.2a). As previously described, in the absence of stimulation, the percentage of PBMC producing IL-10 in vitro decreased over time in infected and uninfected individuals (Figure 4.2a, light grey bars). The uninfected group had the highest frequency of IL-10 producing cells ex vivo (Figure 4.2a, black bars), however, after 16 hours incubation in lymphocyte medium alone, IL-10⁺ cell frequency fell to similar levels in all groups (Figure 4.2a, light grey bars). The mean frequencies were slightly lower in the uninfected and HCV-infected groups compared to the HIV-infected and HIV/HCV-coinfected groups (Figure 4.2a, light grey bars, 0.12% and 0.12% versus 0.25%, and 0.24% respectively) but not significantly different. The pattern of IL-10 production in response to HCV proteins differed substantially between groups (Figure 4.2a, dark grey bars). After 16 hour

exposure to HCV proteins, the average frequency of IL-10⁺ cells rose to 1.63% (n=12; range 0.1% to 6.6%) in PBMC from a subgroup of HIV-infected individuals, 1.02% (n=6; range 0.02% to 3%) in uninfected individuals, 0.27% (n=10; range 0.01% to 1.56%) in HIV/HCV-coinfected individuals, and 0.18% (n=3; range 0.06% to 0.26%) in HCV-infected individuals. The number of IL-10⁺ cells detected by flow cytometry following 16 hour exposure to the HCV proteins was significantly higher in the HIV-infected group compared to the HCV-infected (H=33, p=0.03) and HIV/HCV coinfecting (H=15.5, p=0.001) groups (Figure 4.2a). The effect of 16 hour exposure to HCV proteins on the number of IL-10⁺ PBMC was substantial (5-10 fold increase) in HIV-infected and uninfected control groups, while the increase in HCV-infected individuals, with or without HIV coinfection, was marginal (Figure 4.2a). If all the chronic HCV-infected individuals from the HIV/HCV and HCV monoinfected groups are combined into one group (n=13), there is still a significant difference between IL-10 induction in the HIV monoinfected group and the combined HCV chronic infection group (p=0.016). IL-10 induction when HCV proteins were added together or individually was not different (Figure 4.2b).

There were two clear populations of IL-10 positive cells based on the level of CD36 expression. Approximately 30% of the IL-10 positive cells had a CD36 mean channel fluorescence (MCF) greater than 100, and the remainder were between 10 and 100 (Figure 4.3a). Cells with higher CD36 mean channel fluorescence (MCF) were more likely to be IL-10 positive, with 100% of CD36^{hi} cells IL-10⁺ versus 50-70% CD36^{low} cells IL-10⁺ in various individuals (Figure 4.3a). There is a statistically significant

difference in the mean channel fluorescence of the CD36^{lo}IL-10^{lo} group compared to the CD36^{hi}IL-10^{hi} group (Figure 4.3b; Fisher's exact test, $p < 0.000$). This association raises the possibility of a mechanistic link between high CD36 expression and IL-10 production.

4.4.3. IL-10 induction is specific to HCV proteins, and independent of T cells and lysosomal antigen processing

To determine whether induction of IL-10 was selective for HCV proteins, we also exposed PBMC from 5 uninfected and 5 HIV-infected individuals to HIV p24 protein. Unlike the HCV proteins, HIV p24 did not increase IL-10 production in PBMC relative to the β -galactosidase controls (Figure 4.4a, black bars). Therefore, HCV core, NS3 and NS4 selectively induce IL-10 production in PBMC. To confirm that IL-10 induction did not result from endotoxin contamination of the HCV antigen preparations, we incubated PBMC from 5 uninfected and 3 HIV/HCV coinfecting individuals with HCV proteins in the presence of polymyxin B, an endotoxin blocker. Inclusion of polymyxin B in the assays had no effect on induction of IL-10 in HCV-exposed PBMC (Figure 4.4a, light grey bars). To test whether antigen processing of the HCV proteins was required for induction of IL-10, we inhibited the lysosomal antigen processing pathway with chloroquine in PBMC from uninfected, HIV-infected and HIV/HCV-coinfecting individuals exposed to HCV proteins ($n=3$, $n=4$, $n=4$ respectively). Chloroquine treatment did not significantly affect the IL-10⁺ cell frequency in PBMC from uninfected, HIV-infected or HIV/HCV-coinfecting groups exposed to HCV proteins (Figure 4.4b, grey bars with diagonal hatch).

Since the HCV proteins had the greatest effect on IL-10 induction in PBMC from HIV-infected individuals and reduced CD4⁺ and total T cell numbers is an integral aspect of HIV infection, we decided to test if the absence of T cells affected the IL-10 response to HCV proteins. Therefore, we depleted CD3⁺ T cells from the PBMC of 2 individuals in each of the HIV-infected and HIV/HCV-coinfected groups (Figure 4.4b, horizontal hatch bars) and assessed IL-10 production with and without exposure to HCV proteins (white or grey background bars respectively). The percentage of PBMC producing IL-10 constitutively or in response to HCV proteins was essentially unaffected by T cell depletion after correction for T cell depletion.

4.4.4. Relationship between IL-10 induction and HCV-specific T cell proliferation

Since IL-10 is an immunomodulatory cytokine that suppresses Th1 type adaptive immunity, we investigated the relationship between IL-10 induction by HCV proteins and HCV-specific T cell proliferation in 10 HIV/HCV-coinfected individuals. A positive HCV-specific proliferation response (stimulation index greater than or equal to 3) was observed in only one individual (018, Figure 4.5a, black bar), who demonstrated a positive response to HCV core but not NS3 and NS4. After 16 hour exposure to HCV antigens, 6 of the 10 coinfecting individuals tested demonstrated an HCV protein-induced increase in the number of IL-10⁺ cells (grey bars, Figure 4.5b). Of note, subject 018 was one of the four individuals who did not have increased numbers of IL-10⁺ cells following exposure to HCV proteins.

4.5. Discussion

In this study, we demonstrated that HCV core, NS3 and NS4 proteins specifically and selectively induce IL-10 production when incubated for 16 hours with PBMC. The cells producing IL-10 in response to HCV proteins were identified by flow cytometry as CD14⁻CD36⁺ with lymphoid light scatter characteristics. We previously reported constitutive ex vivo production of IL-10 by this PBMC subset and that this production rapidly stops in vitro (359). These cells do not fit into one of the major subpopulations of circulating immune cells, but can be reliably isolated from all healthy individuals tested at a frequency of up to 1%. The ex vivo frequency of IL-10-producing PBMC was generally equivalent in the four distinct groups we studied, indicating that the differences seen after 16 hour culture were caused by differential responses to the HCV proteins. While 16 hour culture with HCV proteins increased the number of IL-10-producing cells in all groups, the frequency of IL-10⁺ cells was higher in PBMC from HIV-infected individuals and uninfected controls compared to PBMC from HIV/HCV coinfecting individuals or HCV-infected individuals. This demonstrates a propensity for HCV-induced IL-10 production by PBMC from uninfected individuals that is exaggerated by HIV infection and attenuated by chronic HCV infection, even in the setting of HIV coinfection.

We used recombinant HCV core, NS3 and NS4 to investigate cytokine production in response to HCV proteins. Although these three proteins have little sequence or structural similarity and their known functions in HCV replication are very different (360, 361), we found that, collectively or individually, HCV core, NS3 and NS4 proteins

induced IL-10 production in CD14⁺CD36⁺ PBMC. Others have also reported that recombinant HCV core, NS3 and NS4 proteins all induce IL-10 production (294, 295, 362). However, in these studies, monocytes were identified as the responding cells and, in direct contrast to our results, there was a greater effect on monocytes from HCV-infected individuals than on monocytes from uninfected controls (294, 363). These studies utilized monocytes isolated by overnight adherence to plastic, rather than freshly isolated PBMC. Despite varying protocols and different characteristics of the responding cells, the central point is that multiple distinct HCV proteins stimulate IL-10 production by PBMC. There are other examples of redundancy in HCV protein effects, as mice transgenic for hepatic HCV structural or non-structural polyproteins develop hepatic steatosis characteristic of chronic HCV infection (364). Such functional redundancy of viral proteins is not unique to HCV, as three different HIV proteins (nef, tat and vpr) have all been reported to induce IL-10 production by various PBMC subsets (365-369).

In our study, structural and non-structural HCV proteins induced IL-10. Having both structural and non-structural proteins with redundant immunosuppressive functions may be important in sustaining infection. The availability of these proteins in circulation is temporally separated, and not concurrent. Non-structural proteins are only expressed in actively infected cells, while the core protein can be found in plasma at moderately high levels throughout chronic infection (370, 371). Having multiple proteins that induce IL-10 supports production of an immunosuppressive cytokine whether or not HCV replication is upregulated or infectious virions are present.

In parallel with the redundancy in HCV protein-induced IL-10 production, additional HCV escape mechanisms within both the innate and adaptive immune system have been extensively described. Subversion of the eukaryotic anti-viral interferon-inducible system is a potent evasion strategy employed by many viruses, and several HCV proteins suppress host proteins in this system. HCV E2 inactivates PKR (372), and the HCV serine protease NS3/4A causes proteolysis of Toll-IL-1 receptor domain-containing adaptor inducing IFN- β (TRIF) (242), and inhibits phosphorylation of IRF-3, preventing nuclear translocation and IFN- β gene activation (241). HCV E2 has also been associated with decreased NK cell function and HCV core stabilizes HLA-E expression and subsequently downregulates NK-mediated cytotoxicity (300, 301, 373). HCV core, E1, NS3, and NS4 are all associated with impaired dendritic cell maturation, thus potentially modulating the function of this integral link to adaptive cellular immunity (294, 363, 374). HCV core also downregulates in vitro anti-HCV T cell responses (298, 353, 375). While the exact mechanism of HCV protein interaction is unclear, IL-10 inhibits dendritic cell maturation, resulting in an immature phenotype similar to that seen after exposure to HCV core (376). Not only do HCV proteins modulate immune responses, it is clear that more than one protein has evolved to share similar functions. Production of IL-10 by the cell population described in this paper may stunt dendritic cell maturation, and impair development of anti-HCV cellular immunity through this pathway as well as through a direct effect on responding T cells.

The mechanism for IL-10 induction in PBMC by HCV proteins is unknown, but processing of soluble HCV antigens through the endocytic pathway is unnecessary as

blocking antigen processing had no effect on constitutive or induced IL-10 production. A higher percentage of cells from HIV-infected individuals than uninfected controls produced IL-10 in response to HCV proteins. This appears unrelated to lower T cell function in HIV infection as depletion of CD3⁺ PBMC also did not affect constitutive or HCV-induced IL-10 production by the CD14⁻CD36⁺ PBMC.

The direct correlation between intracellular IL-10 production and intensity of cell surface CD36 expression supports a possible connection between these molecules. Ligands of CD36, a scavenger receptor, include oxidized low density lipoproteins (oxLDL), thrombospondin, collagen types I and IV, apoptotic cells, long chain fatty acids and Plasmodium-infected erythrocytes (377-385). At least 2 of these ligands, thrombospondin and apoptotic cells, have immunosuppressive or anti-inflammatory effects (334, 386). In addition, peroxisomal proliferator associated receptor- γ (PPAR- γ) mediated differentiation of monocytes towards a less inflammatory status is accompanied by upregulation of CD36, which has been used to clinical advantage in cerebral malaria (387).

While there is circumstantial evidence for a link between CD36 and induction of IL-10, there has been no reported evidence of interactions between HCV or any of its individual components and CD36. HCV associates with low density lipoproteins in circulation, and uses the LDL receptor and CD81 to enter cells (21). CD36 is not an HCV cellular receptor however, recent studies suggest that HCV and the CD36 ligand oxLDL do interact (388). HCV proteins may indirectly activate IL-10 production through a CD36 related mechanism.

IL-10 is an important immunomodulatory cytokine, and recent experiments in the LCMV model of chronic viral infection showed that manipulating levels of IL-10 alone was sufficient to dictate LCMV clearance or persistence (389). However, studies comparing plasma IL-10 levels in HCV-infected and healthy individuals have been less definitive, with some studies describing control levels of serum IL-10 in chronic HCV infection, while other groups describe a marked increase in IL-10 in chronic infection (390-392). This raises questions about the value of serum IL-10 measurements, as IL-10 has important localized paracrine effects that are not reflected by these generalized IL-10 determinations. As well, none of these studies determine IL-10 levels during acute infection when it may be pivotal in the establishment of chronic infection. We demonstrated that exposure to HCV antigens in chronically infected individuals produced less IL-10 than seen in HCV naïve individuals. Longer term exposure to HCV antigens apparently conditions the CD36⁺ population of IL-10⁺ cells, attenuating HCV protein specific IL-10 induction. Thus, IL-10 levels during acute infection may be more relevant to the establishment of persistent infection than to maintaining chronic infection.

The transience of IL-10 responses to HCV proteins may allow emergence of cellular immune responses that are pathological in the context of chronic HCV infection. Several studies indicate a protective role for IL-10 in the immunopathology of HCV infection including a clinical trial of exogenous IL-10 administration, accumulation of IL-10 producing CD5⁺ B cells in HCV-infected individuals with mild disease and an association between high IL-10 gene polymorphism and decreased rates of HCV-related liver fibrosis (130, 393-395). The diminution in IL-10 induction seen in chronic HCV

infection after HCV protein exposure appears to parallel, and possibly support, insidiously progressive liver disease.

Coinfection with HIV is an important issue in HCV infection because of its relatively high prevalence, higher HCV virus loads in HIV/HCV coinfecting individuals and because of increased HCV-related morbidity in HIV coinfecting individuals (396-398). The higher level of IL-10 induction in PBMC from HIV-infected individuals may allow the establishment of more aggressive HCV infection, with downregulation of anti-HCV responses permitting increased HCV replication and higher HCV virus loads. However, this could depend on the order of HCV and HIV infection, with stronger anti-HCV cellular immunity developing when HCV infection precedes HIV-associated CD8⁺ T cell activation during the asymptomatic phase of HIV infection. Enhanced CD8⁺ T cell immunity against HCV in HCV/HIV coinfecting individuals is likely to accelerate liver disease, while those who acquire HCV following the generalized decline in cellular immunity associated with progressive HIV infection are likely to have especially weak HCV-specific cellular immunity. Thus, there are 2 distinct groups of HIV/HCV coinfecting individuals with respect to HCV-specific cellular immunity and its potential role in viral suppression and in the pathogenesis of liver disease.

Chronic HCV infection mitigates IL-10 production induced by HCV proteins. This is true in the group of three HCV mono-infected individuals, as well as the 10 HIV/HCV coinfecting people. Though the mono-infected group is small, the two groups with chronic HCV infection have a total of thirteen individuals, and both have similar results. The limited IL-10 response is even more marked considering that the HIV

monoinfected group has the highest IL-10 induction of all groups. Therefore, the lack of IL-10 induction in the setting of chronic infection is likely not an artifact of the small sample size, but a real phenomenon that can even supplant the expected HIV-associated IL-10 induction. The mechanism is unclear, and perhaps reflects an 'exhaustion' of this cell type in the context of chronic stimulation.

Consistent with previous reports, we found that HCV-specific T cell proliferation is rare in chronic HCV infection with HIV coinfection (284, 399). In fact, the only individual with anti-HCV proliferative responses is also one of the individuals with the least number of IL-10 producing cells after HCV protein exposure.

We have demonstrated the presence of a novel population of PBMC that act differentially in response to HCV proteins in uninfected and HIV- or HCV-infected individuals. This differential IL-10 response to HCV antigens is consistent with the generation of an immunoregulatory environment that promotes viral persistence in acute exposure while allowing disease progression in the chronic state. The behaviour of this CD36⁺CD14⁻ population offers insight into how HCV establishes viral persistence through decreased cellular immune responses and how this is exacerbated by HIV infection.

4.6. Acknowledgements

We greatly appreciate the participation of clients from the Infectious Diseases Clinic in St. John's NL as well as that of clients of the Hepatitis Clinic in Halifax, NS. Thanks to Ian Bradbury for helpful discussion on the statistical treatments, Jessica Rose (PHA and PMA/ionomycin) and Chris Little (IL-12 and IL-10) for assistance with the positive cytokine controls. This research was supported by the Canadian Institutes of Health Research (CIHR) and Health Canada through an operating grant to MG (EOP41541) and a CIHR MD/PhD scholarship awarded to LB.

Table 4.1. Patient characteristics

	HIV	HIV/HCV	Chronic HCV
Number (n)	24	10	3
Age (yrs±SD)	41.7±7.0	39.2±3.85	49±15
Sex (%)			
M	75	80	66.7
F	25	20	33.3
HCV Genotype (%)			
1	N/A	100	0
Non-1	N/A	0	0
Unknown	N/A	0	100
HCV status (%)			
Chronic infection (HCV RNA +)	N/A	80	100
Clearance (HCV RNA -)	N/A	20	0
HCV Risk Factor (%)			
IVDU	N/A	30	66.7
Transfusion	N/A	20	33.3
Tattoo	N/A	0	0
Endemic area	N/A	0	0

Table 4.1. (con't)

	HIV	HIV/HCV	Chronic HCV
Unknown	N/A	50	0
Estimated duration of infection (yrs±SD)¹			
HIV	18±5.1	10±2.1	N/A
HCV	N/A	18±3.4	24±8
Antiviral therapy (%)			
HIV	58	90	N/A
HCV	N/A	0	0
Suppressed viral load² (%)			
HIV	75	70	N/A
HCV	N/A	20	Unk.
CD4⁺ T cell count³ (median cells/μL, interquartile range)			
	409, 422	608, 80	N/A
CD4⁺ T cell nadir⁴ (median cells/μL, interquartile range)			
	252, 296	247, 350	N/A

Abbreviations: Avg average; N/A not applicable; SD standard deviation; Unk. unknown.

¹The time since clinically confirmed infection or, in the case of HCV, high risk activity.

²Undetectable viral load by Roche Amplicor RT-PCR test for HIV and in house PCR for HCV at the time of participation. This also includes those who have spontaneously cleared the virus in the case of HCV.

³Measured on the day of participation. ⁴Lowest documented count over the course of followup.

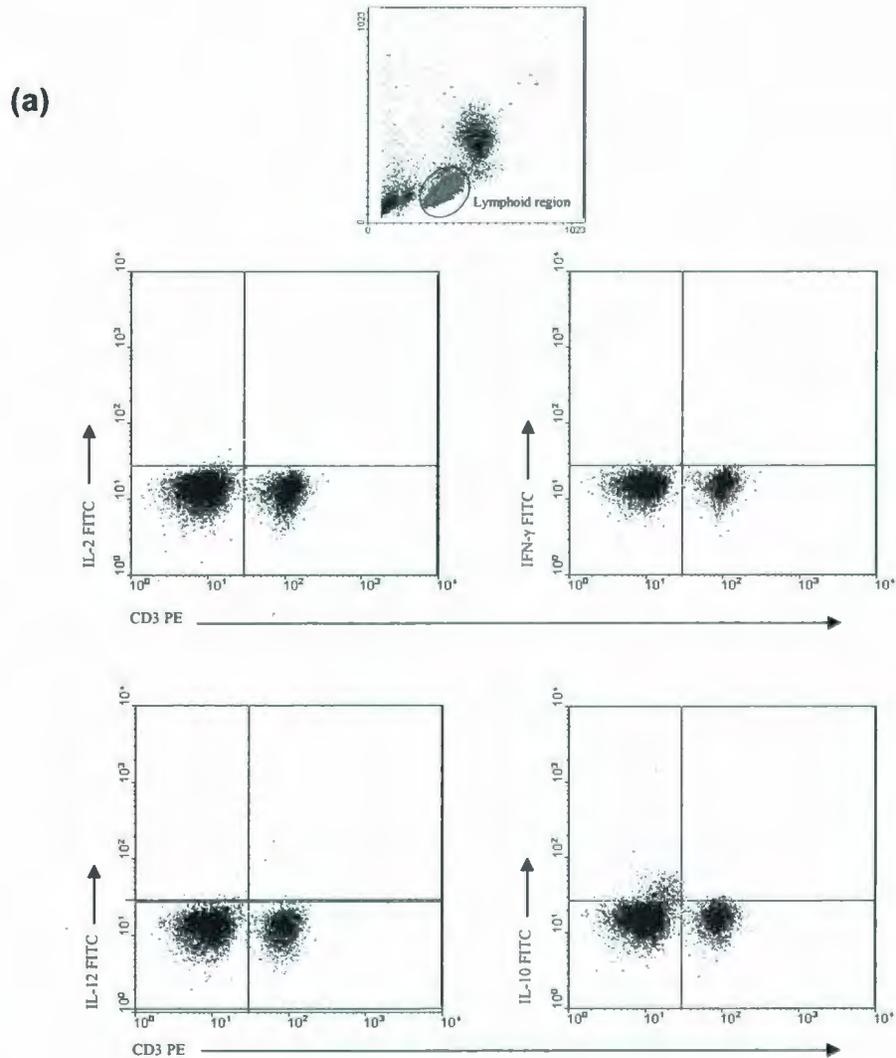


Figure 4.1. Cytokine production following 16 hour exposure of PBMC to HCV proteins. (a) Freshly isolated PBMC from HIV-infected, HCV-infected, HIV/HCV coinfecting and uninfected controls were incubated for 16 hours with HCV proteins, then analyzed by intracellular flow cytometry for cytokine production as shown in representative plots. Selective IL-10 production by a small subset of CD3⁺ PBMC with lymphoid scatter characteristics occurred in each case.

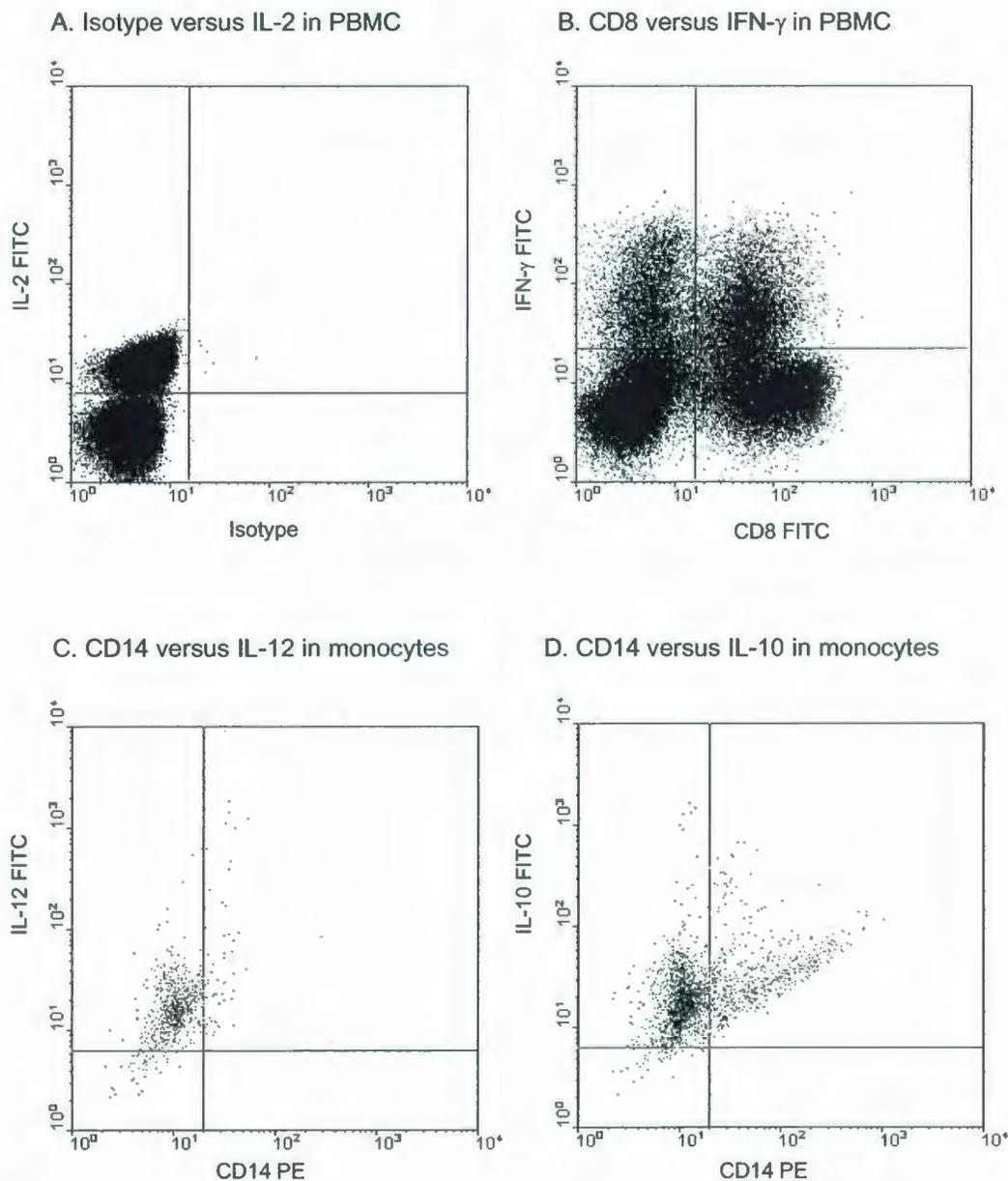


Figure 4.1. (con't) (b) Positive controls for cytokine production. (A) IL-2 production in total PBMC after PMA/ionomycin treatment. (B) IFN- γ production by CD8⁺ and CD8⁻ lymphoid gated PBMC after phytohemagglutinin treatment. (C) IL-12 production in CD14⁺ cells after LPS stimulation. (D) IL-10 production in CD14⁺ cells after LPS stimulation.

(a)

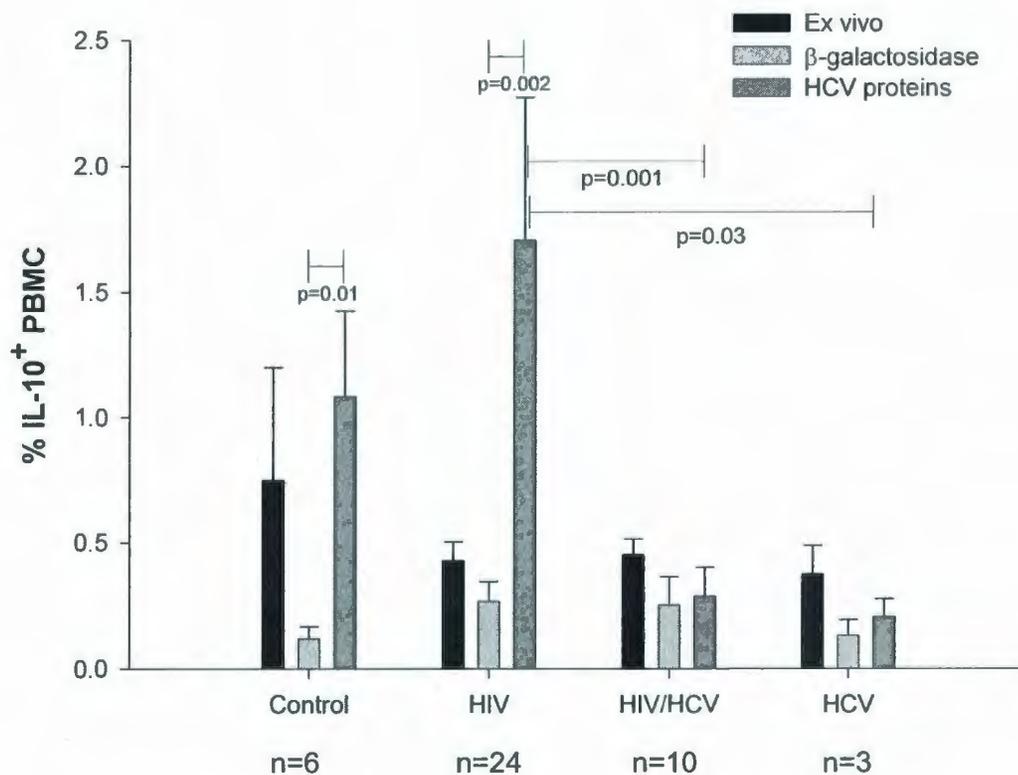


Figure 4.2. Mean percentage of PBMC from different groups expressing IL-10 ex vivo and following 16 hour incubation with HCV proteins. (a) The percentage of IL-10 producing cells (mean±SE) was assessed by intracellular flow cytometry in freshly isolated PBMC and following 16 hour incubation with HCV proteins. Black bars represent fresh PBMC, dark grey bars represent PBMC incubated for 16 hours with recombinant HCV core, NS3 and NS4 and lighter grey bars represent the same PBMC incubated for 16 hours with β-galactosidase control protein. Statistical comparisons are made within groups using the Mann-Whitney U test and between groups using the Kruskal-Wallis test.

(b)

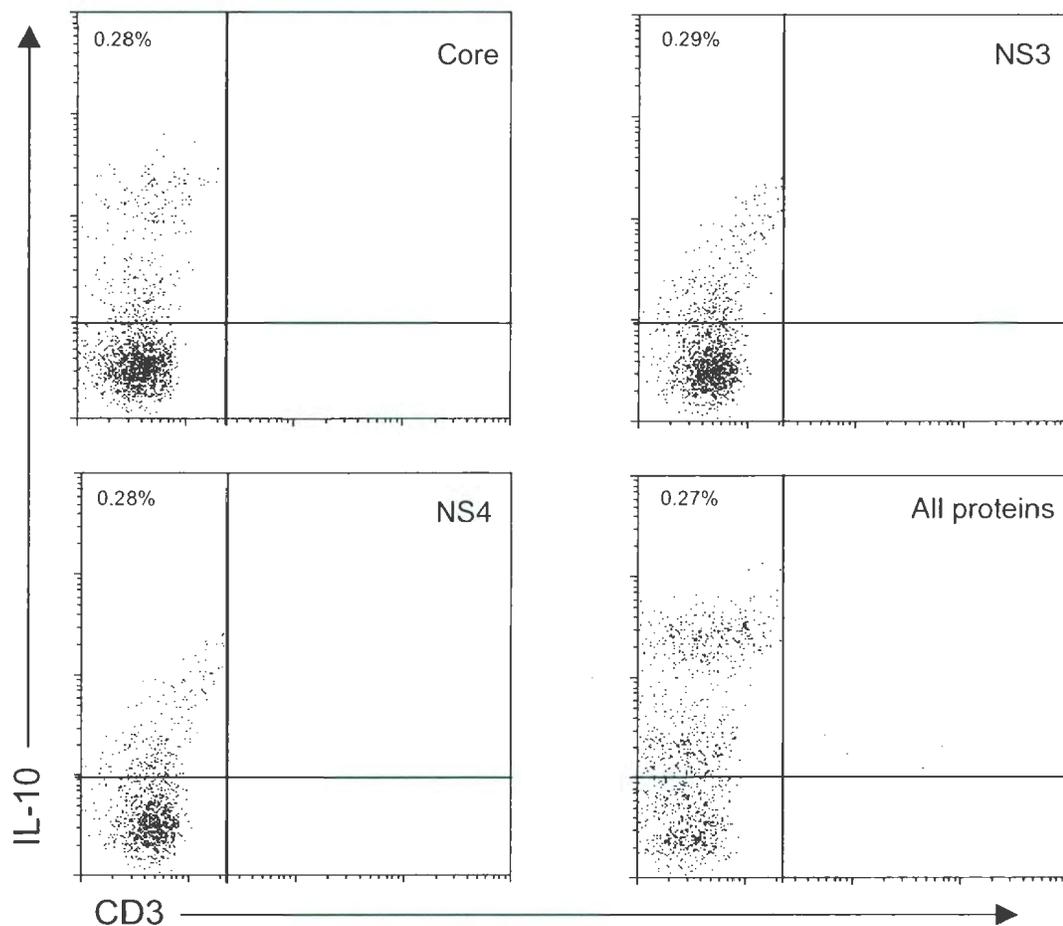


Figure 4.2. (con't) (b) Representative flow plots of IL-10 production in PBMC from an HIV/HCV-coinfected individual after incubation of PBMC with individual HCV proteins at 2.5 $\mu\text{g}/\text{mL}$. Numbers represent the percentage of IL-10 positive PBMC.

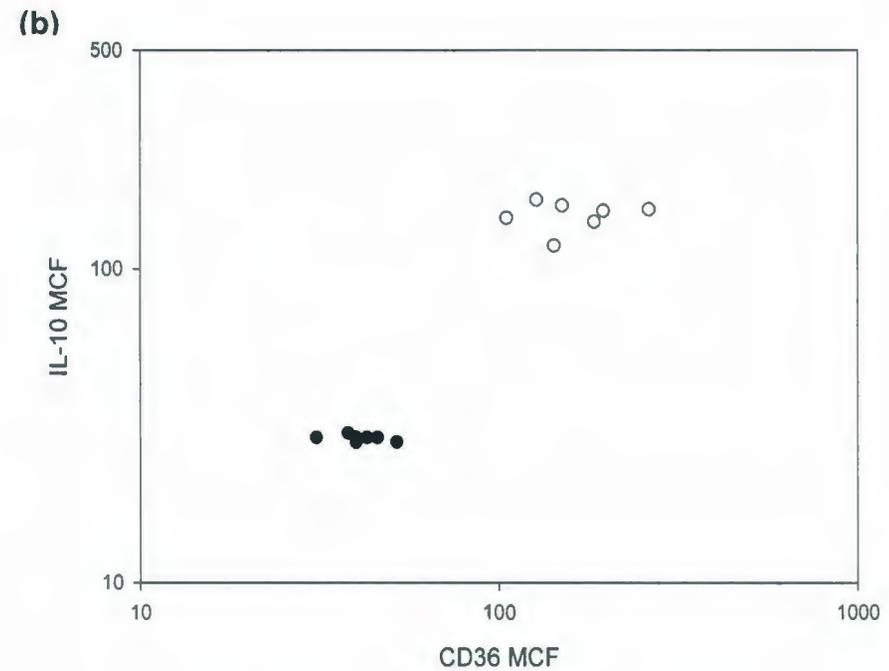
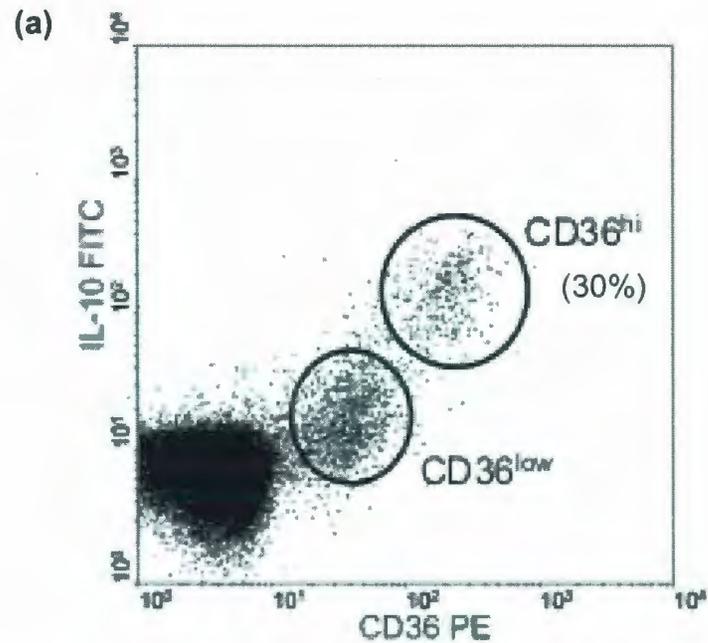


Figure 4.3. Relationship between CD36 expression and ex vivo IL-10 production by PBMC. (a) Representative flow cytometry plot showing IL-10 production in freshly-isolated CD36⁺ PBMC cells with lymphoid light scatter characteristics. The percentage of CD36⁺ cells that are IL-10^{hi} is indicated. (b) Mean channel fluorescence (MCF) for CD36 plotted against IL-10 MCF for seven individuals. Open circles represent the CD36^{hi} subset and dark circles represent the CD36^{low} subset as shown in Figure 4.3a.

(a)

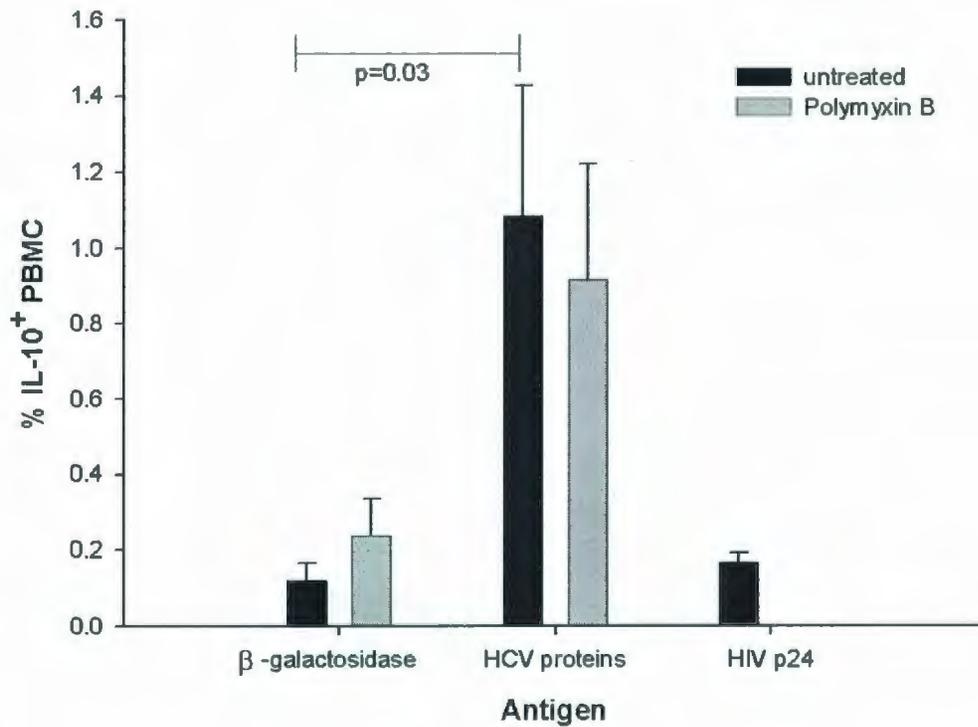


Figure 4.4. Effect of polymyxin B, chloroquine and T cell depletion on IL-10 production by PBMC exposed to recombinant HCV proteins. (a) Cells from uninfected individuals (n=5) were pretreated with polymyxin B (grey bars) before incubation with recombinant HCV proteins to block the effects of any endotoxin contamination of the recombinant proteins. The effect of 16 hour exposure to recombinant HIV p24 on IL-10 induction was also assessed, but incubation with polymyxin B was not performed.

(b)

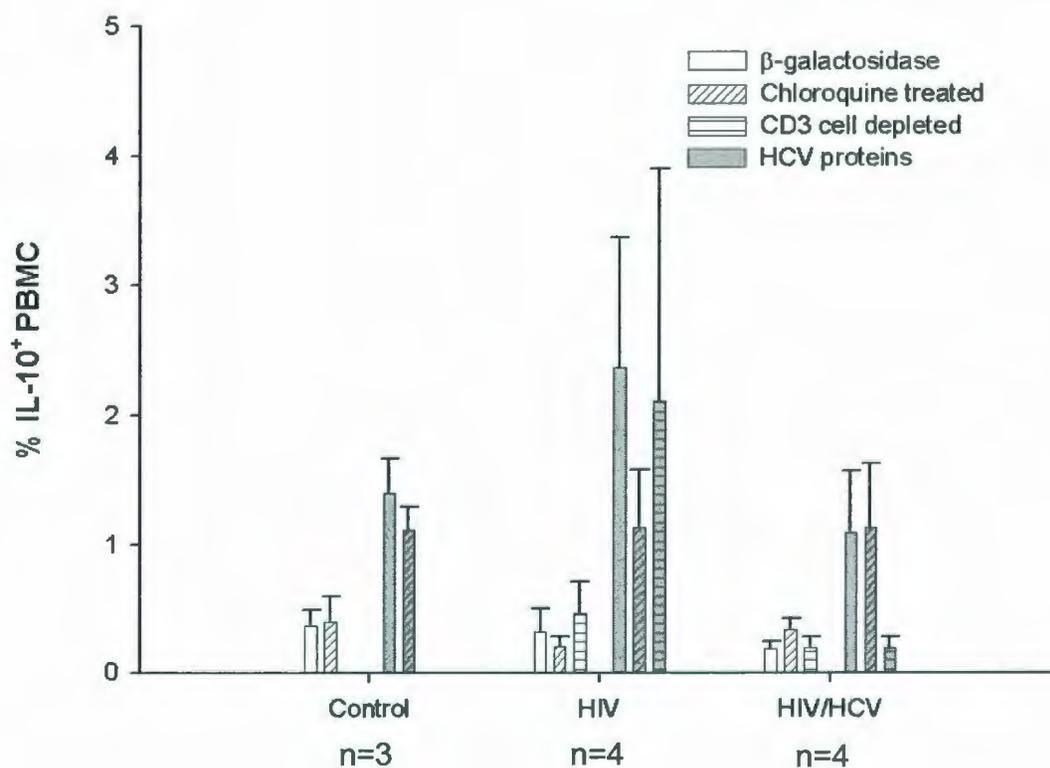


Figure 4.4. (con't) (b) The mean percentage PBMC expressing IL-10 (\pm SE) from control, HIV-infected and HIV/HCV coinfecting individuals was assessed after exposure to either β -galactosidase (white background bars) or HCV proteins (grey background bars). Effects of treating PBMC with chloroquine (diagonal hatch) or depleting CD3⁺ T cells (horizontal hatch) are also shown for comparison with the HCV protein group (grey bars). Each individual was tested on at least 2 different visits.

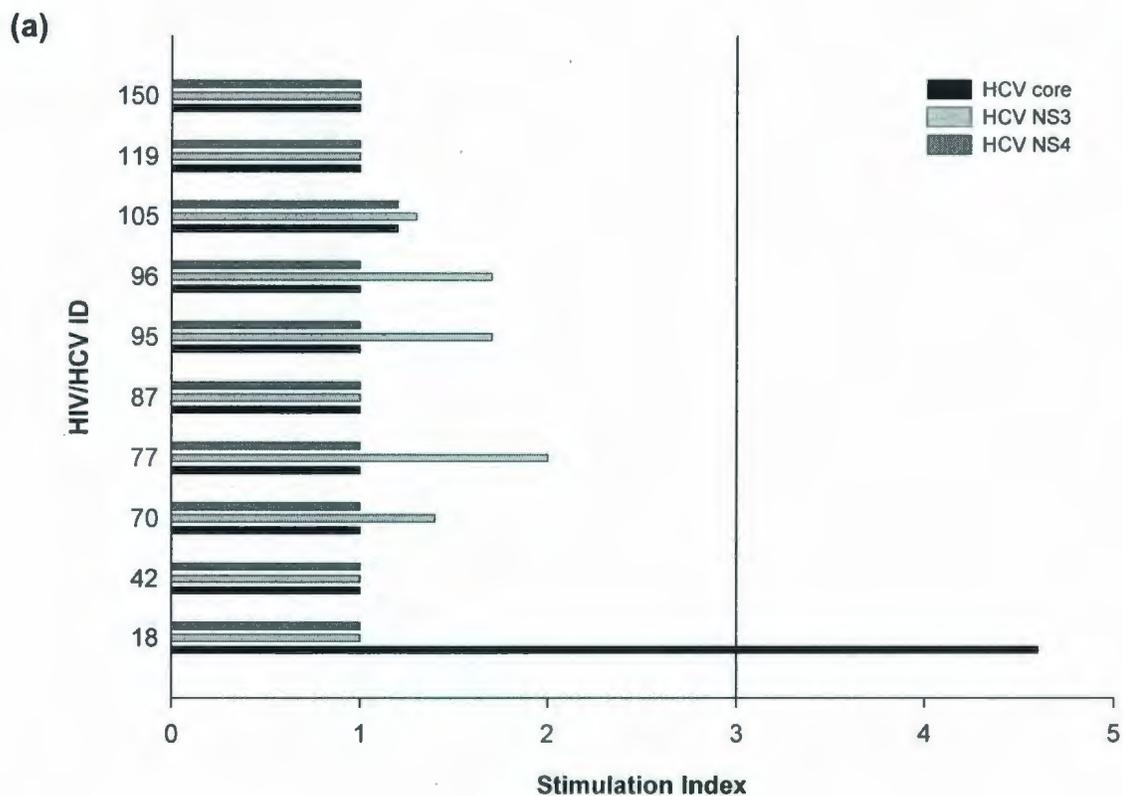


Figure 4.5. Anti-HCV cellular responses and IL-10 induction in HIV/HCV coinfected individuals. (a) ^3H -thymidine incorporation by PBMC from 10 HIV/HCV coinfected individuals incubated for 5 days with HCV core (black bar), NS3 (medium grey bar), or NS4 (dark grey bar). A stimulation index of ≥ 3 was considered positive. Individuals 18 and 150 were anti-HCV positive but HCV RNA negative at the time of testing.

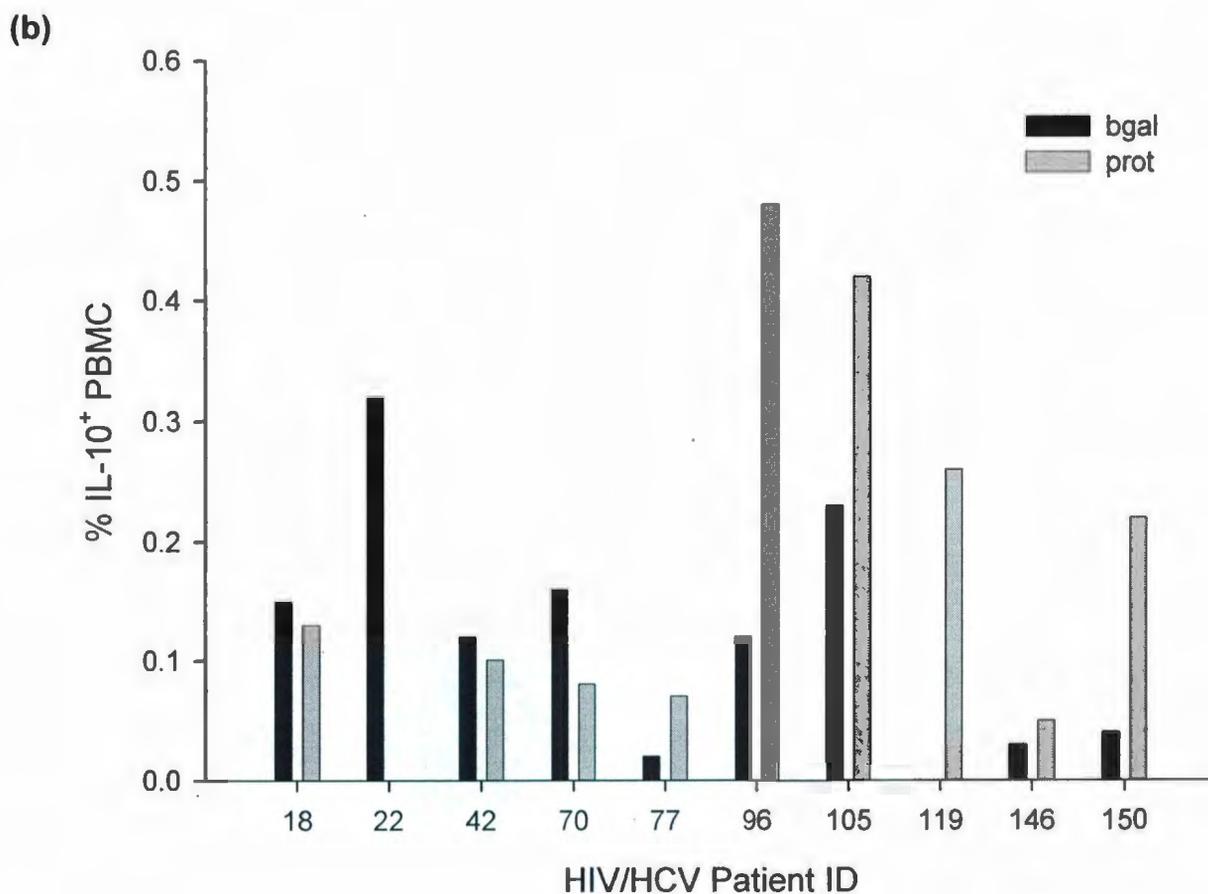


Figure 4.5. (con't) (b) IL-10 production in 10 HIV/HCV coinfecting individuals after 16 hour incubation with either β -galactosidase or HCV proteins. The percentage of IL-10⁺ PBMC within the lymphoid gate is represented on the y axis. Testing was done on the same day as the proliferation data was collected. Individuals 18 and 150 were anti-HCV positive but HCV RNA negative at the time of testing.

CHAPTER 5**INCREASED MAGNITUDE ANTI-HCV CD8⁺ T CELL RESPONSES IN
HIV/HCV COINFECTION COMPARED TO HCV MONOINFECTION**

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5.1. Abstract

Hepatitis C virus (HCV) is a common chronic infection associated with human immunodeficiency virus (HIV) infection through similar routes of transmission. Clinically apparent liver disease arises within years in HIV/HCV coinfection, compared to decades in HCV monoinfection. In this study, we compared frequency, magnitude, breadth, specificity and vigor of peripheral blood CD4⁺ and CD8⁺ T cell responses between HCV monoinfected and HIV/HCV coinfecting individuals as well as between HIV/HCV coinfecting subgroups with variable anti-HCV antibody and RNA status. Coinfection with HIV marginally decreased the frequency and breadth of anti-HCV CD8⁺ T cell responses overall, but when present, responses were stronger than in monoinfection. This suggests two distinct groups of HIV/HCV coinfecting individuals bracketing monoinfected individuals in terms of the strength of their HCV-specific CD8⁺ T cell responses. HCV-specific CD4⁺ T cell responses were rare and weak in both groups independent of either nadir or concurrent CD4⁺ T cell counts of HIV-infected individuals. Although CD8⁺ T cell responses occurred in isolation, the magnitude of HCV-specific CD4⁺ and CD8⁺ T cell responses was positively correlated in HIV/HCV coinfection. Subgroup analysis demonstrated restricted breadth of CD8⁺ HCV-specific T cell responses and lower B cell counts in HIV/HCV coinfecting individuals with no detectable anti-HCV antibodies. The greatest difference between HIV/HCV coinfecting and HCV monoinfected groups was stronger HCV-specific CD8⁺ T cell responses in the HIV coinfecting group, which may relate to the accelerated liver disease in this setting. Factors

beyond breadth and specificity of anti-HCV CD8⁺ T cell responses likely play an important role in disease course and low HCV clearance rates of in HIV coinfection.

5.2. Introduction

Hepatitis C virus (HCV) is a global health concern, with over 170 million people infected worldwide. The insidious nature of early HCV infection and high rate of chronic infection dictate that a large percentage of infected individuals are unaware of their status. As they share similar routes of transmission, HIV infection often coexists with HCV infection. In this context, the clinical course of HCV disease is accelerated and response to HCV therapy may be compromised. With the increased life expectancy afforded in HIV infection by highly active antiretroviral therapy (HAART), HCV-related disease has become a significant cause of morbidity and mortality in HIV/HCV coinfecting individuals.

A substantial body of research has addressed the role of the immune system in HCV clearance, chronicity and disease. Multiple studies in humans (198-200, 229) and chimpanzees (210, 400) link viral clearance to strong anti-HCV CD4⁺ and CD8⁺ T cell responses in acute infection, suggesting a central role for adaptive immunity in viral clearance. Vigorous and broadly directed HCV-specific CD4⁺ and CD8⁺ T cell responses occur during spontaneous clearance (199, 401, 402), while in chronic HCV infection, these responses appear much less robust. Most studies found HCV-specific CD4⁺ T cell responses to be rare (202, 403) in persistent infection, both by tetramer and ELISPOT analysis, and that CD8⁺ T cells have impaired proliferative capacity (404). There is some evidence that CD4⁺CD25⁺ regulatory T cells suppress anti-HCV CD8⁺ T cell responses and promote chronic HCV chronic infection (272, 273, 405).

HCV-specific CD4⁺ and CD8⁺ T cell responses in the context of HIV coinfection have been investigated by several groups (252, 282, 284, 285, 399, 406). In short, peripheral T cell responses are weak and narrow, but somewhat stronger in the liver. No apparent differences in the specificity of the responses have been reported. Some studies report similar frequencies of CD4⁺ and CD8⁺ T cell responses in monoinfection and HIV/HCV coinfection (282), while others report very rare (284, 407) CD4⁺ T cell responses in the presence of weak but detectable CD8⁺ T cell responses in HIV/HCV coinfection. The exact role of CD4⁺ and CD8⁺ T cell responses in this coinfecting group remain unclear. In addition, there are several subgroups within the overall HCV exposed group based on plasma HCV RNA and anti-HCV antibodies. There are HCV exposed, anti-HCV antibody positive, HCV RNA negative individuals (spontaneous clearers), chronic infections (anti-HCV positive, HCV RNA positive) and chronically HCV infected people without detectable anti-HCV antibodies (serosilent group). These subgroups have not been thoroughly studied in the context of HIV infection, and may have distinct adaptive immune responses unappreciated through global analysis.

In this study, we compared the frequency, magnitude, breadth and specificity of HCV-specific CD4⁺ and CD8⁺ T cell responses between HIV/HCV coinfecting individuals and chronically infected HCV individuals. We also investigated the character of CD8⁺ T cell responses in HIV/HCV subgroups. HIV coinfection slightly decreased the frequency and breadth of anti-HCV CD8⁺ T cell responses compared to chronic HCV monoinfection but the magnitude of responses we observed was higher than previously reported. Strong CD8⁺ T cell responses that correlated with CD4⁺ T cell response

magnitude were observed in coinfection but not in monoinfection. CD4⁺ T cell responses were rare and weak in both groups, independent of CD4⁺ nadir or CD4⁺ T cell count for HIV-infected individuals. Not all coinfecting groups were similar, with subgroup analysis indicating narrow anti-HCV CD8⁺ T cell response breadth and lower B cell counts in serosilent coinfecting individuals.

HIV coinfection was associated with stronger HCV-specific CD8⁺ T cell responses in this group of HCV coinfecting individuals. Vigorous immune responses in the context of persistent infection may contribute to rapid progression of liver disease in this group.

5.3. Materials and Methods

5.3.1. Study participants

HIV-infected and HIV/HCV-coinfected individuals were recruited from the St. John's General Hospital Infectious Disease Clinic, St. John's, NL, Canada. HCV-infected individuals were recruited from the Capital Health Queen Elizabeth Hospital Hepatitis Clinic in Halifax, NS, Canada. Ethics approval for this project was obtained from the Human Investigation Committee at each institution, and all subjects provided informed written consent for blood collection and immunological studies.

5.3.2. Identification of HCV infection

HCV exposure was ascertained by testing for serum anti-HCV antibodies using second or third generation EIA assays from Ortho Diagnostics.

To demonstrate the presence of HCV RNA, total nucleic acids were extracted from plasma samples, using NucliSens[®] Lysis Buffer and the NucliSens[®] Isolation Kit (Organon Teknika, Durham, NC). Briefly, 200 μ L of plasma was added to 1 mL of lysis buffer. Silica beads were added to bind free nucleic acids, followed by successive washes with 70% ethanol, 95% ethanol, and acetone. Nucleic acids were removed from the silica at 56°C with the elution buffer provided. Thirty μ L of eluant was frozen at -20°C if not used immediately for cDNA synthesis.

Complementary DNA (cDNA) was produced from isolated RNA using a first-strand cDNA synthesis kit (Amersham Biosciences, Baie d'Urfé, Québec). Eight μ L of extracted nucleic acid was denatured at 65°C for 5 minutes and added to a reaction mix containing random hexamer primer, deoxyribonucleotides, and Moloney Murine

Leukemia Virus reverse transcriptase (MMLV-RT). Reactions were placed at 37°C for 1 hour followed by MMLV-RT inactivation at 95°C for 5 minutes. cDNA was stored at –80°C until needed.

cDNA was amplified by polymerase chain reaction (PCR) with primers specific for the highly conserved HCV 5'-untranslated region. Primers and PCR conditions were modified from Shindo et al. (357) as follows: forward primer 5'-GGCGACACTCCACCATAGATC-3' and reverse primer 5'-GGTGCACGGTCTACGAGACCT-3'. The expected amplicon size was 324 bp. Final reactions included 20 mM TRIS-HCl buffer (pH 8.4), 50 mM KCl, 0.5 mM MgCl₂, 0.025 U/μL DNA polymerase (Invitrogen, Burlington, Ontario), and 0.5 μM MG18 and MG321 primers (Invitrogen, Burlington, Ontario) in 50 μL. Samples were initially denatured at 95°C for 2 minutes, followed by thirty cycles of amplification (95°C for one minute, 60°C for one minute, and 72°C for one minute). PCR products were analyzed on 2% agarose gels with ethidium bromide visualization and stored at –20°C.

5.3.3. PBMC isolation

Acid citrate dextrose (ACD) treated whole blood was obtained by venipuncture from each individual and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-HyPaque Plus (GE Health Care, Baie d'Urfé, PQ, CA) density gradient centrifugation. Cells were washed, counted and suspended at 1x10⁶/mL in lymphocyte medium (RPMI supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1% penicillin/streptomycin, and 2x10⁻⁵ M 2-mercaptoethanol; all from Invitrogen,

Burlington, ON, CA) until use. In some cases, cells were frozen at -80°C until use. Samples with less than 80% viability when thawed were discarded.

5.3.4. Peptide preparation

A set of 18-mer peptides spanning the entire HCV genotype 1a polyprotein was obtained from the NIH AIDS Research and Reference Reagent Program. A total of 441 peptides overlapping by 11 amino acids were reconstituted in 80-100% sterile dimethylsulphoxide (DMSO), diluted to $200\ \mu\text{g}/\text{mL}$ in lymphocyte medium, and then pooled together in groups of 10 consecutive peptides at a concentration of $20\ \mu\text{g}/\text{mL}/\text{peptide}$. Peptides were stored at -80°C until use, and added to cells at a final individual concentration of $10\ \mu\text{g}/\text{mL}$. Control wells contained 0.5% DMSO. The composition of the peptide pools is found in Appendix A.

5.3.5. ELISPOT for IFN- γ production

The number of IFN- γ producing cells in PBMC was determined by ELISPOT. Flat bottom polyvinylidene difluoride (PVDF)-coated 96-well plates (Millipore, Bedford, MA) were prewet with $100\ \mu\text{L}$ 70% ethanol/well (Sigma Chemical Co.), washed with PBS, and coated either overnight at 4°C or for one hour at 37°C with $1.5\ \mu\text{g}/\text{well}$ anti-IFN- γ antibody (clone 1D1K, Mabtech, Stockholm, Sweden) in PBS. Plates were washed five times with PBS and $1.0\text{-}2.0 \times 10^5$ intact or CD4^+ depleted PBMC were added to each well in duplicate. Peptide pools were added to each test well, and $2\ \mu\text{g}/\text{mL}$ PHA or 100 IFN- γ positive C10/MJ cells (NIH AIDS Research and Reference Reagent Program, Popovic et al. (408)) served as a positive control. Plates were incubated at 37°C , 5% CO_2 for 16 hours, washed with PBS, and incubated for 2 hours with 0.1

$\mu\text{g/well}$ of biotinylated anti-IFN- γ antibody (clone 7-B6-1, Mabtech). Plates were washed, and 100 μL of a 1:1000 dilution of streptavidin-alkaline phosphatase (Mabtech) was added for 1 hour. After washing, 100 μL freshly prepared 3 mg/mL nitro blue tetrazolium chloride (NBT)/1.5 mg/mL 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine (BCIP) substrate (BioRad, Hercules, CA) in Tris buffer (pH 9.5) was added to each well for approximately 20 minutes. Plates were washed with distilled water to stop the color reaction and air-dried overnight before spot enumeration using the high resolution Zeiss reader system and associated KS software (Carl Zeiss Canada, Ontario, Canada). The number of IFN- γ producing cells per million PBMC was determined by multiplying the average number of spots/duplicate well $\times 10$ or $\times 5$, depending if 1×10^5 or 2×10^5 cells were used. A peptide pool response was considered positive if the number of spots was greater than 10 spot forming cells (SFC), and twice the background count. CD4⁺ depletion was done with 2 coinfecting samples; there was no difference in the number of peptide induced IFN- γ ⁺ spots with or without depletion (data not shown). As each spot represents one cell, the area of the spot is related to IFN- γ production per cell (409, 410). Average spot area in arbitrary units was used as a relative measure of IFN- γ production per cell. Spot area is determined electronically by calculating the number of pixels in a well with color.

5.3.6. Proliferation assays

Cellular immune responses were measured by standard 5 day thymidine incorporation assay. PBMC were washed twice with proliferation medium (lymphocyte medium with 10% human AB serum (Atlanta Biologicals, lot number M0102) substituted

for FBS) and 1×10^5 cells/well were incubated in triplicate with either plain medium, 5 $\mu\text{g/mL}$ phytohemagglutinin (ICN Biomedicals Inc., Costa Mesa, CA), 2.5 or 5 $\mu\text{g/mL}$ *Candida albicans* (Greer Laboratories Inc., Lenoir, NC), or 2 $\mu\text{g/mL}$ HCV core, NS3, NS4 or HIV p24 (all Virogen) for 5 days. One μCi of tritiated (^3H) thymidine (Perkin Elmer Life Sciences, Boston, MA) was added to each well on day 4, and the assay was harvested onto glass fiber filter mats 18 hours later using a semi-automated harvester (Tomtec Harvester 96 Mach M III, Hamden, CT). Incorporated ^3H -thymidine was measured by a 96 well scintillation counter (TopCount, Packard, Meriden, CT). Stimulation indices were calculated as follows:

$$\text{Stimulation Index} = \frac{\text{cpm}_{\text{antigen}}}{\text{cpm}_{\text{background}}}$$

5.3.7. Statistical analyses

All statistical analyses were performed using SPSS version 9 (SPSS Inc., Chicago, IL). Means were compared using either the Mann-Whitney U test or Student's *t*-test. All graphs indicate average values, and error bars are the standard error of the mean. Pearson's correlation coefficients were used to assess relationships between independent continuous variables by linear regression.

5.4. Results

5.4.1. Study participants

Twelve individuals coinfecting with HIV and HCV were recruited and 22 people with chronic HCV infection. Demographic and baseline clinical parameters are reported in Table 5.1. Mean age (40 years in the HIV/HCV groups and 47 years in the HCV group) and gender distribution (75% and 77% male respectively) did not differ significantly between the two groups.

Most HCV genotypes were unknown, but those tested were predominantly subtype 1, as expected for North American populations. Intravenous drug use (IVDU), blood product transfusion and organ transplant were predominant risk factors in both groups. Estimated duration of HCV infection was similar in both groups with approximately 20 years as the average time since infection. Liver enzymes were reported as elevated if the plasma level was greater than twice the upper limit of normal. All individuals in the coinfecting group and 68% in the chronic group had normal plasma liver enzyme levels on the sampling date.

Overall, the monoinfected and HIV/HCV coinfecting groups were similar in demographics and HCV-related clinical parameters.

5.4.2. HCV does not affect HIV related immune parameters

Table 5.2 distinguishes three groups of HIV infected individuals exposed to HCV: those who were HCV seropositive and HCV RNA positive (chronic infection, n=5); those who were HCV seropositive and HCV RNA negative (spontaneous clearers, n=3), and

those who were HCV seronegative and HCV RNA positive (serosilent HCV infection, n=4).

HIV-associated parameters are described for the HIV/HCV coinfecting group in Table 5.2. HCV related parameters are discussed above. All subgroups were similar in age, gender distribution, duration of HIV infection, and type of antiretroviral therapy at the time of testing. Only one individual, HIV-087, was not receiving antiretroviral therapy at the study time point. There were no statistically significant differences in either CD4⁺ T cell nadir or CD4⁺ T cell count. HIV viral load was not significantly different between groups, and ranged between undetectable (<50 or <400 copies/mL) and 130,000 HIV RNA copies/mL plasma. All liver function tests and plasma liver enzyme levels were normal on the test date. There was no significant difference in HIV related parameters such as CD4⁺ T cell count, CD4⁺ T cell nadir or HIV plasma viral load in the serosilent group (lack of anti-HCV antibodies), spontaneous clearance group (plasma HCV RNA negativity), or chronic HCV infection group.

We compared HIV related clinical data for the group of 12 HIV/HCV coinfecting individuals with a group of 75 HIV monoinfected individuals enrolled concurrently for HIV immunology studies (Figure 5.1a). Mean CD4⁺ T cell counts were comparable (370±245 in the HIV group, 380±259 in the HIV/HCV group) as were CD4⁺ T cell nadir (181±175 and 255±197 respectively), HIV viral loads (3.27±1.1 and 3.8±1.2 respectively) and CD8⁺ T cell counts (886±445 and 892±582 respectively). B cell counts (CD20⁺) were also similar in the HIV/HCV group and HIV monoinfected group (248±163 vs. 194±134; respectively). Of note, the 4 HCV seronegative, HCV RNA

positive individuals had B cell counts that were below the HIV/HCV seroevident coinfecting group mean (Figure 5.1b; 306 ± 187 versus 134 ± 65). HCV infection does not appear to affect HIV-related clinical laboratory parameters in our study group, except for a trend toward lower B cell counts associated with a selective lack of HCV antibodies.

5.4.3. HCV NS3 is immunodominant in HIV/HCV coinfection, chronic HCV mono-infection and in HIV coinfecting spontaneous clearers.

We assessed the specificity, breadth and magnitude of anti-HCV CD8⁺ T cell responses in PBMC from HIV/HCV coinfecting and HCV mono-infected individuals using an overlapping set of 441 18-mer peptides spanning the entire HCV polyprotein in an IFN- γ ELISPOT assay. Peptides were grouped in 44 sequential pools of 10 beginning from the amino terminus and running through the carboxy terminus of the HCV polyprotein. Results are expressed in terms of pools and protein regions, with different numbers of peptide pools in relation to the protein size: 3 pools for core; 2 for E1; 6 for E2; 1 for p7; 3 for NS2; 9 for NS3; 2 for NS4A; 3 for NS4B; 7 for NS5A; 8 for NS5B.

Eighteen of the enrolled 22 chronic HCV infected individuals and 11 of the 12 HIV/HCV coinfecting individuals were tested. Figure 5.2 illustrates differences in the specificity and frequency of HCV-specific CD8⁺ T cell responses between groups, depicted as the proportion of individuals from each group responding to each individual peptide pool. More than 40% of the coinfecting group responded to NS3 pool 18 and at least 30% responded to NS3 pool 16, NS3 pool 22 and NS5B pool 44. There were no responses to pools 2, 10-13, 15, 17, 31, 34, 36, 37, and 41. In the chronic HCV mono-infected group (Figure 5.2, panel 1), 40% of individuals responded to core pool 2

and NS3 pool 19. More than 30% responded to NS3 pool 16, NS3 pool 17, NS4A pool 25 and NS5A pool 30. There were no responses to pools 10, 36, and 38. Of note, no responses to pools 10 or 36 were detected in either group.

5.4.4. Restricted breadth anti-HCV CD8⁺ T cell responses in HIV/HCV coinfection and chronic HCV infection

Breadth is the percentage of peptide pools to which an individual responds. The proportion of individuals responding to at least one HCV peptide pool was similar between groups, as 8/11 HIV coinfecting (72%) and 14/18 HCV-infected individuals (78%) had anti-HCV IFN- γ responses. On average, the HIV coinfecting group had responses to 11% of the peptide pools (range 0-38%) compared to 17% (range 0-64%) for the HCV monoinfected group, suggesting a somewhat restricted breadth to the HCV-specific CD8⁺ T cell responses in the HIV coinfecting group. When responses were compared on the basis of HCV proteins (Figures 5.3a and 5.3b), there was little difference between HIV coinfecting and HCV infected groups. In the HIV coinfecting group, we observed responses against 9/10 proteins, however, the proportion of individuals with responses to any one region was generally low (<50%). The exception was the NS3 region, where 82% of coinfecting individuals responded (Figure 5.3a, dark bars). Responses were detected against 10/10 proteins in the chronic HCV group (Figure 5.3b, dark bars). As in the coinfecting group, the highest proportion of individuals responded to NS3 (67%), with greater than 53% of individuals also responding to NS4A. Since the size of the protein is one factor affecting the probability of generating immunogenic peptides, we adjusted for the effect of protein size. After this correction

(grey bars of Figure 5.3b), it was clear that one of the reasons NS3 was so dominant was because it is the largest region in the genome. The response breadth did not change with size correction but differences in proportions between groups equalized. E1 became the most frequent response on a per peptide basis in HIV coinfecting group, and NS4A in the chronic group.

Figure 5.4 illustrates differences in the specificity and frequency of anti-HCV IFN- γ responses between HIV coinfecting subgroups. In the HIV chronic HCV group (n=5), there were responses to all regions except p7 and NS2 (black bars) with responses most frequently against NS3 and NS5B. In the HCV serosilent group (n=2), responses were limited to NS3 and NS5A. The spontaneous clearers (n=2) had responses against all proteins except p7, with both individuals demonstrating responses against NS3.

In summary, NS3 region T cell responses are found in all of those who spontaneously cleared the virus. NS5 region T cell responses were common in both HIV/HCV coinfection and HCV monoinfection, however NS2 and NS4A T cell responses were more prevalent in the monoinfected group. Responses to NS3 are most frequent, in part because of its large size. However, the most commonly recognized peptide pool for all groups is in the NS3 region, suggesting that there is a true immunological hotspot in NS3.

5.4.5. High magnitude anti-HCV CD8⁺ T cell responses in HIV/HCV coinfection

Magnitude is the number of IFN- γ producing cells elicited by HCV peptides. The average magnitude of anti-HCV responses per peptide pool, as well as cumulative magnitude, was compared between HIV coinfecting and HCV-infected groups after

background subtraction. More IFN- γ -producing cells/positive pool were observed in the HIV coinfecting group (72 ± 22 SFC/ 10^6 PBMC versus 21 ± 4.2 SFC/ 10^6 PBMC, $p=0.02$; Figure 5.5a). The cumulative magnitude of the response was significantly higher in the HIV coinfecting group (1050 ± 369 SFC/ 10^6 PBMC versus 162 ± 47 SFC/ 10^6 PBMC $p=0.003$; Figure 5.5b).

5.4.6. HCV response vigor

Vigor is measured in arbitrary units relative to IFN- γ spot size that correlates with IFN production per cell. Larger spot size indicates increased IFN- γ production, therefore, spot size was analyzed in this study as a measure of the vigor of the IFN response. Spot area was measured in arbitrary units, and the average IFN- γ spot area per group determined. There was no significant difference in average spot area between the HIV coinfecting group and the HCV-infected group, with large standard deviations in each group (Figure 5.6a). We then assessed whether spot area was associated with the cumulative magnitude of the response in each group. Response magnitude correlated with spot area in the HIV coinfecting group ($R^2=0.42$; $p=0.0001$) but not the HCV infected group (Figure 5.6b). In the coinfecting group, cells from individuals with higher magnitude responses produce more IFN- γ per cell than those with lower magnitude responses.

5.4.7. CD4⁺ T cell response magnitude, frequency and specificity

CD4⁺ T cell responses against mitogen, recall antigen and HCV viral antigens were assessed in all groups by standard 5 day proliferation assay. An HIV monoinfected group ($n=75$) was included to compare mitogen and recall antigen responses to those of

the HIV/HCV coinfecting group. The average magnitude of the mitogen responses, shown on the right hand axis of Figure 5.7, was not significantly different between HIV-infected and HIV/HCV coinfecting groups (160 ± 21.1 and 129 ± 37.1 , respectively). The HCV monoinfection group average (473 ± 242) was higher than either HIV-infected group but the difference was not significant, with large high standard deviations. This suggests overall $CD4^+$ T cell function in the HIV/HCV group is marginally reduced compared to the HCV monoinfected group. The antigen specific response magnitude measured against Candida antigen differed between groups in a pattern similar to the PHA responses (Figure 5.7). The HIV/HCV coinfecting group had a somewhat lower average stimulation index in response to low dose recall antigen (mean \pm SE; 10.9 ± 4.6) than the HCV-infected group (27.5 ± 9.4 ; $p=0.06$). The same trend was seen with the high dose Candida response. HCV antigen-specific responses were only seen against HCV core, and these were of low magnitude.

In summary, the frequency and magnitude of $CD4^+$ T cell responses was decreased in HIV/HCV coinfecting individuals compared to HCV monoinfected individuals but similar to the HIV monoinfected group. Anti-HCV-specific responses were rare, and seen only to HCV core and NS3.

5.5. Discussion

HCV evades eradication by the immune system and establishes chronic infection in a majority of exposed individuals. In the context of HIV coinfection, HCV infection not only becomes chronic more frequently, but also accelerates development of liver disease. The role of immunity against HCV, particularly in the context of HIV coinfection, is not well understood, and few studies have used complete sets of HCV peptides to examine T cell responses in this setting. We investigated whether HIV coinfection affects the overall character of the cell mediated immunity against HCV by comparing the breadth, frequency, specificity and magnitude of anti-HCV responses in HCV-infected and HIV/HCV coinfecting individuals with a comprehensive set of peptides spanning the entire HCV polyprotein. CD4⁺ T cell responses were rare in both groups, and CD8⁺ T cell response breadth was reduced in a subgroup of serosilent coinfecting individuals. Otherwise, the frequency, breadth and specificity of HCV-specific CD8⁺ T cell responses in the 2 groups did not differ significantly. The coinfecting individuals had significantly stronger responses, which may amplify the immunopathogenesis of HCV in HIV coinfection.

Approximately 70% of individuals in both the HCV-infected and HIV/HCV coinfecting groups had detectable HCV-specific CD8⁺ T cell responses, which was a higher frequency than reported in some previous studies. Dutoit et al. found approximately 40% of individuals produced IFN- γ responses (282), and Kim et al. described anti-HCV CD8⁺ T cell responses in only 50% of HIV/HCV coinfecting individuals (252). An earlier study reported HCV-specific CD8⁺ T cell responses in only

2/32 chronically infected individuals and 0/11 HIV/HCV coinfecting people (399). All groups used similar experimental conditions to this study, however, their definition of a positive response required a minimum 20-50 spots/ 10^6 PBMC, and greater than 3 times background levels. This absolute value would correspond to a frequency of 0.002% of PBMC, which is 100 fold less than that found in other viral infections (eg. EBV with up to 0.2%). On the assumption that extrahepatic CD8⁺ anti-HCV T cell responses are rare in chronic low level infection, we decided this high threshold may miss responses relevant to overall outcome. We chose a threshold of greater than twice background and an absolute minimum of 10 spots as our cutoff. If we applied the criteria from other studies, responder frequency was unaffected in the HIV coinfecting group, but fell from 70% to 50% in the HCV monoinfected group. The responses of the HCV monoinfected individuals were broadly directed but low magnitude, and often twice, but not three times, background. Therefore, with the more exclusive criteria previously used, our coinfecting group still had more frequent responses than previously reported and more frequent responses than the HCV monoinfected group.

The ELISPOT method we used may underestimate CD8⁺ T cell responses by relying only on ability to produce IFN- γ . Functionally cytotoxic anti-HCV CD8⁺ T cells can have a stunned phenotype and produce less IFN- γ in both acute (411) and chronic (349) infection. The estimate is further limited by the use of a consensus sequence to generate peptides, and not endogenous sequences found in individual patients.

As reported previously, we found the breadth and specificity of CD8⁺ T cell responses similar in coinfecting and chronic monoinfected groups (252). Before

correction for size, NS3 responses were most common on a per protein basis, as previously described by many groups. However, after correction for size, the hierarchy of response frequency in each group shifts to smaller protein regions. The subgroup of HIV coinfecting individuals who appear to have spontaneously cleared their HCV infection all have responses to NS3, reinforcing suggestions that anti-NS3 responses may play a role in viral clearance (412, 413). One particular region of NS3 (pool 18/19) had a large number of responders in each group. These pools contain amino acids 1240-1390, where 12 human CTL epitopes have been defined (Los Alamos HCV database, <http://hcv.lanl.gov>), therefore, a high frequency of responders would be expected in this area. Even after the effect of size is removed, the number of epitopes defined is higher in NS3 region than other HCV proteins, suggesting immunological hotspots within that region. Maintenance of NS3 immunodominance in HIV coinfection further underscores the importance of this region in efforts to develop vaccine strategies.

In contrast to previous studies (252, 282, 284), the magnitude of HCV-specific CD8⁺ T cell responses was elevated in HIV coinfection compared to chronic HCV infection, independent of either current or nadir CD4⁺ T cell count. This may significantly contribute to disease pathogenesis in the context of persistent infection. Several mechanisms have been proposed to explain the decrease in effective clearance of HCV despite the presence, and perhaps even higher than usual, levels of anti-HCV-specific CD8⁺ T cells. These include impaired effector function (282), incomplete differentiation (213), and decreased CD28-mediated costimulation in the context of HIV coinfection (414). Our study indicates the number of HCV-specific CD8⁺ T cells is

higher in HIV coinfection than chronic HCV infection, but still low in absolute numbers compared to other chronic viral infections. This may reflect hepatic localization of HCV-specific CD8⁺ T cells.

The frequency of CD4⁺ T cell responses was low in both the coinfecting and HCV chronic infection groups. Core specific responses were most frequent in the monoinfected group, while NS3-specific responses were more common in the HIV/HCV group. The magnitude of the HCV core response in the coinfecting group was lower than that of the monoinfected group. A recent study of HCV-specific CD4⁺ T cells in HIV coinfection noted attenuated IFN- γ production independent of CD4⁺ T cell count (407). In our study, proliferation of HCV-specific CD4⁺ T cells was not related to the absolute CD4⁺ T cell count as long as the number of PBMC/well of those individuals with <200 CD4⁺ T cells was increased to control for total CD4⁺ T cell numbers. The proliferation assay reflects IL-2 production more than IFN- γ production, therefore, parallel attenuation in each assay would not necessarily be expected based on differential cytokine production attributed to various T cell memory subsets. This study was cross sectional and only provided CD4⁺ T cell counts and HCV-specific CD4⁺ T cell reactivity at one time point in a disease process that spans decades. The status of CD4⁺ T cell responses change over time from acute through chronic HCV infection depending on viral replication and stage of liver disease, as well as the state of the host immune system as affected by concurrent HIV infection. The lack of CD4⁺ T cell responses in the periphery in the context of detectable CD8⁺ T cell responses may be a result of enhanced CD8⁺ T cell responses favored by the high CD8⁺ T cells counts characteristic of HIV infection,

compartmentalization of CD4⁺ T cells to the liver, subset exhaustion with constant antigenic stimulation, or very low frequency that is not detectable by proliferation assay.

HIV-associated immune dysregulation affects the vigor and magnitude of the HCV-specific CD8⁺ T cell responses and also affects HCV-specific humoral responses. There were 3 individuals with plasma HCV RNA in the absence of detectable anti-HCV antibodies. These individuals had detectable but extremely narrow anti-HCV CD8⁺ T cell responses, and no detectable CD4⁺ T cell responses. Thus, they have a different profile from the other HIV/HCV coinfecting individuals in terms of both humoral and cellular HCV-specific immunity and provide an opportunity for further study into HCV disease processes in a novel immunological setting. They may have a different course of clinical disease and need selective monitoring, while also providing a 'natural' experiment to assess the viral and host factors that may impact their impaired immune response.

Our studies were conducted on peripheral blood samples. The frequency of HCV-specific CD4⁺ and CD8⁺ T cells is many fold higher in the liver of HCV-infected individuals (406, 415, 416), and this also holds true in the context of HIV coinfection (285). HCV-specific T cell responses in the periphery differ not only quantitatively from liver infiltrating lymphocytes but also qualitatively. Intrahepatic T cells produce less IFN- γ and have a phenotype consistent with T cell exhaustion (PD-1^{high}, CD127^{low}) compared to the effector phenotype observed in periphery (PD-1⁺, CD127⁺) (276, 417). This may reflect differences in HCV antigen levels or the different characteristics of viral quasispecies within intra- and extra-hepatic compartments, although a recent study

showed the same viral sequences predominate in the liver and peripherally in chronic HCV mono-infection (276). Therefore, responses in the peripheral blood may not directly relate to intrahepatic environments.

Cells outside the liver that have been associated with autonomous HCV replication and infection include PBMC (418) and neutrophils (187), and higher levels of lymphoid infection have also been associated with HIV coinfection (30, 419). This potential for increased extrahepatic viral antigen in the context of HIV coinfection may be the stimulus for greater magnitude anti-HCV CD8⁺ T cell responses observed in our group. Neither treatment-associated 'cure' nor spontaneous clearance of HCV infection appears to truly eradicate HCV (125) from the extrahepatic compartment. In these cases, peripheral T cell responses may be good indicators not only of ongoing viral replication but also provide direction in developing vaccines to target quasispecies that come to predominate in the post-treatment era.

We have demonstrated an increase in the magnitude of anti-HCV CD8⁺ T cell responses in HIV coinfection that is consistent with accelerated hepatic immunopathology. This further highlights that HIV related immune dysregulation and immune reconstitution can lead to overly vigorous responses to some pathogens. Further studies to elucidate the mechanisms of HCV persistence and disease pathogenesis in the context of the HIV coinfection will be necessary to fully treat individuals with residual infection in the IFN- α era.

5.6. Acknowledgements

We gratefully acknowledge the contributions of the HCV-infected and HIV/HCV coinfecting individuals who volunteered for this study. In addition, we thank Maureen Gallant for her excellent technical assistance with the proliferation assays. The C10/MJ cell line was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: C10/MJ from Dr. Dean Mann and Dr. Miklaus Popovic.

Table 5.1. Baseline demographics and characteristics in the HIV/HCV coinfection and HCV monoinfection groups

	HIV/HCV	Chronic HCV
Number (n)	12	22
Age (yrs)	40±5	47±10
Sex (%)		
M	75	77
F	25	23
HCV Genotype (%)		
1	100	40
Non-1	0	5
Unknown	0	55
HCV Risk Factor (%)		
IVDU	25	50
Transfusion	17	14
Tattoo	0	5
Endemic area	0	14
Unknown	58	23
Est. duration of infection (yrs)		
HIV	11±5	N/A
HCV	Unk	26±10
Liver enzymes (%)		
Normal	100	68
>2ULN ¹	0	27
Unknown	0	5

¹2ULN Twice the upper limit of normal

Table 5.2. Cohort demographics and baseline characteristics in HIV/HCV coinfecting subgroups.

Patient ID	Age (yrs)	Sex	Anti-HCV ab	HCV RNA	HCV genotype	HCV risk factors	Est. duration HIV/HCV infection (yrs)	HIV treatment	CD4 count (cells/ μ L)	CD4 nadir (cells/ μ L)	HIV viral load (copies/mL)	Liver enzymes or fxn (AST ⁴ /ALT ⁵ /Alb ⁶)
HCV exposed, RNA negative, n=3												
HIV-018	33	M	+	-		IVDU	12/unk	2 PIs ¹ , 1 NRTI ²	608	92	<50	N
HIV-095	43	M	+	-			unk/unk		627	580	<400	N
HIV-150	34	M	+	-		Transfusion	16/unk	1 PI, 1 NRTI, 1 NNRTI ³	644	468	<50	N
Mean \pmSD	36\pm5								626 \pm 18	380\pm 255		
Chronic HCV infection, n=5												
HIV-042	40	M	+	+		IVDU	14/unk	2 PIs, 2 NRTIs	308	70	<50	N
HIV-070	36	M	+	+		IVDU	6/9	2 NRTIs, 1 NNRTI	580	264	<400	N
HIV-077	46	M	+	+		Transfusion	18/4	2 NRTIs	660	81	4600	N
HIV-087	38	M	+	+		IVDU	18/unk	2 NRTIs, 1 NNRTI	360	162	3000	N

Table 5.2. (con't)

Patient ID	Age (yrs)	Sex	Anti-HCV ab	HCV RNA	HCV genotype	HCV risk factors	Est. duration HIV/HCV infection (yrs)	HIV treatment	CD4 count (cells / μ L)	CD4 nadir (cells / μ L)	HIV viral load (copies/mL)	Liver enzymes or fxn (AST/ALT/Alb)
HIV-167	44	F	+	+			unk/unk			264		N
Mean\pmSD	40\pm4								477\pm169	168\pm94		
Serosilent chronic HCV infection, n=4												
HIV-096	40	F	-	+			12/unk	2 NRTIs	780	442	<50	N
HIV-105	41	M	-	+			6/unk	None	608	490	130,000	N
HIV-119	41	M	-	+			5/unk	2 PIs, 2 NRTIs	736	229	<50	N
HIV-153	51	F	-	+			4/unk	3 NRTIs	400	198	<400	N
Mean \pmSD	43\pm5								633\pm170	340\pm147		
Overall Mean \pmSD	40\pm5	75% M					11\pm5.5/unk		573 \pm 152	278\pm 175	12640 \pm 38952	

¹PI-protease inhibitor ²NRTI-Nucleoside reverse transcriptase inhibitors ³NNRTI-Non-nucleoside reverse transcriptase inhibitors ⁴AST-Aspartate aminotransferase ⁵ALT-Alanine aminotransferase ⁶Alb-Albumin

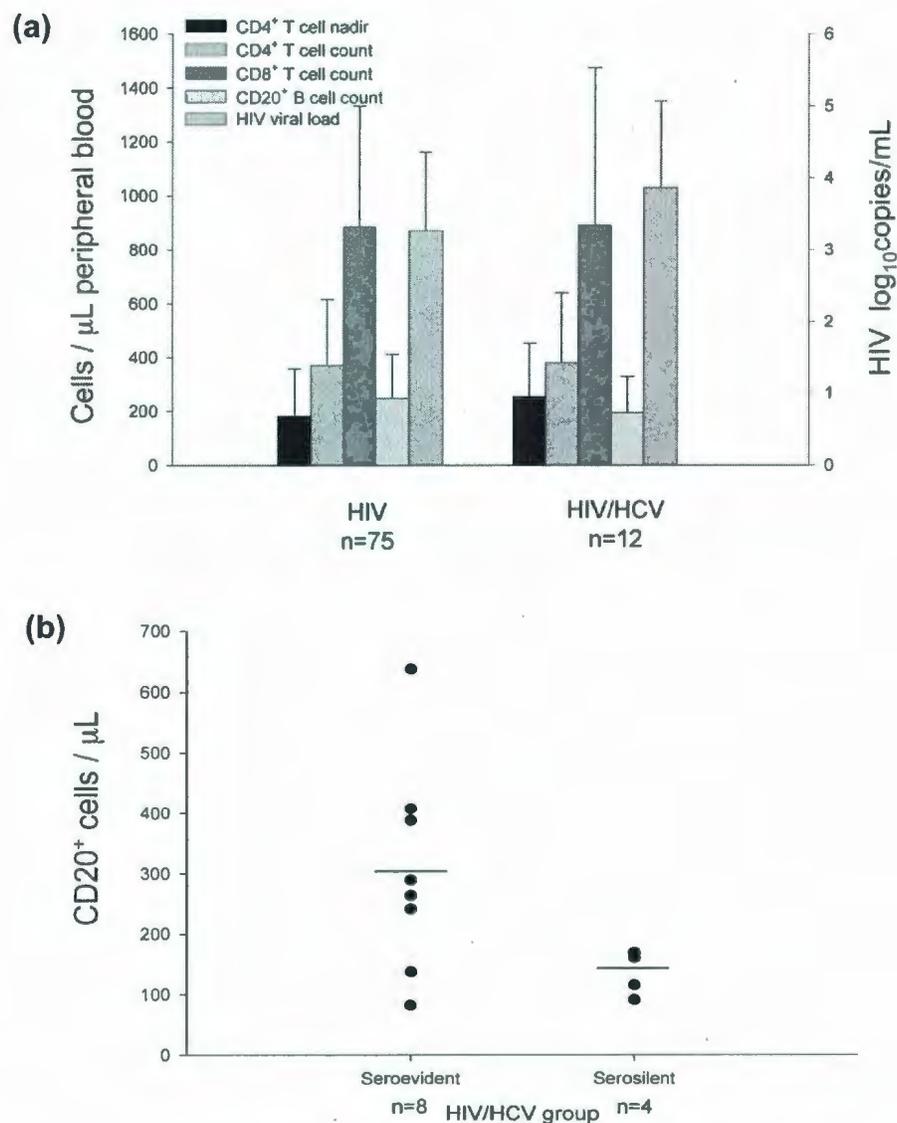


Figure 5.1. Comparison of immunological parameters in HIV-infected and HIV/HCV coinfecting study groups. (a) CD4⁺ and CD8⁺ T cell counts, B cell counts and HIV plasma virus loads (right sided axis) are shown for the peripheral blood sampling date. The CD4⁺ T cell nadir was the lowest CD4⁺ T cell count documented at any time in clinical records. Vertical bars represent the mean value for each parameter compared between study groups with error bars showing standard error of the mean. (b) B cell counts are shown for each HCV seropositive and seronegative HCV-exposed, HIV coinfecting individual with horizontal bars denoting the mean B cell count for each group.

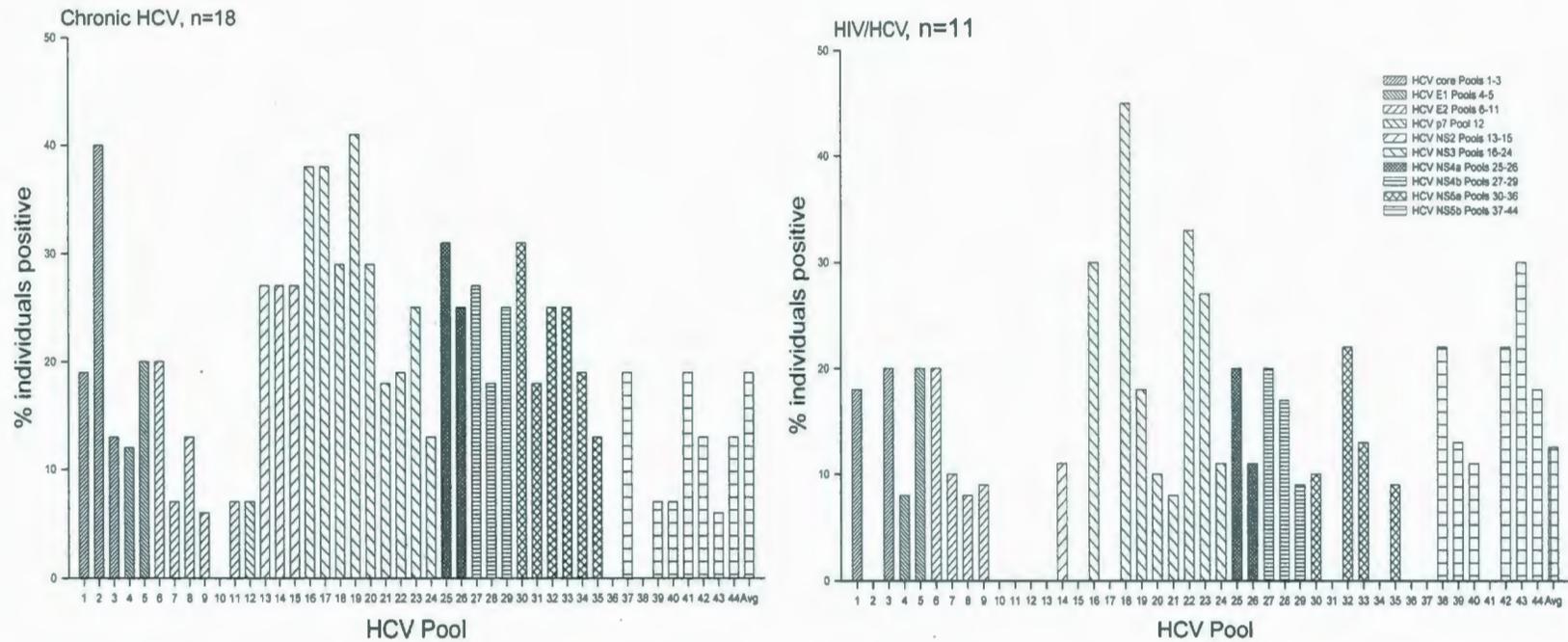


Figure 5.2. Distribution of HCV-specific CD8⁺ T cell responses in HCV and HIV/HCV coinfecting study groups. Peripheral blood mononuclear cells were incubated with pools of ten overlapping 18-mer HCV peptides for 18 hours and IFN- γ producing cells enumerated by ELISPOT. The percentage of each group responding to individual pools is indicated bars above pool number and the average response rate indicated by the final bar. Pools corresponding to each protein region are distinguished by different hatching.

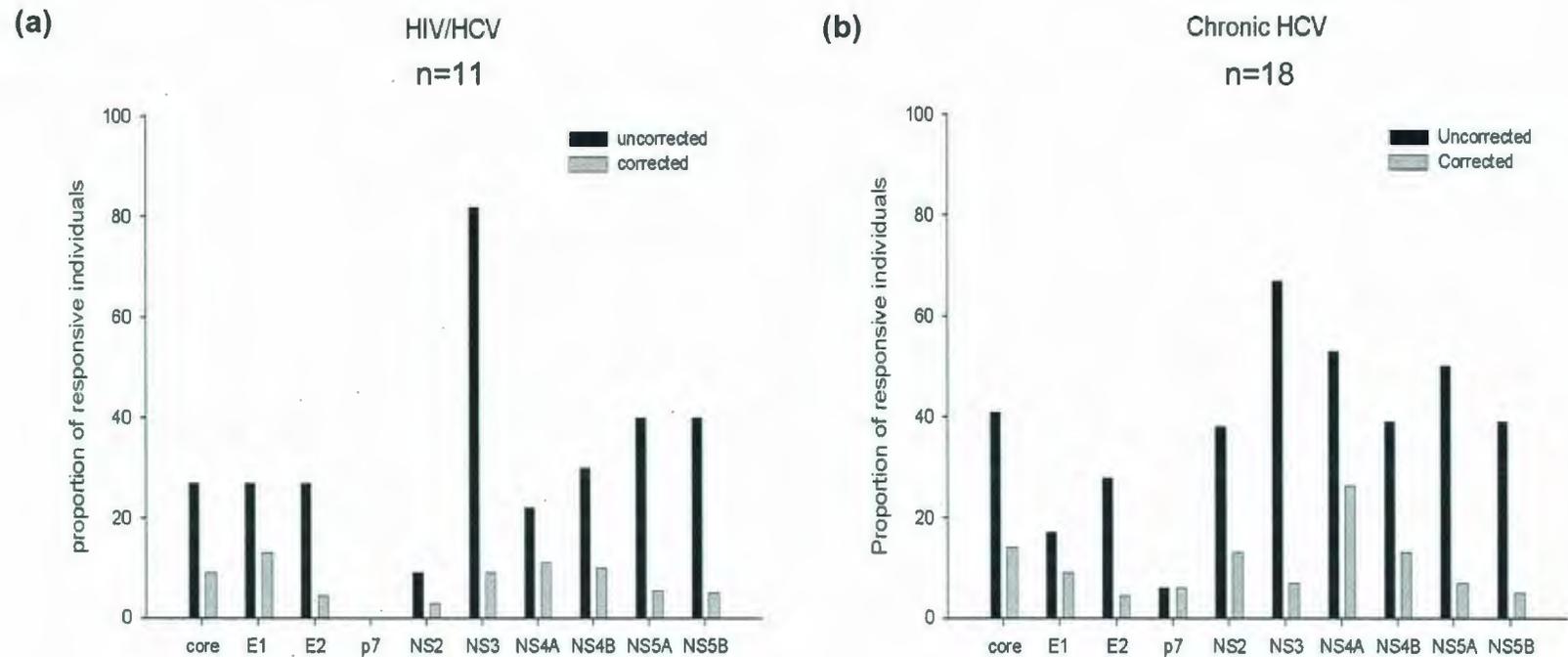


Figure 5.3. Distribution of HCV-specific CD8⁺ T cell responses by region in HCV and HIV/HCV coinfecting study groups. Peripheral blood mononuclear cells were incubated with pools of ten overlapping 18-mer HCV peptides for 18 hours and IFN- γ producing cells enumerated by ELISPOT. (a) The percentage of HIV/HCV coinfecting individuals responding to peptides from each HCV protein are shown in black bars with grey bars showing percentages adjusted for protein size (number of peptide pools/region). (b) The percentage of HCV monoinfecting individuals responding to peptides from each HCV protein are shown in black bars with grey bars showing percentages adjusted for protein size by dividing each proportion by the relative number of pools represented by each protein region.

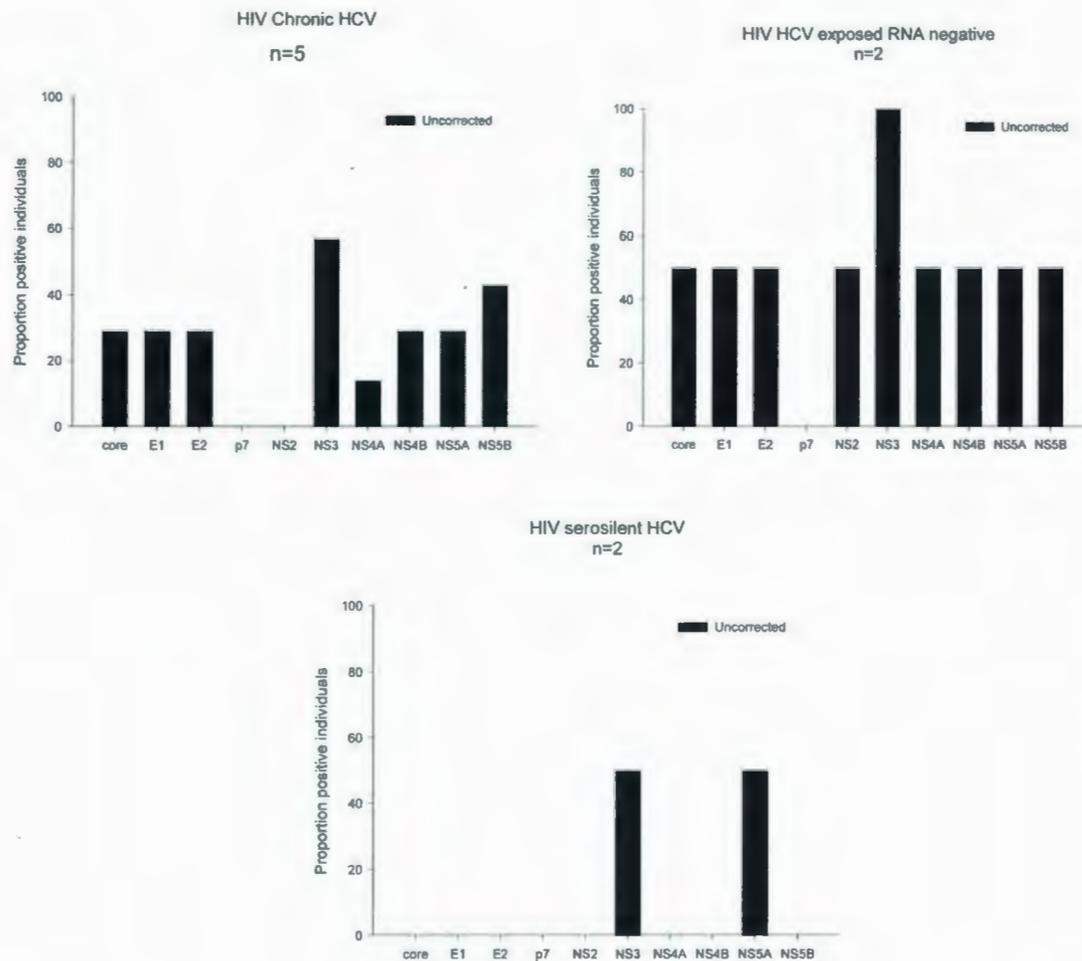


Figure 5.4. Distribution of HCV-specific CD8⁺ T cell responses in HIV/HCV coinfecting subgroups. HIV-infected, HCV-exposed individuals were divided into chronic HCV infection (top left panel), chronically infected but HCV seronegative (serosilent, bottom panel), and HCV seropositive but HCV RNA negative (top right panel). The percentage of individuals in each group with positive responses against each protein region is shown by black bars.

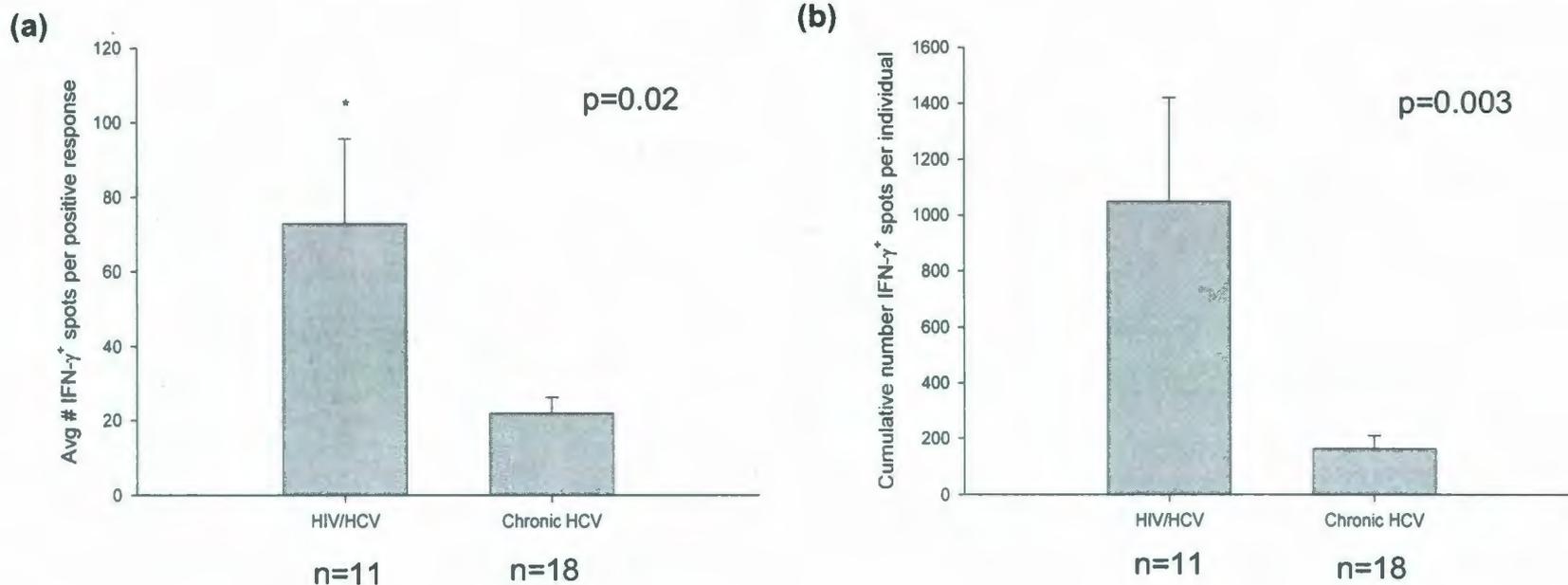


Figure 5.5. Comparison of pool-specific and cumulative magnitude of HCV-specific CD8⁺ T cell responses in HIV/HCV coinfecting and HCV-infected individuals. Peripheral blood mononuclear cells were incubated with pools of ten overlapping 18-mer HCV peptides for 18 hours and IFN- γ producing cells enumerated by ELISPOT. (a) Grey bars indicate the mean number of IFN- γ producing cells responding to each positive pool in HIV/HCV coinfecting and HCV-infected groups with standard error of the mean shown. (b) Grey bars indicate the mean cumulative number of IFN- γ producing cells responding to the entire set of HCV peptides in HIV/HCV coinfecting and HCV-infected group.

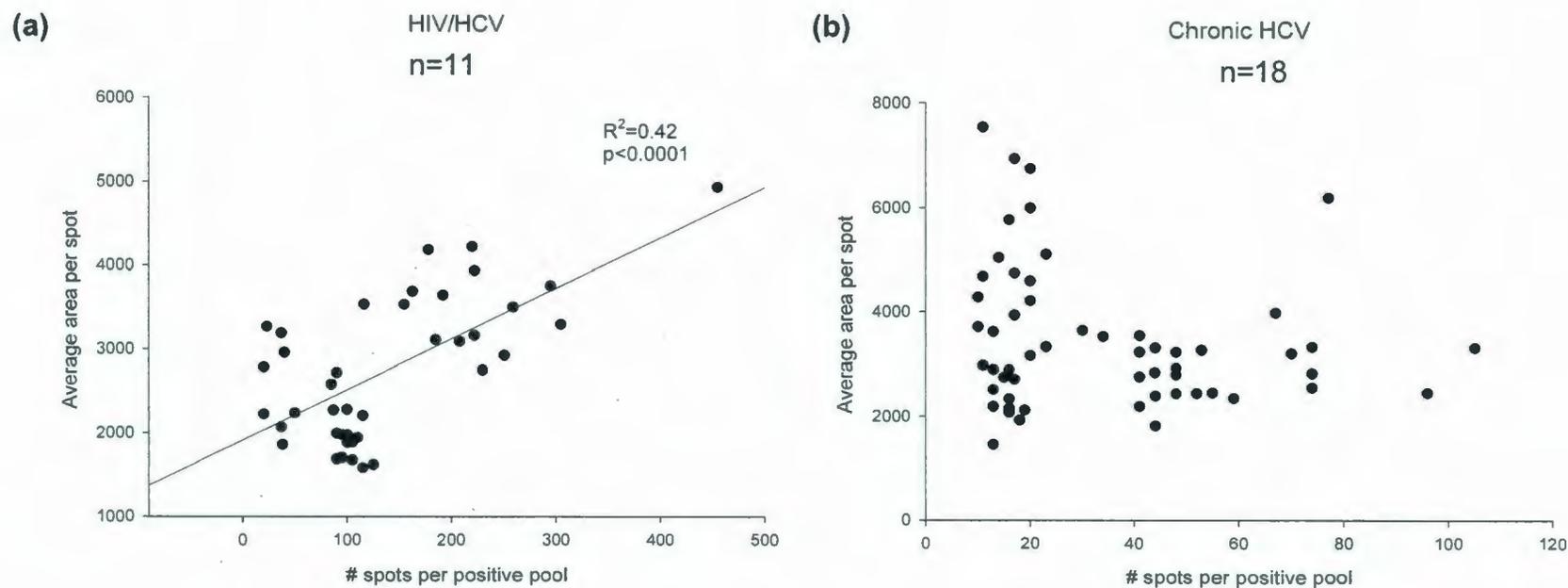


Figure 5.6. Relationship between IFN- γ spot size and cumulative magnitude of the HCV-specific CD8⁺ T cell response.

Peripheral blood mononuclear cells were incubated with pools of ten overlapping 18-mer HCV peptides for 18 hours and IFN- γ producing cells enumerated by ELISPOT. The magnitude of the IFN- γ response was plotted against mean spot size for the HIV/HCV coinfectd (a) and HCV-infected (b) groups and correlation assessed by linear regression.

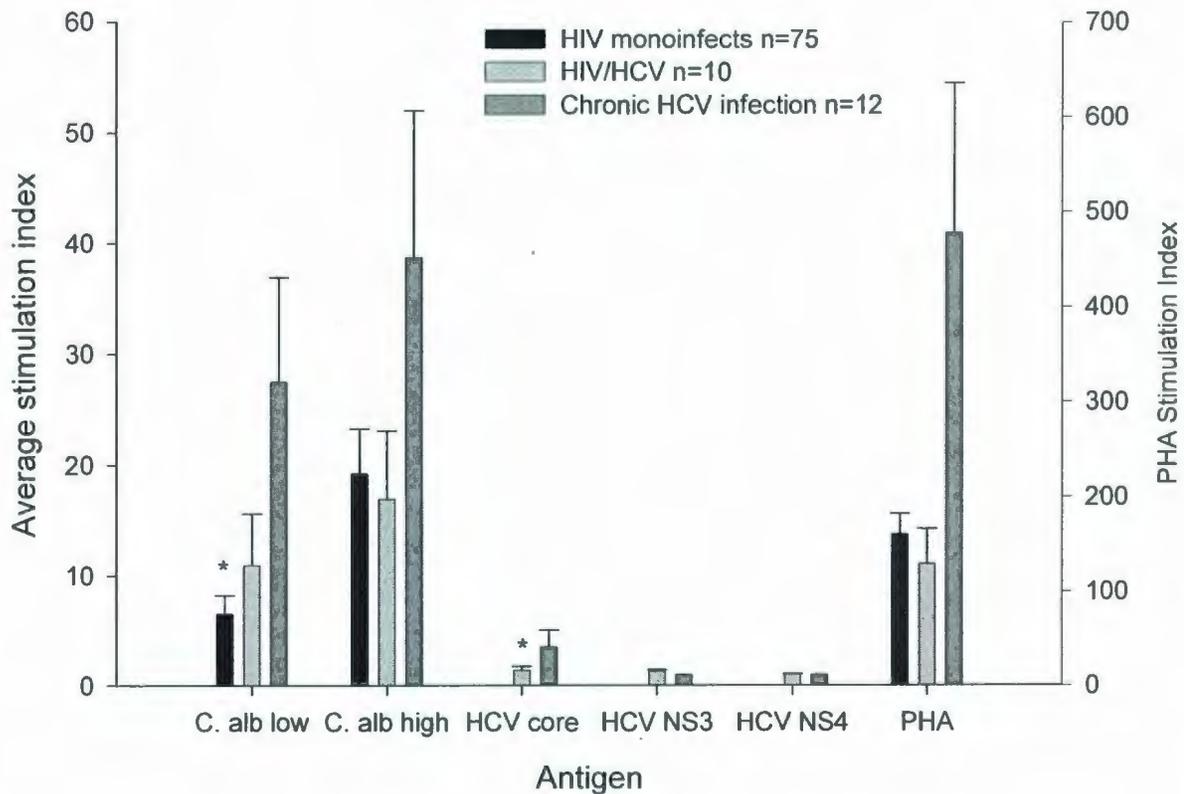


Figure 5.7. HCV-specific CD4⁺ T cell responses to mitogen, recall antigen, and HCV proteins. Peripheral blood mononuclear cells were incubated in triplicate for 5 days with phytohemagglutinin, *Candida albicans* antigen or HCV proteins and proliferation measured by ³H thymidine uptake. Responses were reported as stimulation indices calculated as the ratio of ³H counts in test wells divided by background ³H incorporation. Each bar shows the average for each group (SE). * - statistically significant differences between groups.

CHAPTER 6**DIFFERENTIAL CHARACTER OF HCV-SPECIFIC T CELL RESPONSES IN
SPONTANEOUS VERSUS TREATMENT-INDUCED VIRAL CLEARANCE**

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6.1. Abstract

Hepatitis C virus (HCV) is a common infection with approximately 170 million individuals infected worldwide. Most exposed individuals develop chronic infection and even with the newest antiviral therapies, only 50-75% of treated individuals sustain virologic responses. The role of adaptive immunity, particularly cell-mediated immunity, is well established in other viral infections, but is unclear in HCV infection. This makes design and development of prophylactic and therapeutic vaccine strategies difficult. We compared the character (breadth, specificity, vigor, and magnitude) of anti-HCV T cell responses in four groups of HCV exposed individuals: 18 chronically HCV-infected (HCV antibodies and HCV RNA); 5 spontaneous clearers (HCV antibodies, no HCV RNA); 16 sustained virologic responders (HCV antibodies, no HCV RNA post treatment); and 5 non-responders (HCV antibodies and HCV RNA post treatment). Interferon-gamma (IFN- γ) ELISPOT revealed relatively low numbers of HCV-specific CD8⁺ T cells in peripheral blood, especially in the non-responders. Anti-HCV CD4⁺ T cell responses, measured by ³H-thymidine proliferation, were rare, weak, and restricted to HCV core in all groups. The overall character of the anti-HCV CD8⁺ T cell response was strongest in virus clearers and weakest in those who failed treatment. However, individual components of character differed between spontaneous clearers and treated responders. The character of the anti-HCV T cell response differs between chronic infection and clearance and also between spontaneous and treatment-associated HCV

clearance. Therefore, therapeutic and prophylactic vaccine strategies may need to engage different characteristics of cell-mediated immunity against HCV.

6.2. Introduction

Hepatitis C virus is a global health problem, with over 170 million chronically infected people worldwide. The often insidious nature of early HCV infection and the high rate of chronic infection results in a large percentage of infected individuals who are unaware of their status. Approximately 80% of exposed individuals develop chronic infection, with detectable antibodies against HCV and detectable HCV RNA. The other 20%, termed spontaneous clearers, have anti-HCV antibodies, but no detectable serum HCV RNA. Standard of care treatment for chronic HCV infection is 24-48 weeks of pegylated interferon-alpha (PEG-IFN- α) and ribavirin. Depending on the HCV genotype they carry, between 50% and 75% of treated individuals have sustained virologic responses, meaning they are HCV RNA negative at treatment weeks 24 and 48 and remain so afterwards. Thus, the natural history of HCV infection and different treatment outcomes create four distinct groups of HCV exposed individuals: those who clear the virus spontaneously; those who develop chronic HCV infection; those who clear the virus with treatment, and treated individuals who fail to clear the virus.

Cell-mediated immunity is integral to clearance of viral infections. Not only is activation and expansion of virus-specific CD4⁺ and CD8⁺ T cell responses important, but the overall character of the cell-mediated response also appears key to the outcome of infection. People who spontaneously clear HCV have robust, broadly directed HCV-specific CD8⁺ and CD4⁺ T cell responses (199, 229, 347), while highly attenuated CD4⁺ and CD8⁺ T cell responses develop in chronic infection (198, 216, 411, 420). Several

studies have shown augmentation of CD4⁺ and CD8⁺ T cell responses during successful HCV treatment with IFN- α and ribavirin (351, 421, 422); however, reciprocal interactions between pharmacological and immunological suppression of HCV within the setting of successful treatment remain unclear.

Chronic HCV infection leads to significant morbidity and mortality, therefore, a prophylactic vaccine and more efficient immunotherapy would be highly beneficial. Since CD4⁺ and CD8⁺ T cell responses are associated with viral clearance, and may be augmented during immunostimulatory treatment with IFN- α , further delineation of the role HCV-specific T cell responses play in spontaneous and treatment-induced HCV clearance is required.

Most previous studies used individual peptides for assessing HCV-specific CD8⁺ T cell responses and did not directly compare the character of anti-HCV CD8⁺ T cell immune responses in spontaneous versus treatment-induced clearance. We used sets of peptides spanning the entire HCV polyprotein to assess HCV-specific T cell response character as a composite of four features: breadth, specificity, magnitude, and vigor. We then compared the composite character of anti-HCV CD8⁺ T cell responses in HCV persistence, treatment-induced clearance and spontaneous clearance. The composite character of the CD8⁺ T cell responses was stronger in the two groups that cleared the virus, but different components of character predominated in each group.

6.3. Materials and Methods

6.3.1. Study participants

HCV-infected individuals were recruited from the Capital Health Queen Elizabeth Hospital Hepatitis Clinic in Halifax, NS, Canada. Ethics approval for this project was obtained from the Human Investigation Committee at the institution and all subjects provided written informed consent for blood collection and immunological studies. Liver enzymes (AST and ALT) as well as liver function tests (INR, total bilirubin and albumin) were obtained on the same day as PBMC were collected for the study. Elevated AST and ALT is indicative of hepatic inflammation, while increased INR and total bilirubin or decreased albumin is suggestive of impaired hepatic function.

6.3.2. Identification of HCV infection

HCV exposure was determined using second or third generation EIA assays from Ortho Diagnostics to detect serum anti-HCV antibodies. HCV RNA viral load testing was performed using the Cobra Amplicor HCV Monitor v2.0 system (Roche Diagnostics, Laval, QC, Canada).

6.3.3. PBMC isolation

Acid citrate dextrose (ACD) treated whole blood was obtained by venipuncture from each individual and peripheral blood mononuclear cells (PBMC) isolated by Ficoll-HyPaque Plus (GE Health Care, Baie d'Urfé, PQ, CA) density gradient centrifugation. Cells were washed, counted, and frozen at -80°C until use. Samples with less than 50% viability when thawed were discarded.

6.3.4. Peptide preparation

A set of 18-mer peptides spanning the entire HCV genotype 1a polyprotein was obtained from the NIH AIDS Research and Reference Reagent Program. A total of 441 peptides overlapping by 11 amino acids were reconstituted in 80-100% sterile dimethylsulphoxide (DMSO), diluted to 200 µg/mL in lymphocyte medium, and then pooled together in groups of 10 consecutive peptides at a concentration of 20 µg/mL/peptide. Peptides were stored at -80°C until use, and added to cells at a final individual concentration of 10 µg/mL. Control wells contained 0.5% DMSO. The composition of each peptide pool is detailed in Appendix A.

6.3.5. ELISPOT for IFN- γ production

The number of IFN- γ producing cells in PBMC was determined by ELISPOT. Flat bottom polyvinylidene difluoride (PVDF)-coated 96-well plates (Millipore, Bedford, MA) were prewet with 100 µL 70% ethanol/well (Sigma Chemical Co.), washed with PBS, and coated either overnight at 4°C or for one hour at 37°C with 1.5 µg/well anti-IFN- γ antibody (clone 1D1K, Mabtech, Stockholm, Sweden) in PBS. Plates were washed five times with PBS and $1.0-2.0 \times 10^5$ intact or CD4⁺ T cell depleted PBMC were added to each well in duplicate. Peptide pools were added to each test well, and 2 µg/mL PHA or 100 IFN- γ positive C10/MJ cells (NIH AIDS Research and Reference Reagent Program, Popovic et al. (408)) served as a positive controls. Plates were incubated at 37°C, 5% CO₂ for 16 hours, washed with PBS, and incubated for 2 hours with 0.1 µg/well of biotinylated anti-IFN- γ antibody (clone 7-B6-1, Mabtech). Plates were washed, and 100

μL of a 1:1000 dilution of streptavidin-alkaline phosphatase (Mabtech) was added for 1 hour. After washing, 100 μL freshly prepared 3 mg/mL nitro blue tetrazolium chloride (NBT)/1.5 mg/mL 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine (BCIP) substrate (BioRad, Hercules, CA) in Tris buffer (pH 9.5) was added to each well for approximately 20 minutes. Plates were washed with distilled water to stop the color reaction and air-dried overnight before spot enumeration using the high resolution Zeiss reader system and associated KS software (Carl Zeiss Canada, Ontario, Canada). The number of IFN- γ producing cells per 10^6 PBMC was determined by multiplying the average number spots/duplicate well x 10 or x 5, depending if 1×10^5 or 2×10^5 cells were used. A peptide pool response was considered positive if the number of spots was greater than or equal to 10 spots and twice the background count. CD4⁺ T cell depletion done with two samples produced no difference in the number of peptide-induced IFN- γ ⁺ spots (data not shown). As each spot represents one cell, the area of the spot is related to IFN- γ production per cell (409, 410). Average spot area in arbitrary units was used as a relative measure of IFN- γ production per cell.

We assessed anti-HCV CD8⁺ T cell response character using four components: breadth, specificity, magnitude, and vigor. Breadth is the percentage of peptide pools to which an individual has a positive response. An individual peptide pool or HCV protein region was considered as a positive contributor to specificity if greater than 50% of individuals in the group had positive responses to that individual pool or within that HCV protein region. Vigor was measured in arbitrary units related to IFN- γ spot size that

correlate with IFN- γ production, and magnitude was the number of IFN- γ producing cells elicited by HCV peptides, per individual pool and cumulatively for the 44 pools. These factors were graded relative to the other groups, and the sum total used as a semi-quantitative assessment of anti-HCV T cell character.

6.3.6. Proliferation assays

CD4⁺ T cell immune responses were measured by standard 5 day ³H thymidine incorporation assays. PBMC were washed twice with proliferation medium (lymphocyte medium with 10% human AB serum (Atlanta Biologicals, lot number M0102) substituted for FBS) and 1x10⁵ cells/well were incubated in triplicate with either plain medium, 5 μ g/mL phytohemagglutinin (ICN Biomedicals Inc., Costa Mesa, CA), 2.5 or 5 μ g/mL *Candida albicans* (Greer Laboratories Inc., Lenoir, NC), or 2 μ g/mL HCV core, NS3, or NS4 (all from Virogen) for 5 days. One μ Ci ³H thymidine (Perkin Elmer Life Sciences, Boston, MA) was added to each well on day 4, and the assay was harvested onto glass fiber filter mats 18 hours later using a semi-automated harvester (Tomtec Harvester 96 Mach M III, Hamden, CT). Incorporated ³H-thymidine was measured by a 96 well scintillation counter (TopCount, Packard, Meriden, CT). Stimulation indices were calculated as follows:

$$\text{Stimulation Index} = \frac{\text{cpm}_{\text{antigen}}}{\text{cpm}_{\text{background}}}$$

6.3.7. *Statistical analyses*

All statistical analyses were performed using SPSS version 9 (SPSS Inc., Chicago, IL). Means were compared using either the Mann-Whitney U test or Student's *t*-test. All graphs indicate average values, and error bars represent standard error of the mean. Pearson's correlation coefficients were used to assess relationships between independent continuous variables for linear regression.

6.4. Results

6.4.1. Study participants

Eighteen individuals were chronically HCV-infected, 5 were spontaneous clearers, and 22 were treated for HCV infection (Table 6.1). Seventeen of the treated 22 had sustained virologic responses while 5 did not have a virologic response. All individuals in each group were assessed for HCV-specific cell-mediated immunity, except for sustained virologic responders where only 16/17 were available for assessment. Age and gender distribution were similar in the 5 groups, except for the spontaneous clearers, of whom four of five were female. All other groups were predominantly male. Similar proportions of individuals in the treated groups received each type of therapy. Forty-one percent of treated responders and 40% of the treated non-responders received PEG-IFN- α and ribavirin while 53% of the responders and 60% of the non-responders were treated with IFN- α and ribavirin. Risk factors for HCV infection were unknown for the majority of individuals in all groups; however, intravenous drug use (IVDU), blood product transfusion and organ transplant were the predominant risk factors identified in all groups. HCV viral load testing was done in treated individuals to monitor response to therapy, but only some of these results (8/17 treatment responders and 3/5 non-responders) were available. The variance was large and there was no significant difference in baseline HCV viral loads. Estimated duration of HCV infection was similar in all groups (mid-20 year range).

The majority of individuals in all except the treated non-responder group had normal liver enzymes on the sampling date. Two of five treated non-responders (40%) had abnormally elevated liver enzymes compared with 6% of treated responders and 27% of the chronic HCV group. Average AST and ALT levels were above normal limits in the chronically infected and the treated non-responder group. INR was normal in all groups, however albumin was decreased in the treated non-responder group. Overall, the treated non-responders had more liver enzyme elevation and decreased albumin, suggesting worse liver function. Available liver biopsy data indicated that the average stage and grade of liver pathology was similar in the treated non-responder and treated responder groups (data not shown).

6.4.2. Breadth of HCV-specific CD8⁺ T cell responses

We assessed the breadth of HCV-specific CD8⁺ T cell responses in PBMC from HCV exposed individuals using 44 pools of 10 overlapping 18-mer peptides spanning the entire HCV polyprotein in an IFN- γ ELISPOT assay. Results are expressed in terms of protein regions, which have different numbers of corresponding peptide pools depending on the protein size: 3 pools for core; 2 for E1; 6 for E2; 1 for p7; 3 for NS2; 9 for NS3; 2 for NS4A; 3 for NS4B; 7 for NS5A; 8 for NS5B.

The proportion of individuals responding to HCV peptides was not significantly different between groups. Only 14/18 (78%) chronic HCV individuals had HCV peptide-specific IFN- γ responses compared to 15/16 (94%) treated responders, 5/5 (100%) treated non-responders, and 5/5 (100%) spontaneous clearers. We compared the breadth of

HCV-specific responses by calculating the average percentage of positive pools in each of the 4 groups (Figure 6.1). There was no statistically significant difference between the groups, however, there was a trend toward broader responses in the spontaneous clearer (n=5; 26%±7.23%) and treated responder groups (n=16; 21.5%±4.2%). The chronically infected individuals had more restricted responses (n=5; 18%±4.57), while treated non-responders had very restricted responses (n=5; 8.6%±2.97%). The difference between spontaneous clearer and treated non-responder groups approached statistical significance (t=2.22, p=0.0567). All spontaneous clearers had responses against one or more of the 9 peptide pools spanning NS3. At the opposite end of the spectrum, peptide pools from p7 were rarely recognized by CD8⁺ T cells from any group. A hierarchy of response breadth was apparent, with broader HCV-specific responses associated with HCV clearance and the narrowest responses occurring in treated non-responders (Table 6.2).

6.4.3. *HCV peptide pool and region specificity of CD8⁺ T cell responses*

Figure 6.2 illustrates differences in the specificity of the CD8⁺ T cell response between groups at both the individual HCV peptide pool and HCV region levels. Regions and pools with greater than fifty percent responsive individuals were considered positive. Core, E1 and E2 are the 3 structural HCV proteins. All groups had responses to core and E2, however, neither the spontaneous clearers nor non-responders had detectable E1-specific responses (Figure 6.2a). All five non-structural proteins elicited responses from all groups except for NS4A, with no observed reactivity from non-responders. p7 is a small protein with unknown function against which only a small proportion (<20%) of

individuals in the chronic infection and treated responder groups reacted. All spontaneous clearers responded against at least one NS3 pool and the majority of spontaneous clearers also responded to 4 other regions (core, NS4B NS5A and NS5B). Treated responders had frequent responses to 3 regions (core, NS3, and NS5A). Chronically infected individuals responded to 2 regions, NS3 and NS4A. Responses against the NS2 region were most common in treated non-responders, though no regions met the positivity criterion.

To compare fine specificity between groups, we compared the frequency of responses to individual peptide pools. Those with greater than 50% of the individuals responding were considered positive (Figure 6.2b). Spontaneous clearers had frequent responses to 7 peptide pools overall: core pool 3; NS3 pools 16, 19, 20 and 22; NS4B pool 29; and NS5A pool 31. Treated responders had frequent responses to 1 peptide pool overall: NS5A pool 30. In the chronically infected and treated non-responder groups, no individual pools were positive in greater than 50% of individuals. In summary, NS3 responses were found in all who spontaneously cleared the virus (Table 6.2), and responses against pool 18/19 were particularly common among those who cleared the virus. Similar regions are frequently recognized among those who clear HCV, either through treatment or spontaneously. Treated non-responders lacked core and NS5 responses relative to the groups with spontaneous or treatment-induced HCV clearance.

6.4.4. HCV-specific CD8⁺ T cell response magnitude

The average magnitude of CD8⁺ T cell responses per peptide pool, as well as the cumulative magnitude, was calculated from ELISPOT results. Background was

subtracted from the responses seen to individual peptide pools and results for the magnitude presented as spots per 10^6 PBMC (Figure 6.3a). The magnitude of the average response per pool was not significantly different between the chronically infected HCV, spontaneous clearer, treated responder and treated non-responder groups (average \pm SE; 21 ± 4.2 , 23 ± 3.1 , 32 ± 11 , and 27 ± 6.5 respectively). All responses were weak, with a mean of less than 50 antigen specific cells in most cases. There was a trend toward higher magnitude responses for a given pool in the treated responder group.

The cumulative magnitude of the CD8⁺ HCV-specific T cell response was also examined (Figure 6.3b). A similar trend was seen as per pool. Cumulative responses were highest in the treated responder group, followed by spontaneous clearers and the chronic HCV group (average \pm SE; 441 ± 281 , 250 ± 77 and 162 ± 47 , respectively). Cumulative magnitude was least in the treated non-responders group (60 ± 13), but not significantly so. Some individuals were tested with only a subgroup of peptide pools due to limited PBMC numbers, and in those cases pools that had been shown to have frequent responses were used. The magnitude of the anti-HCV CD8⁺ T cell response to any given pool was low regardless of whether there was ongoing chronic infection or not (Table 6.2). The cumulative magnitude trends toward being least in the treated non-responders, and successively higher in the chronic infection, spontaneous clearer and treated responder groups respectively.

6.4.5. *HCV-specific CD8⁺ T cell response vigor*

Larger spot size in the IFN- γ ELISPOT indicates more IFN- γ production, therefore, spot size was analyzed in this study as a measure of response vigor. Spot area was measured in arbitrary units, and the average spot area per group calculated (Figure 6.4a). Average spot area was greatest in the treated responder group (4126 ± 211), and the range of values in this group was noticeably higher than in other groups. Maximum spot areas in the other 3 groups never exceeded 8000 while maximum values in the treated responders groups reached 12000 (Figure 6.4b). Although the treated responders trended toward a more vigorous response, there were no significant differences between the mean IFN- γ spot areas of the different groups overall. We also tested for associations between IFN- γ spot area and response magnitude in each group (Figure 6.4b). Average area per spot ranged from 1730 to 3980 in treated non-responders, 1451 to 7531 in chronically infected, 1805 to 11926 in treated responders, and 1787 to 5213 in the spontaneous clearers. There was more variance in spot area in the lower magnitude responses, with some of the largest spot areas associated with low magnitude responses. Anti-HCV CD8⁺ T cell response vigor in terms of IFN- γ production/responding cell was not associated with overall response magnitude in any group. Our results indicate that even in low cumulative magnitude responses, IFN- γ production/HCV-specific CD8⁺ T cell is not compromised. Table 6.2 summarizes the relationship between the groups with respect to vigor.

6.4.6. $CD4^+$ T cell responses

$CD4^+$ T cell responses against mitogen (PHA), recall antigen (*C. albicans*) and HCV viral antigens (core, NS3 and NS4) were assessed by standard 5 day proliferation assay. The frequency of $CD4^+$ T cell responses to each form of stimulation was determined in all groups except treated non-responders, and presented as the percentage of individuals with stimulation indices >3 (Figure 6.5a). Almost all individuals had PHA and recall antigen responses. However, only core protein stimulated HCV-specific proliferative responses, and the frequency was low in all groups. One of the two spontaneous clearers responded (light grey bars, 50%), compared to three of eight treated responders (dark grey bars, 38%) and 3 of 12 chronically infected individuals (black bars, 25%). The mean stimulation indices for responses to mitogen, and specific antigen for each group are shown in Figure 6.5b. Mitogen-induced proliferation did not differ significantly between groups. Responses against *Candida* were lower in spontaneous clearers (average \pm SE; 4.75 ± 0.25) than in the chronically infected group (27.5 ± 9.4). Core-specific responses were marginal for all groups tested (range of 3.5-5.5), compared with the vigorous recall antigen (range 4-41) and mitogen (range 373-641) responses. Mean HCV core-specific stimulation indices were not significantly different between the chronic HCV, spontaneous clearer, and treated responder groups (mean \pm SE; 3.5 ± 1.5 , 5.5 ± 3.5 , and 4.1 ± 1.8 , respectively). In general, HCV-specific $CD4^+$ T cell responses were rare, weak and detectable only against core. The relative magnitude of the responses did not differ significantly between groups (Table 6.2).

6.4.7. Overall T cell response character

Table 6.2 summarizes HCV-specific CD4⁺ and CD8⁺ T cell response characteristics in the four groups tested, and semi-quantitatively compares the character of the groups. CD4⁺ T cell responses were not assessed in the treated non-responder group, therefore, overall character was assessed both with and without CD4⁺ T cell responses to allow comparison across all groups. The character of HCV-specific T cell responses was strongest in the treated responder and spontaneous clearance groups (14+ and 13+ cumulatively) and least in the treated non-responder group (4+).

6.5. Discussion

HCV is a prevalent cause of liver disease and is associated with high rates of morbidity and mortality. It is rarely cleared by the immune system, and even the newest therapies remain ineffective for many chronically infected individuals. While it appears clear that cell-mediated immunity is important to viral clearance and control in other infections, its role in HCV infection is not well defined. Cases of spontaneous and treatment-induced viral clearance offer an opportunity to examine different roles and differential evolution of HCV-specific CD4⁺ and CD8⁺ T cell responses in these distinct settings. In this paper, we compared the character of the HCV-specific T cell immune responses in individuals who spontaneously cleared the virus, treatment responders, treatment non-responders, and untreated individuals with chronic HCV infection. The strength of individual anti-HCV CD8⁺ T cell features used to assess character (breadth, specificity, magnitude, vigor) differed between persistent and resolved infection, as well as between HCV-negative groups that cleared the virus. However, the overall strength was greater in the HCV RNA negative groups than the HCV RNA positive groups.

Spontaneous clearance of acute HCV infection is associated with broadly directed, high magnitude HCV-specific CD8⁺ T cells responses (224, 229). Although CD8⁺ T cell responses are stronger and more frequent shortly after viral clearance than years later, they remain detectable by ELISPOT (423) at an average frequency of 0.15% of circulating CD8⁺ T cells even in those with resolved infection. In the case of treatment associated viral clearance in acute and chronic infection, the kinetics of CD8⁺ T cell

responses vary between studies. Some studies report augmentation of peripheral CD8⁺ T cell responses (290, 351) while other studies describe a rapid decline in HCV-specific CD8⁺ T cells after treatment response (350, 424, 425). Our results support higher magnitude CD8⁺ T cell responses after successful treatment than in chronic infection. Treated responders are likely immunologically different than spontaneous clearers, as they are unable to clear HCV without IFN and ribavirin therapy. It may be that treated responders have relatively weak pre-treatment CD8⁺ T cell responses that are boosted indirectly by IFN- α treatment. It is also possible that these individuals benefit from the direct viral suppression of pharmacologic HCV treatment, and reduced viral antigenemia allows otherwise 'stunned' HCV-specific cells to respond.

We utilized spot area as a measure of IFN- γ secretion per reactive cell to estimate response vigor. Vigor was greatest in treated responders and appeared somewhat blunted in spontaneous clearers. At the individual cell level, IFN- γ response magnitude may not be critical for clearance, but may more closely reflect recent viremia, as vigor is higher in chronically infected patients. Also contributing may be that CD8⁺ T cells observed in the longstanding spontaneous clearers are central memory cells, which produce less IFN- γ than the effector memory cells more likely found in those with ongoing infection. This also suggests that persistent infection is not just a product of impaired IFN- γ production by HCV-specific cells, but also involves other defects in the cellular response, as detailed by other groups (347, 411).

Overall, the HCV-specific CD8⁺ T cell response was greatest in those clearing the virus, but herein, breadth was the distinguishing feature in spontaneous clearers, while magnitude and vigor were the distinguishing features in treated responders. Our results suggest that in varied settings, different aspects of the immune response can be of selective value towards HCV clearance and the overall character of the response, as opposed to any of just magnitude, breadth, specificity or vigor alone is key. For example, a CD8⁺ T cell response diminished in one component (breadth, specificity, magnitude or vigor) may effectively clear the virus if other components compensate. The T cell response overall was strongest in the groups that were HCV RNA negative, reinforcing the perceived role for HCV-specific CD8⁺ T cells in viral clearance.

We also examined CD4⁺ T cell responses in the different groups, as these are important components of immunity and aid in the development of effective CD8⁺ T cell responses. Previous studies compared the character of CD4⁺ T cell responses in acute infection before viral clearance, with the CD4⁺ T cell character observed in chronic HCV infection. Responses to NS3 were associated with clearance in one study (413), while core responses were more frequent in another (426). These studies used recombinant HCV proteins and proliferation assays similar to ours to assess CD4⁺ T cell responses. However, they observed a higher frequency, magnitude and breadth of responses in spontaneous clearers than we did (stimulation indices of 14 and 20 compared to 3 and 4 in our study). Results for the chronic group were similar to ours with respect to magnitude, but not specificity.

Another group used a combination of proliferation assays, ex vivo intracellular IFN- γ staining, and bulk in vitro stimulation to examine CD4⁺ T cell responses in spontaneously resolved infection (348). The three spontaneous clearers in this study had cleared HCV relatively recently (between 7 months and 2.5 years), and a 5-fold higher recombinant HCV protein concentration was used for proliferation assays than in our study. They observed broad reactivity against all HCV regions, with stimulation indices frequently greater than 20 and as high as 120. This is in stark contrast to our findings of limited specificity and magnitude CD4⁺ T cell responses, even in spontaneous resolution. This may relate to the duration since viral clearance and to the very high HCV protein concentrations used. The mean time since HCV infection in our study was greater than 20 years in each group, significantly longer than most other studies. Not all studies publish estimates of the time of infection or of probable spontaneous clearance, and therefore, it is difficult to estimate how different our cohort is in this regard. The low numbers of spontaneous clearers available for comparison may also be an issue. One study of HCV-exposed health care workers that followed HCV-specific proliferative responses over the course of acute infection and resolution (200) found that CD4⁺ T cell responses peaked 12 weeks after exposure and decreased to <25% of maximum by 27 weeks (only 4 weeks post clearance). Another study followed 2 individuals with the same known exposure and either spontaneous or treatment-induced HCV clearance (427). The person with spontaneous clearance had broad, but low magnitude CD4⁺ T cell responses extending to 24 months, while low magnitude CD4⁺ T cell responses in the

treated responder were only detected after treatment. Other viral infections, such as the highly informative and influential lymphocytic choriomeningitis virus (LCMV) model, have documented declining CD4⁺ T cell memory over time (428), which may reflect the situation after viral clearance in HCV infection as well.

Treatment with IFN- α and ribavirin appears to augment HCV-specific CD4⁺ T cell responses measured by either proliferation (421) or IFN- γ production (351). One study compared treatment-associated clearance with spontaneous clearance prospectively by proliferation and ELISPOT assay (429). PEG-IFN- α plus ribavirin treatment was associated with increased breadth, frequency and magnitude of HCV-specific CD4⁺ T cell responses compared to those chronically infected, but the character of the response was similar to spontaneous clearers. The subjects in this study had an unusually high treatment response rate, probably reflecting a preponderance of HCV genotype 4, while HCV genotype 1 is most common among our study subjects. The absolute magnitude of the CD4⁺ T cell response was higher in our spontaneous clearance group, and lower in the treated responders group, even though some of the individuals were just finishing treatment and likely at the peak of the CD4⁺ T cell response according to published CD4⁺ T cell response kinetics (429). Even at their maximum, the responses we observed were lower in magnitude and breadth, suggesting that in cleared infection, regardless of the route, HCV-specific CD4⁺ T cell responses do not have to be maintained at readily detectable levels in peripheral circulation for continued viral suppression.

There are certain limitations to this study. While comprehensive in that we examined peptides spanning the entire HCV polyprotein, we used peripheral cells only, and it is clear that intrahepatic T cell responses may differ in magnitude and specificity (415, 416). Even so, we would expect differences between groups to be maintained in the periphery. Also, our peptides were derived from HCV genotype 1a consensus sequence and although most of our individuals were identified as HCV genotype 1, it is possible that autologous sequences were different enough that we missed some immunologically significant T cell responses. This would be most likely in non-genotype 1a individuals or those who are chronically infected and have high virus loads where ongoing viral replication and speciation are common. Duration since clearance is also significantly different between the spontaneous and treated clearer groups in our study. Therefore, the magnitude of the response may be relatively reduced in the spontaneous clearer group just because of greater degeneration time. It will be interesting to compare the evolution of immune responses in spontaneous clearers with those of treatment-induced clearers from an equivalent starting point over the course of years.

Chimpanzee studies have demonstrated the importance of CD8⁺ T cell responses in HCV infection (400), and a recent T cell directed vaccine trial in chimpanzees linked viral clearance after challenge with robust T cell responses (430). When designing vaccines, research often focuses on a few highly immunogenic target regions. Our results suggest that there may be other ways to promote viral clearance in HCV infection. Although overall magnitude and NS3 reactivity appear most important, the general

character of the response may hold the key to clearance. We found qualitative and quantitative differences in the character of HCV-specific CD8⁺ T cell responses in comparing individuals who effectively cleared the virus by two different mechanisms. This raises the possibility of multiple effective ways to augment HCV-specific T cell responses. HCV mutates rapidly and as such, fine specificity-based vaccines may have limited applicability. Our data suggest that the individual epitopes targeted may be less important than a strong overall CD8⁺ T cell response, and that there may be multiple ways to effectively produce a sterilizing immune response.

6.6. Acknowledgements

We thank the individuals attending the QEII Hepatitis Clinic for participating in this study.

Table 6.1. Baseline clinical parameters

	Chronic HCV	HCV Exposed, RNA Neg	HCV Tx responders	HCV Tx non-responders
Number (n)	18	5	17	5
Age (yrs)	47±10	49±11	49±7.5	48±3
Sex (%)				
M	77	20	53	80
F	23	80	47	20
HCV Genotype (%)				
1	40	0	59	60
Non-1	5	0	6	0
Unknown	55	100	35	40
HCV Risk Factor (%)				
IVDU	50	40	52	20
Transfusion	14	40	29	20
Tattoo	5	0	0	20
Endemic area	14	0	0	20
Unknown	23	20	23	40
Est. duration of infection (yrs)				
HCV	26±10	24±6	24±8.5	ND

Table 6.1. (con't)

	Chronic HCV	HCV Exposed, RNA Neg	HCV Tx responders	HCV Tx non-responders
HCV Treatment (%)	N/A	N/A		
IFN- α			6	0
IFN- α + Ribavirin			53	60
PEG-IFN α + Ribavirin			41	40
HCV baseline viral load (IU/mL)	ND ¹	ND	779,111 \pm 1,332,464	1,913,333 \pm 2,413,344
Labs (Avg)				
AST (IU/L) ²	59	28	31	78
ALT (IU/L) ³	105	22	31	81
INR ⁴	1	1	1.1	1.1
Total bilirubin (μ mol/L) ⁵	11	10	11	16.5
Albumin (g/L) ⁶	40	38	41	31

¹ND, not determined ²AST, aspartate aminotransferase; normal range 10-42 IU/L ³ALT, alanine aminotransferase; normal range 10-41 IU/L ⁴INR, International normalized ratio normal range 0.9-1.1 ⁵Total bilirubin normal range 0-16 μ mol/L

⁶Albumin normal range 38-50 g/L. Bold numbers are outside the normal range.

Table 6.2. Comparison of HCV-specific T cell response character in different groups. Responses from each of the groups are graded relative to the other 3 groups and summarized as a measure of the strength of the overall anti-HCV T cell response.

	Chronic Infection	HCV exposed RNA negative	Treated Responders	Treated Nonresponders
CD8⁺ T cell response				
<i>Specificity</i> ¹	NS3, NS4A (++)	core, NS3, NS4B, NS5A, NS5B (+++++)	core, NS3, NS5A (+++)	None (0)
<i>Relative Breadth</i> ²	++ ⁵	++++	+++	+
<i>Relative Magnitude</i> ³	++	+++	++++	+
<i>Relative Vigor</i> ⁴	+++	+	++++	++
CD4⁺ T cell Response	+	++	+	NT ⁶
Total ⁷	10+/9+	15+/13+	15+/14+	ND ⁸ /4+

¹Regions with greater than 50% of individuals responding are indicated. ²Breadth is the percentage of peptide pools to which an individual has greater than twice the number of background spots. ³Magnitude is the average cumulative number of IFN- γ positive spots per pool. ⁴Vigor is the average spot size per group. ⁵Relative response with respect to the four groups: + is least and +++++ is maximum. ⁶NT, not tested. ⁷Sum of the number of pluses per group. The first number is calculated with CD8⁺ and CD4⁺ T cell responses; the second number is CD8⁺ T cell responses alone. ⁸ND, not done.

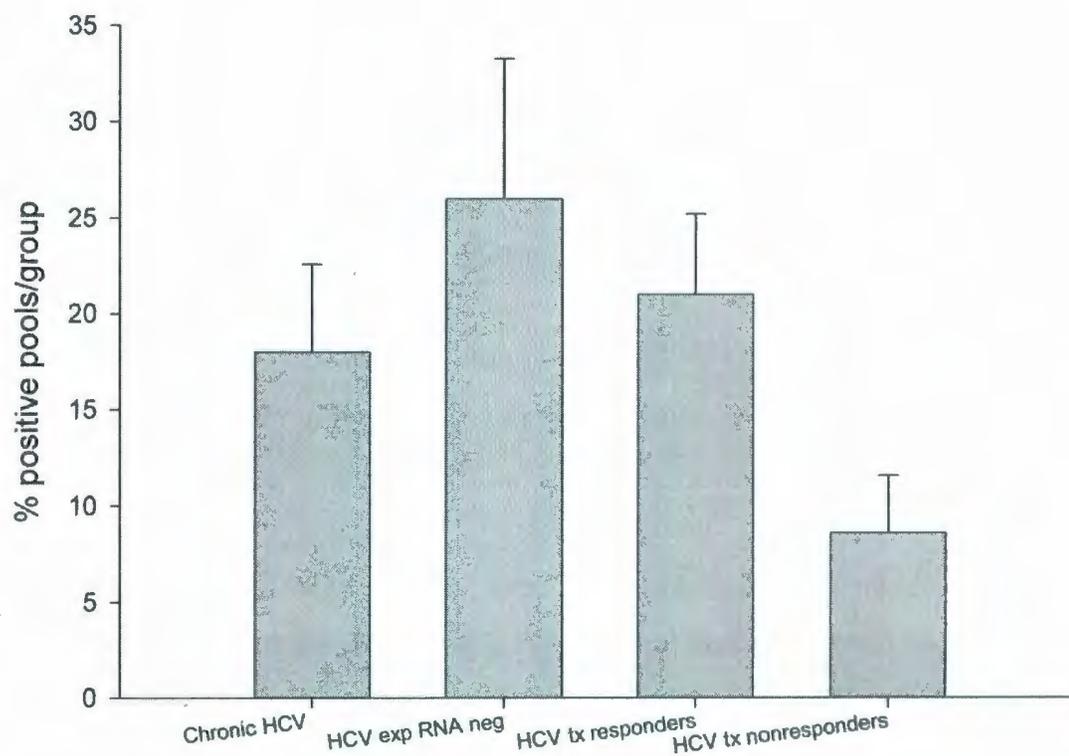


Figure 6.1. HCV-specific CD8⁺ T cell response breadth. After overnight incubation of PBMC with one of 44 peptide pools, each containing 10 overlapping HCV 18-mers, IFN- γ producing cells were visualized and enumerated by ELISPOT. Responses were considered positive if the number of spots in wells with HCV peptides was more than twice background. Bars represent the mean percentage of positive pools for individuals in each group (\pm SE). There were 18, 5, 16, and 5 individuals in the HCV chronic, spontaneous clearer, treated responder, treated non-responder groups respectively.

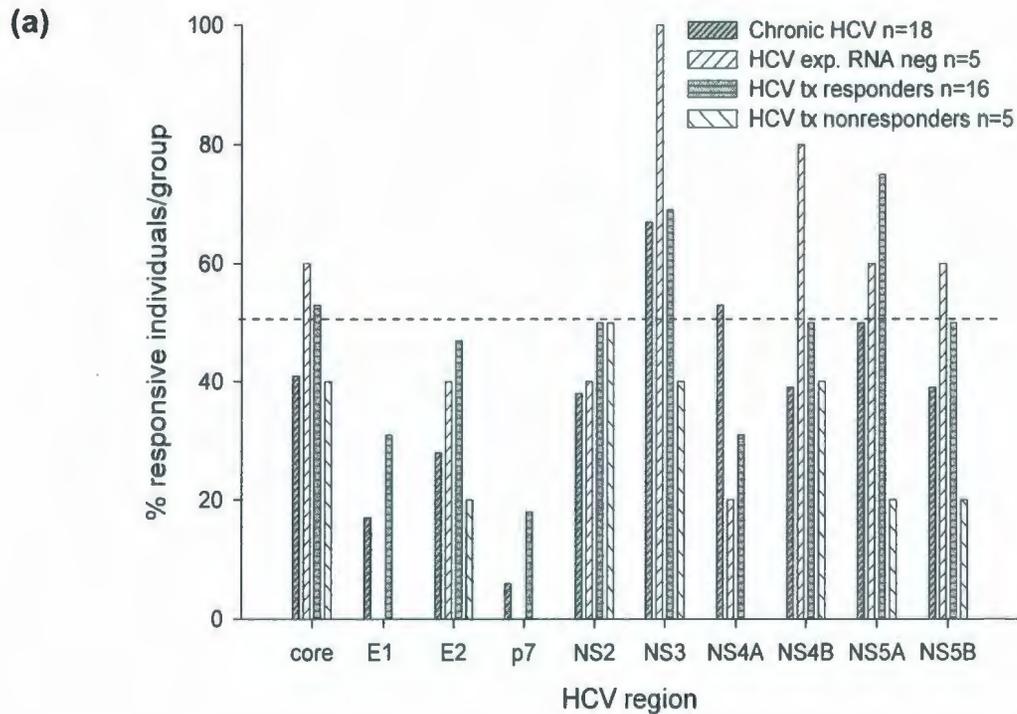


Figure 6.2. HCV region and pool-based specificity CD8⁺ T cell responses. (a) The percentage of individuals with positive responses to each HCV region is shown. Dark, fine hatched bars represent chronic infection (n=18), white hatched bars for spontaneous clearers (n=5), dark horizontal hatch bars for treated responders (n=16) and white left slanted bars for treated non-responders (n=5). The horizontal line indicates the threshold for a positive response.

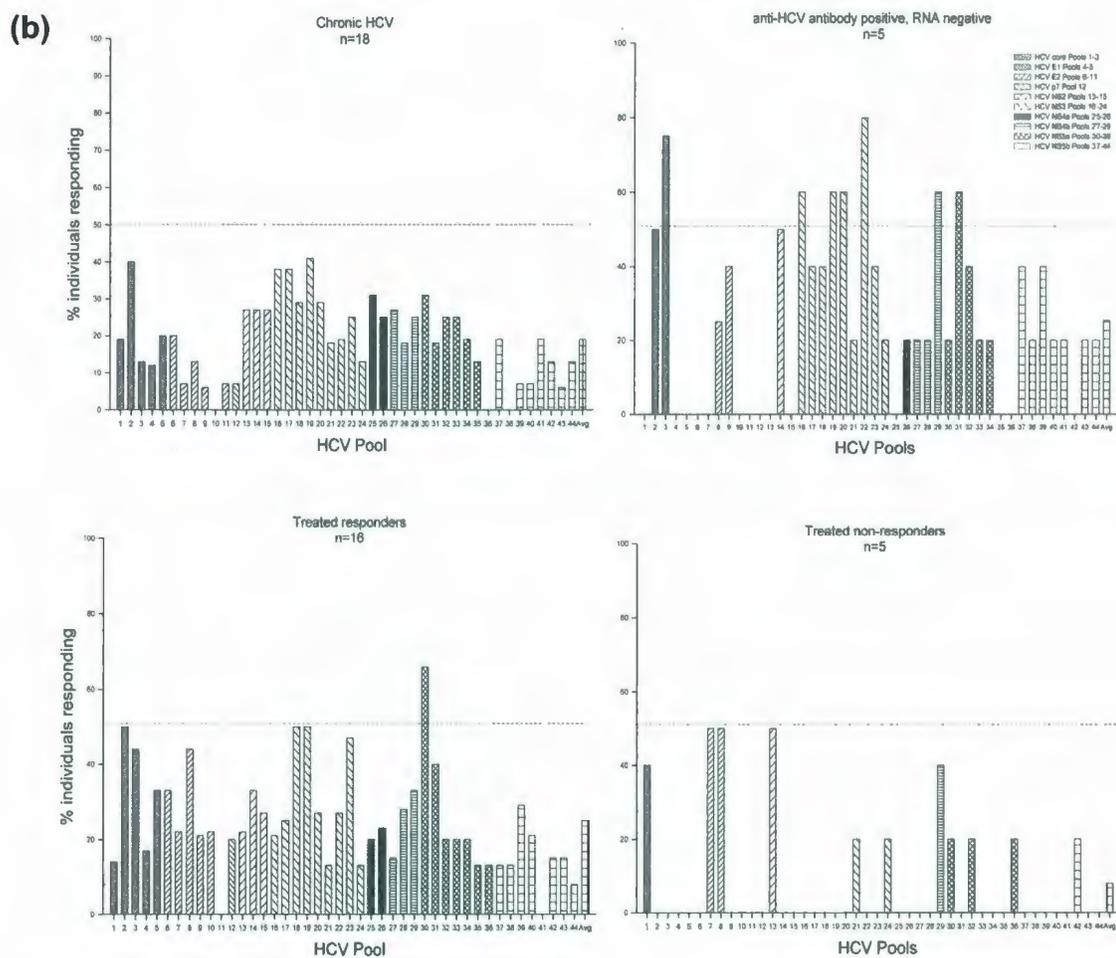


Figure 6.2. (con't) (b) The percentage of individuals with positive HCV responses to each HCV pool is shown. An HCV region or pool was considered a significant target for a group if more than 50% of individuals in that group responded. The horizontal line indicates the threshold for a positive response.

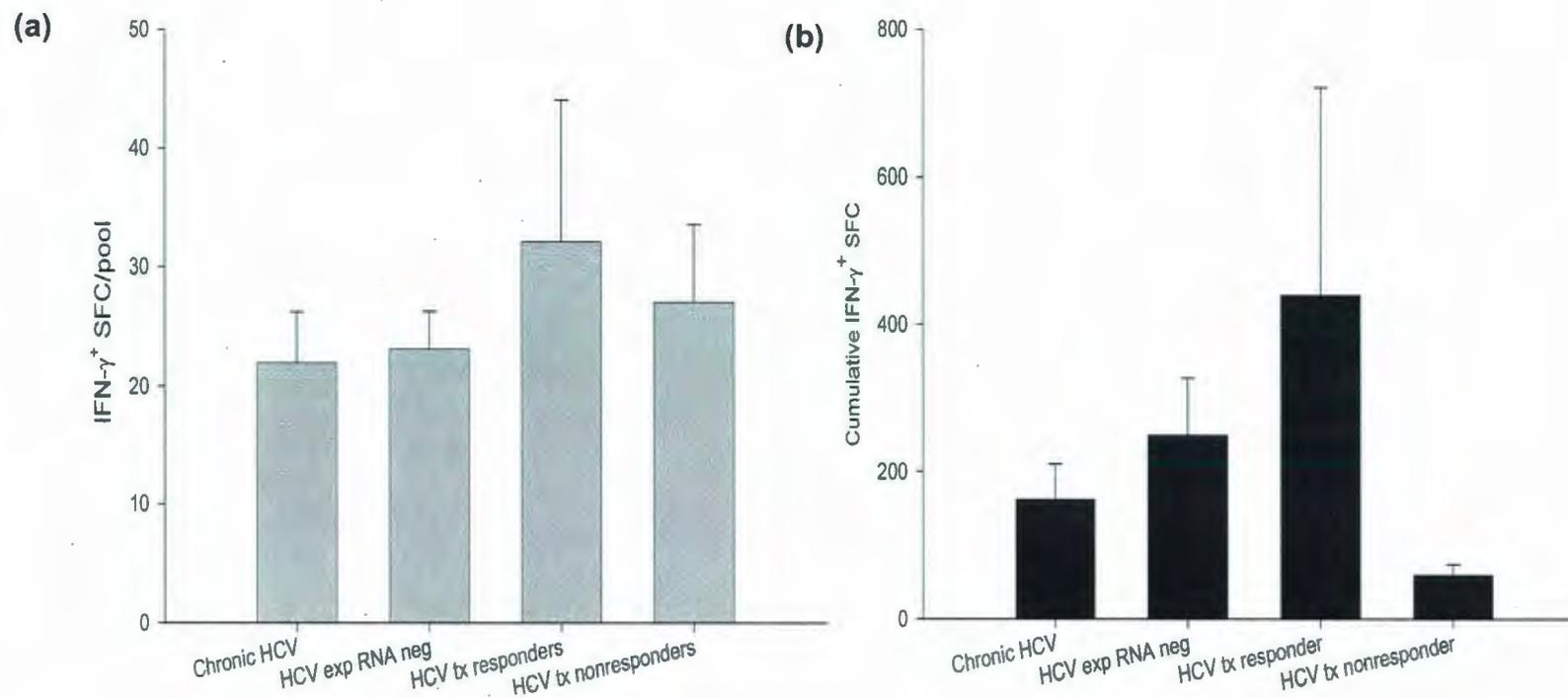


Figure 6.3. HCV-specific CD8⁺ T cell response magnitude. (a) Grey bars indicate the mean (\pm SE) number of IFN- γ producing cells per million PBMC in positive peptide pools for each group. The Mann-Whitney U test was used to assess significant differences between groups. (b) Black bars indicate the mean (\pm SE) cumulative number of IFN- γ producing cells stimulated by all peptide pools in each group. The Mann-Whitney U test was used to test for differences between groups.

(a)

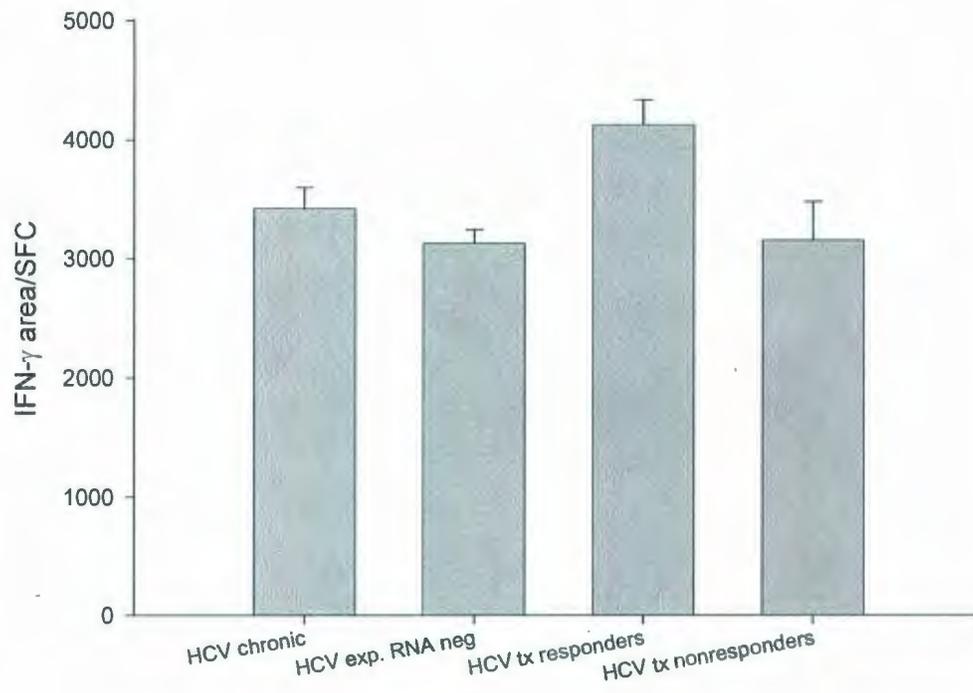


Figure 6.4. HCV-specific CD8⁺ T cell response vigor. (a) The amount of IFN- γ produced per cell was assessed by measuring spot size in arbitrary units (termed vigor), and the mean \pm SE for each group is shown in grey bars.

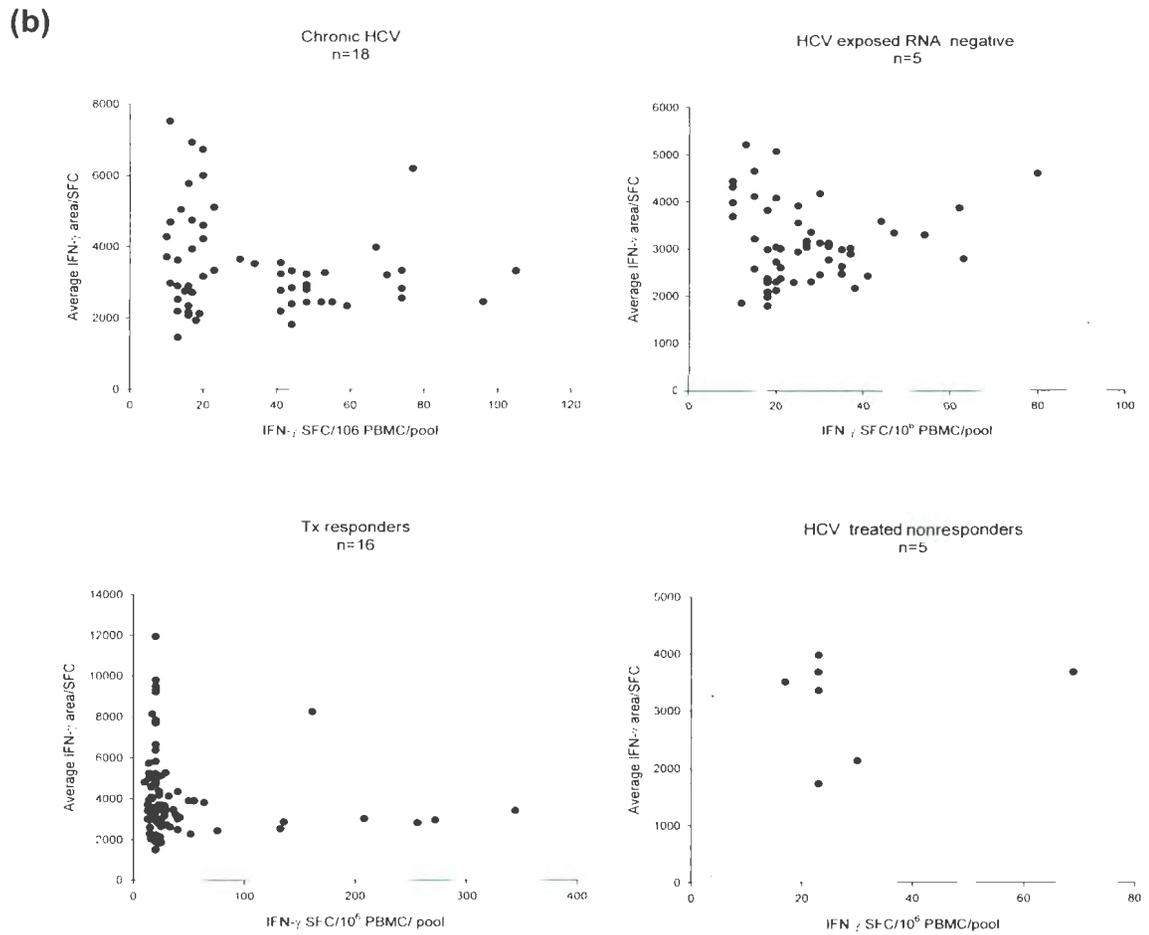


Figure 6.4. (con't) (b) Response magnitude was plotted versus the vigor of the response in each group. Correlation was assessed by linear regression.

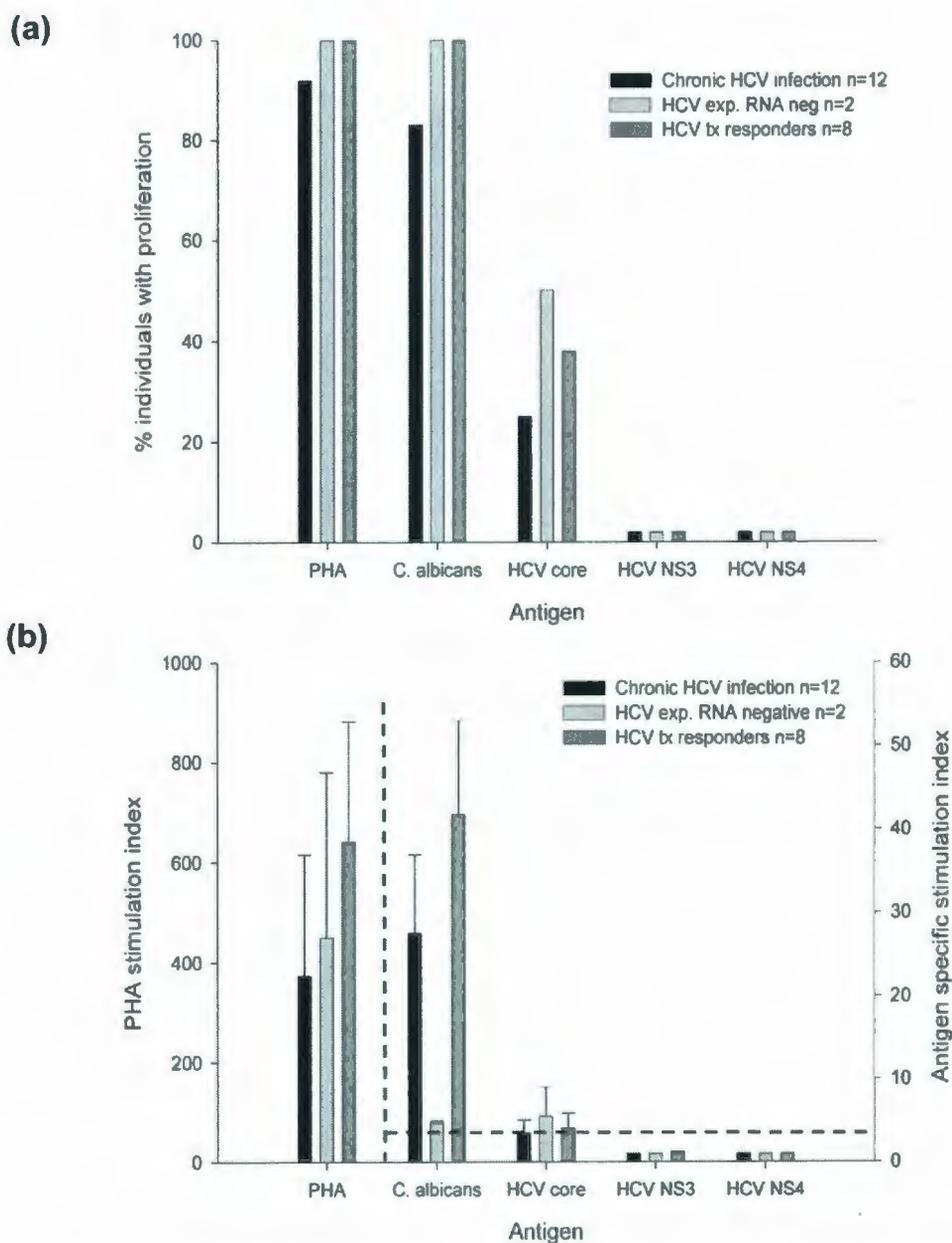


Figure 6.5. CD4⁺ T cell responses. (a) The frequency of CD4⁺ T cell responses to mitogen (PHA), recall antigen (*Candida albicans*), and HCV antigens (core, NS3, NS4) was determined by proliferation assay for members of the chronic infection (n=12), spontaneous clearer (n=2) and treated responder (n=8) groups. (b) The average magnitude (\pm SE) of CD4⁺ T cell responses to mitogen (left hand axis), recall antigen and HCV antigen (right hand axis) is shown for each group. A stimulation index greater than 3 (indicated by the horizontal dashed line) was considered positive.

CHAPTER 7

SUMMARY AND DISCUSSION

Persistent infections are generally deleterious to the host, causing both immediate and long term pathology that cause increased morbidity and accelerated mortality. However bad it is for the infected person, persistent infection offers the opportunity to use an unsuccessful response to better understand the sensitivities or weak spots of the immune system in its unsuccessful interactions with various invaders. The list of weapons incorporated into the pathogen arsenal in order to frustrate sterilizing immunity continues to grow. Viruses have been particularly adept at evolving mechanisms to subvert the immune response. In this work, we investigated whether HCV gene products induce IL-10, an immunomodulatory cytokine that facilitates chronic infection. We identified and phenotyped a novel cell population producing this immunomodulatory cytokine, not just in those infected with HCV but in all healthy controls tested. The production of IL-10 by this new cell type may have a role in immune system homeostasis through immune regulation, but may also contribute to viral persistence. More of these previously undescribed cells produce IL-10 when exposed to HCV proteins, an effect that is accentuated when HCV-naïve cells from HIV-infected individuals are exposed to HCV proteins. We demonstrated more robust anti-HCV T cell responses with viral clearance, and distinguished features of anti-HCV T cell responses associated with spontaneous versus treatment-induced clearance. We also described distinct HCV-specific T cell responses in HIV coinfection. Together, these findings emphasize the role of the immune system in HCV clearance, as well as in control of chronic infection and illustrate the

complex interactions between chronic pathogens in terms of their effects on the immune system.

Our first objective was to identify cells that may be involved in modulating the immune response to HCV. While we anticipated that HCV proteins might induce some regulatory cytokines such as IL-10, we did not anticipate that the cells producing it would be a previously undescribed peripheral blood mononuclear leukocyte population. Surface antigens expressed on these cells are consistent with bone marrow origin, however, they do not conform to any previously defined lineage. One possibility is that they represent a precursor population that has not yet fully differentiated. This rare population was found in all individuals and produced IL-10 *ex vivo*, suggesting an as yet unidentified *in vivo* stimulus that maintains IL-10 gene expression at some level. Given the link between increased IL-10 and CD36 expression, several of the ubiquitous CD36 ligands such as apoptotic bodies or oxidized LDL are candidates for providing this stimulus. In the context of HCV infection, the potential role of apoptotic bodies in generation of the pro-fibrogenic and tolerogenic cytokine IL-10 holds special interest. Intact HCV and HCV derived proteins have been implicated in induction of apoptotic cell death (431, 432), which may facilitate IL-10 production from our cell population. This could offer an additional mechanism for the hallmark fibrogenesis and viral persistence characteristic of HCV infection.

While we have concentrated on the role of this novel cell population in response to viral infection, it is possible that these cells also have a normal physiological function as homeostatic agents helping to preserve self-tolerance. These cells may also offer the

opportunity to therapeutically modulate overzealous immune responses in autoimmunity by appropriate temporal and spatial direction of their IL-10 production towards specific target sites. Multiple sclerosis, for example, is an autoimmune disease treated in some instances with IFN- β , a type I interferon. Wang et al. demonstrated an IL-10 dependent decrease in IL-12 production in PBMC from IFN- β treated multiple sclerosis patients compared to controls (433). This study suggests a role for IL-10 in disease amelioration, however, the usefulness of exogenous IL-10 administration has been hampered by limited bioavailability, variable serum concentration, and short in vivo half life (434). The cells we have identified may provide a physiological mechanism of IL-10 induction through the use of naturally occurring CD36 ligands that would attenuate autoimmune disease while avoiding the limitations of external IL-10 administration. Further in vitro work on the manipulation of our cell population will be necessary to determine the distribution of this population, as well as the potential for IL-10 induction.

The next steps in harnessing the potential of this population involve more extensive physical and functional characterization together with development of an in vitro culture system. Our current studies have been limited by the relatively low number of IL-10⁺ cells in peripheral blood. By devising a method to culture, expand and possibly transform them in vitro, it may be possible to develop a cell line appropriate for long term in vitro experiments. Access to human lymphoid tissue, perhaps from cadaveric organ donors or leukaphoresis, would provide an excellent opportunity to isolate, sort and culture potentially large numbers of these cells. This would also be useful in allowing large scale microarray analysis to perform a more exhaustive phenotypic description of

the gene expression profile. This would facilitate comparison of the gene 'fingerprint' of our cells with well established populations such as T cells, B cells, monocytes, NK cells, and stem cells in an effort to further delineate the cell lineage and ascertain their function. Exploration of small animal models such as the mouse for a comparable cell population will be another important next step. This would offer a readily manipulable system to study the effects of this population, as well as its frequency and distribution in lymphoid tissue and relative levels in murine models of autoimmunity, immunodeficiency and immunosuppression. Given that CD36 may play a large role, it would be particularly interesting to explore the immune function and response to infection of the already developed CD36 knockout mouse (435).

As described in chapter 4, more mononuclear cells produce IL-10 in response to several HCV proteins in HCV-naïve individuals with HIV infection than in HCV-naïve controls. In contrast, IL-10 induction was not observed in the context of chronic HCV infection, and this lack of IL-10 may contribute to the hepatic immunopathology observed in persistent HCV infection. If there is ongoing antigenemia from chronic viral infection and replication, anti-HCV immune cells are activated and recruited to the liver. Here, cytokines may reduce viral load by eliminating infected hepatocytes, however, they also promote a profibrogenic environment that leads to liver fibrosis and eventually cirrhosis. The presence of IL-10 is anti-inflammatory and may attenuate the detrimental hepatic effects of inflammation. While the number of HCV monoinfected individuals in our comparison was small, the inter-individual results demonstrating little IL-10 produced by PBMC in response to HCV proteins were very consistent. These results

suggest the establishment of persistent infection in the context of increased levels of immunomodulatory cytokines, and subsequent attenuation of the IL-10 induction response that allows progressive disease in chronic infection. Our IL-10 results also correlate with the proliferation studies in HIV/HCV coinfecting individuals, which implies that the HCV-induced IL-10 may be associated with a lack of CD4⁺ T cell proliferation. It will be necessary to extend these studies with more individuals, and determine how the structurally dissimilar HCV core NS3 and NS4 proteins generate a common effect on IL-10 production.

One of the main modulatory effects of IL-10 is indirect attenuation of T cell responses. Based on the results presented in chapter 3, we might expect that attenuated IL-10 production from the CD36 population in the setting of underlying HCV infection may allow anti-HCV T cell responses to develop. In fact, we observed a larger magnitude response in the HIV coinfecting population than the chronically infected HCV individuals, but the breadth of the CD8⁺ T cell response remained limited. This suggests that while the IL-10 production seen in chronic HCV infection may play a role in limiting the magnitude of the T cell response, there are other factors that attenuate the breadth and specificity of the response that may not be IL-10 dependent. Mechanisms of impaired T cell function such as anergy and exhaustion are not likely to be directly dependent on IL-10, however, the indirect effects of IL-10 on APC may play a role. Further phenotyping of both the responsive and non-responsive HCV-specific T cells for markers of effector function and differentiation will provide further insights into specific deficits in T cell function that limit sterilizing immunity in the context of chronic HCV infection.

While studying HIV coinfection offered an opportunity to assess immune responses in the setting of relative immunodeficiency, selecting individuals who clear HCV either spontaneously or after anti-viral therapy allowed comparison of T cell responses in the context of two different forms of successful HCV eradication. Not unexpectedly, we found more robust anti-HCV T cell responses in individuals who cleared the virus compared to those with chronic infection, underscoring the importance of the T cell response in viral clearance. However, there were substantial differences between the character of the CD8⁺ T cell responses generated in spontaneous viral clearance compared with treatment-associated viral clearance. Pharmacologic therapy provides anti-viral activity directly against HCV replication in addition to indirect effects through immune stimulation with IFN-alpha. This seems to produce a different type of T cell response that is as efficacious in some instances as the immune response of spontaneous clearers. Further studies to ascertain the functionality of anti-HCV T cells will be important, and determination of individual epitopes will also provide insight into potential immunogen targets. HLA type was unavailable for these patients, and while they came from a relatively heterogeneous population, it will be important for further studies of epitope mapping to obtain this information. For similar reasons, having HCV genotypes would also be useful.

We have shown that strong CD8⁺ T cell responses are important for viral clearance, and that the character of successful responses differs between various modes of clearance. We have also confirmed that HCV proteins induce IL-10 production, and identified a previously undescribed population of mononuclear cells that can be

manipulated by HCV proteins to produce more IL-10. This work provides insight into what constitutes a successful immune response to HCV, how the virus may cause worse disease in HIV coinfection, and provides a potential target for immune manipulation to ameliorate immunopathogenesis. Further studies to delineate how effector T cell maturation is affected by HCV and to elucidate the lineage of CD36⁺IL-10⁺ cells will provide exciting information that impacts not just HCV infection, but immunology in general.

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APPENDIX A

Appendix A. HCV 18-mer peptide (NIH Research and Reference Reagent Program, Catalogue # 7620) pools. 441 peptides, overlapping by seven amino acids. Each pool consists of 10 adjacent peptides.

Pool number	HCV protein	Amino Acid
1-3	Core	1-192
3-5	E1	192-384
5-11	E2	384-747
11-12	p7	747-810
12-15	NS2	810-1027
15-25	NS3	1027-1658
24-26	NS4A	1658-1712
25-29	NS4B	1712-1973
29-36	NS5A	1973-2421
36-44	NS5B	2422-3011



