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T-Cell Dysfunction by HCV Core Protein Involves PD-1/PD-L1 Signaling

A dissertation

presented to

the faculty of the Department of Internal Medicine and Department of

Microbiology

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Science

by

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May 2007

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ABSTRACT

T-cell Dysfunction by HCV Core Protein Involves PD-1/PD-L1 Signaling

by

Ellis King

In 1989 the hepatitis C virus was identified as a significant cause of post-transfusion hepatitis. Nearly two decades later there is still no vaccine, inadequate treatment options, and limited understanding of how the virus establishes chronicity in the majority of the people it infects. Recent reports suggest that the interaction of a negative co-stimulatory pathway mediated by PD-1 and PDL-1 is associated with persistent viral infection. The role, if any, that PD-1/PDL-1 has in HCV infection is unknown. In this study we report that PD-1 is upregulated in T-cells from persons with chronic HCV infection when compared to healthy donors. In addition, PD-1 and PDL-1 are upregulated on T-cells from healthy donors when exposed to extracellular HCV core protein (a nucleocapsid protein that is immunosuppressive); upregulation of PD-1 is mediated by core's ability to bind to the complement receptor gC1q. We also report that the observed T-cell function can be restored by blocking the PD-1/PDL-1 interaction. Our results indicate that HCV core can upregulate an important negative T-cell signaling pathway that is associated with viral persistence. This upregulation of PD-1/PDL-1 represents a novel and perhaps

shared mechanism that viral pathogens may use to subvert the human immune response. It also represents a potential new treatment option for the millions of people who suffer from chronic hepatitis C infection.

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CHAPTER 1

INTRODUCTION

A Brief History of Post-Transfusion Hepatitis

The possibility and need for large scale transfusion medicine came together in the 1940s. Medical science had advanced sufficiently to provide the possibility and World War II provided the need. So, began the establishment and growth of blood banks, transfusion services, and other related laboratory support. It was quickly realized that hepatitis could be transmitted via blood or blood products when post-transfusion hepatitis (PTH) was first reported in the U.S. by (Beeson 1943). However, the demand for blood continued to grow and by the early 1970s there were over 5000 organizations involved in transfusion medicine with over 12 million units of whole blood transfused per year (Domen 1995). The increased demand brought increased disease transmission; an NIH study during this time reported that the risk of PTH was 6.3% when a patient was transfused with blood from a commercial donor and <0.6% for blood from a volunteer donor (Walsh and others 1970).

In 1965 the first marker for PTH was identified and termed the Australian antigen (Blumberg and others 1965). By 1970 the Australian antigen had been re-named as the hepatitis B surface antigen (HbsAg) (Gocke and others 1969; Gocke and others 1970) and in 1972 the FDA required all blood donations within

the U.S. to be screened for HBsAg (Hoofnagle 1990). These efforts and the FDA decision to disallow commercial donors (persons who donate blood for money) lowered the PTH rate to 7.1% of the prior rate (Alter and others 1972). The next major discovery was in 1973 (Feinstone and others 1973) when the hepatitis A virus (HAV) was identified as an agent of acute hepatitis. However within a few years, it was determined that HAV was not the cause of the non-HBV cases of PTH (Dienstag and others 1977; Stevens and others 1978). The still unidentified agent of hepatitis was termed non-A, non-B hepatitis (NANBH) and represented 90% of residual PTH cases in the 1970s and early 1980s.

It was not until 1988 that a new hepatitis virus was identified by Houghton and associates at Chiron in collaboration with Bradley of the Hepatitis Branch of the Centers for Disease Control (Ezzell 1988). Choo and others then used a cDNA library generated from a patient with NANB hepatitis to screen the serum of a patient with chronic NANBH. A complementary DNA clone was isolated that was shown to encode an antigen associated specifically with NANBH infections (Choo and others 1989). This positive clone and this new virus were associated and named hepatitis C virus (HCV).

Hepatitis C Virus

Since 1998 the Centers for Disease Control and Prevention (CDC) estimate that >40,000 new infections with hepatitis C virus (HCV) occur every year making HCV infection the most common chronic bloodborne viral infection in the United States. The third National Health and Nutrition Examination Survey

(NHANES III) based on data collected from 1988-94, found that within the civilian, non-institutionalized U.S. population 3.9 million American were infected with 2.7 million chronically infected. If this prevalence rate of 1.8% is extrapolated to today's population of 300 million then >5 million Americans are presently infected with nearly 3.5 million chronically infected. These estimates are likely conservative due to the exclusion of incarcerated (infection rates estimated to be 15-40%, CDC) and homeless persons, groups that generally have a high prevalence of HCV infection. Presently, most HCV infected persons are adults between the ages of 30-49 years old (Alter and others 1997; Alter 1997). Given that HCV infection can take years to become clinically significant, most persons are probably infected in their early adulthood. Males are almost twice as likely to be infected compared to females.

As shown in Figure 1.1, the greatest present day risk of HCV infection comes with injecting drug use. Of persons injecting drugs for at least 5 years, 60% to 80% are infected with HCV compared to an approximately 30% infected with HIV.

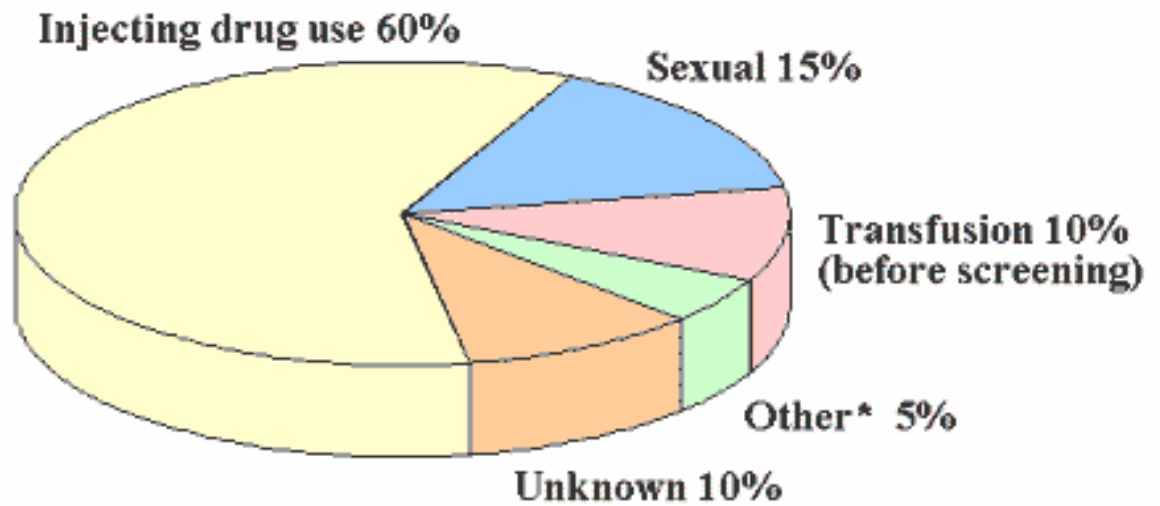


Figure 1.1 Sources of Infection for Persons with Hepatitis C. * Hemodialysis; health-care work; perinatal. Source – CDC

Current screening and testing procedures for donated blood have reduced the likelihood of contracting HCV from a transfusion to less than one per million units transfused. These same procedures, plus virus inactivation procedures, have allowed only one instance of infection from contaminated blood products since the testing/screening procedures were put in place in the U.S. Although the risk from sexual intercourse is low, sex is a common behavior in the general population. So, while other types of exposure have a relatively greater risk (e.g., transfusion from an infected donor), they account for a relatively small proportion of the population in whom these exposures have occurred. The remaining groups at risk – health care worker exposure, vertical transmission from mother to child, and persons undergoing hemodialysis account for approximately 5% of new infections. Approximately 10% of infected persons have no recognized source for their infection.

Preventing and treating HCV is problematic. Currently no vaccine exists and no likely candidates are in the near future. In the U.S., HCV genotypes 1a and 1b are most prevalent (Hoofnagle 1997) and the most resistant to the currently recommended therapy of pegylated interferon alpha and ribavirin (Hoofnagle 2003). Peginterferon alpha and ribavirin will achieve a sustained elimination of HCV infection for at least 6 months in 30% to 40% of patients who complete their therapy. An additional 10% to 20% of patients do not complete the therapy due to the side effects of the medication or have contraindications (such as severe cirrhosis) that prohibit treatment. This combination treatment will also induce a sustained virologic response (SVR) in 42-48% of persons infected with genotype 1a or 1b, whereas the same treatment will induce a SVR in 76-88% of persons infected with genotypes 2 or 3 (Fried and others 2002). Additional contra indicators also exist. IFN alpha is associated with depression and ribavirin can cause neutropenia and can have a teratogenic effect. Current antiviral therapy is not approved for patients below the age of 18.

The study of HCV is made difficult by two key factors. HCV will only reliably infect humans and chimpanzees, thus limiting animal models. Additionally, *in vitro* replication is very poor (although some replicon systems have been made to work in hepatocytes.) Because newly infected persons are usually asymptomatic and, therefore, almost never clinically recognized as infected, it has also been difficult to determine the initial immune responses that correlate with viral clearance. To further compound the issue, it appears that the six major genotypes described (Simmonds and others 1993a) may have different

disease patterns and different responses to treatment (Hoofnagle and di Bisceglie 1997). Finally, in spite of strong evidence linking HCV infection to numerous conditions involving the immune system, diseases associated with immune dysfunction occur frequently in persons without HCV infection, and so it has been difficult to confirm strong associations between HCV and a given disease.

Hepatitis C is a 9.5-kb positive strand RNA virus of the *Flaviviridae* family. The genome consists of ~9500 nucleotides encoding a single polypeptide of ~3000 amino acids that is cleaved into structural and non-structural proteins. It encodes three structural proteins (core, E1, and E2) and at least six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). HCV is classified into 6 distinct but related genotypes; HCV is further divided into over 50 subtypes based on sequence variation in the core and E1 genes and also shows significant geographical variation (Simmonds and others 1993b). Persons with long-term infections also tend to have greater levels of genetic diversity within their genotypes (quasispecies) (Honda and others 1994; Farci and others 1997), most likely due to the poor fidelity of the HCV RNA polymerase. The phenomena of high levels of quasispecies is also associated with poor clinical prognosis (Kanazawa and others 1994), that may be linked to the poor CTL response in chronically infected persons.

A remarkable feature of HCV is its ability to evade the host immune response, resulting in chronic infection for over 80% of persons exposed. The high incidence of persistent infection with HCV suggests that this virus has

evolved one or more mechanisms to evade and possibly suppress the host immune response. Chronic HCV infections are associated with liver cirrhosis and hepatocellular carcinoma and have become a leading indicator for liver transplant in the U.S. (Hoofnagle 1997). Cirrhosis of the liver develops in 10% to 20% of persons with chronic hepatitis C over a period of 2-30 years, and hepatocellular carcinoma (liver cancer) in 1% to 5% of those with cirrhosis. In addition to cirrhosis and carcinoma, persons infected with HCV are also at a significant risk of developing autoimmune diseases such as glomerulonephritis and mixed cryoglobulinemia (Agnello and De Rosa 2004). Hepatitis C disease progression varies greatly from person to person making it not only difficult to predict who will develop chronic disease but also which of the chronically infected will go on to develop significant sequelae. One thing that is known is the impact of alcohol use in infected persons. Chronically infected persons who abuse alcohol have a greater risk of developing cirrhosis and also be refractory to the effect of any anti-viral medication.

While the precise mechanisms for how the disruption of the host immune response by HCV can result in various immune disorders have yet to be defined; however it is apparent that HCV modulates host immunity on several different levels (Moorman and others 2001b). Over the past decade, there have been numerous studies examining different facets of HCV-associated immune dysfunction that have included B-cell, T-cell, NK cell, and dendritic cell dysregulation (King and others 2007). The significance of such immunomodulatory potential appears to be twofold: evasion of the host immune

response leading to viral chronicity, and the potential for development of lymphoproliferative disorders.

It is noteworthy that HCV clearly infects multiple extrahepatic cell types, including peripheral blood mononuclear cells, and this is observed both *in vivo* and *in vitro* (Shimizu and others 1992; Shimizu and others 1997; Lerat and others 1998; Radkowski and others 2000; Radkowski and others 2002; Nowicki and others 2005). It is therefore possible that the immune dysfunction observed in HCV is a function of its ability to infect such cells, although the frequency of this infection appears low (Bronowicki and others 1998). HCV sequences observed in different cellular compartments and in particular peripheral blood mononuclear cells may vary, suggesting viral tropism for specific cells that may be in part affected by the HCV diversity that occurs as quasispecies develop (Lerat and others 1998). The role of a given genotype in targeting hematopoietic cells remains controversial (Kao and others 1997; Lerat and others 1998).

Dysregulation of B Cell Function Mediated by HCV

Because the association between chronic HCV infection and both mixed cryoglobulinemia and B-cell lymphoma was first recognized, there have been an increasing number of studies examining B cell dysfunction in the context of chronic HCV infection. While it has clearly been shown that clonal populations of B lymphocytes are disproportionately present in increased numbers in patients with chronic HCV infection, there are also numerous data detailing specific mechanisms behind B cell clonality. Moreover, such mechanisms offer insight

into the presence of immune dysfunction in patients with chronic HCV infection. Most notably, B cells have become a target of drug development for the treatment of certain HCV-associated immune diseases (Kazkaz and Isenberg 2004).

(Franzin and others 1995), were the first to report the presence of clonal B cell expansion in the peripheral blood of 38 HCV-infected patients. Subset analyses in their study revealed the presence of clonal immunoglobulin gene rearrangements in 100% of HCV-positive patients with type II mixed cryoglobulinemia (MC) as opposed to only 24% in HCV-positive patients without mixed cryoglobulinemia. The reversibility of B cell clonality in chronic HCV infection was similarly assessed in a cohort of 20 patients with HCV-associated MC undergoing interferon alpha therapy (Mazzaro and others 1996). While 80% of these patients (all of whom were noted to have clonal populations of B cells) achieved complete or partial clinical responses, 15% reverted to polyclonal B cell populations following therapy. Recent studies also suggest upregulation of B-lymphocyte stimulator (BLyS) in the setting of MC (Fabris and others 2006; Sene and others 2006), further supporting a significant relationship between clonal expansion of B lymphocytes and HCV-associated mixed cryoglobulinemia. (Zignego and others 2000), found an increased frequency of *bcl-2* gene rearrangement (Mazzaro and others 1996; Nowicki and others 2005) in a prospective study of individuals with HCV and mixed cryoglobulinemia (Antonelli and others 2002), while (Toubi and others 2004), observed enhanced B cell apoptosis in B cells from chronically infected HCV patients. There was relative

resistance to apoptosis seen in the CD95/Fas+ B cell subpopulation, suggesting a potential role for this subpopulation in B cell proliferative disorders.

As the propensity of HCV to induce lymphoproliferation in patients with HCV-associated mixed cryoglobulinemia has been further elucidated, there has been some effort to define the underlying mechanisms. (Machida and others 2005), reported that an HCV E2-CD81 interaction modulates host B cell responses by enhancing activation-induced cytidine deaminase (AID) and hypermutating V(H) immunoglobulin genes in B cells, suggesting a mechanism for HCV-associated B cell lymphoproliferative disorders. HCV E2 was also shown to activate the JNK pathway leading to preferential proliferation of CD27+ B cells (Rosa and others 2005).

The effect of HCV core protein on molecular profiling in human B-lymphocytes was recently examined by (Wu and others 2006), who found dramatic evidence for inhibition of B lymphocyte apoptosis by HCV core in several steps of the apoptotic cascade. The investigators expressed HCV core in an adenoviral vector in a healthy population of human B lymphocytes and observed down regulation of MHC class II molecules and caspase-1 and -4, which are proapoptotic proteins. Up regulation of nuclear factor kappa light peptide inhibitor gene and TATA box protein, both of which are associated with B cell lymphoma, was also observed. These findings underscore the potential of HCV to disrupt antigen presentation and apoptosis and may indicate yet another mechanism for HCV-related autoimmunity and lymphoproliferation.

Oncogenes have also been a focus of investigation in patients with MC. In a prospective study of 37 patients with HCV-associated mixed cryoglobulinemia, (Zignego and others 2000), found that 75.7% had *bcl-2* rearrangements in peripheral blood mononuclear cells, as opposed to 37.6% of patients with chronic HCV infection without mixed cryoglobulinemia (Antonelli and others 2002). (Galli-Stampino and others 2003), further expanded on the relationship between oncogenesis and clonal B cell expansion in patients with HCV-associated mixed cryoglobulinemia by demonstrating the presence of restricted V(H)I gene sequences in peripheral blood, hepatic, and lymph node B lymphocytes in three of four patients (Galli-Stampino and others 2003). These gene sequences are usually associated with B-cell non-Hodgkin's lymphoma (NHL) and their presence in patients with chronic HCV patients may indicate a mechanism of developing NHL through non-malignant, clonally expanded B-cell populations.

Type I Interferon Dysregulation

Interferon-alpha stimulation is a critical and non-specific response to viral infection. Its two main effects are induction of an anti-viral state in infected cells and interferon receptor ligation that results in activation of Janus kinase (Jak) and signal transducer and activator of transcription (STAT). Jak/STAT signaling in turn activates numerous interferon-response genes including 2'-5' oligoadenylate synthetase (OAS), Mx proteins and the double-stranded RNA dependent protein kinase (PKR) (Katze 2002). PKR is induced by double stranded viral RNA and,

in-turn, phosphorylates eukaryotic initiation factor 2 (eIF-2), resulting in inhibition of cellular protein syntheses and viral replication. Two HCV proteins, the envelope glycoprotein E2 and the nonstructural protein NS5A, have been reported as potential inhibitors of the IFN response (Taylor 2000; Pflugheber and others 2002).

Phosphorylated interferon regulatory factor 3 (pIRF-3) is the key transcription regulatory factor for type I interferon (Foy and others 2003). Type I interferon, in turn, upregulates expression of double-stranded RNA-dependent protein kinase (PKR) that is a negative regulator of cell growth. Recently, the HCV protein NS3/4A (a serine protease) was shown to interrupt the IFN signaling pathway. Thus, in the presence of NS3/4A, IRF-3 is never phosphorylated and IFN production is not induced (Foy and others 2003). The importance of IRF-3 was also demonstrated with mutations resulting in dominant negative or constitutively active IRF-3 leading to enhanced or restrained HCV replication, respectively.

Dysregulation of the NK Response

Natural killer (NK) cells play a critical role in innate immunity. NK cells mediate lysis of target cells by releasing cytotoxic granules that contain perforin and granzymes or by binding apoptosis inducing receptors on the target cells. NK cells also secrete numerous cytokines including IFN-gamma and TNF-alpha during inflammation (Poccia and others 2001). Recent reports have implicated

the HCV E2 protein in suppression of NK activity (Crotta and others 2002; Tseng and Klimpel 2002).

The HCV E2 protein binds CD81, which is expressed on the surface of host cells. When NK cells are exposed to immobilized E2 or anti-CD81 (*in vitro*), their function is impaired. The impairment ranges from suppression of cytotoxicity and IL-2 induced proliferation to suppression of IL-2, IL-12, and IL-15 mediated induction of IFN-gamma (Crotta and others 2002; Tseng and Klimpel 2002). Additionally, ligation of CD81 by E2 inhibits CD16-mediated activation of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) in NK cells (Crotta and others 2002). These mechanisms, if employed by HCV, could facilitate HCV persistence. However, this possibility needs further research as NK cells do not play a role in all viral infections.

Dysregulation of Dendritic Cells by HCV Envelope Glycoproteins

One possible cause of an impaired CD8⁺ response to HCV infection may be abnormal dendritic cell (DC) function preventing appropriate antigen processing and presentation. Interestingly, HCV E1 and E2 glycoproteins interact with DC-specific intercellular adhesion molecule 3 (ICAM-3), sequestering nonintegrin (DC-SIGN) and its receptor, DC-SIGNR, in immature DCs (Lozach and others 2003; Pohlmann and others 2003). The role of HCV interactions with DC remains controversial. Numerous studies demonstrate abnormal DC activity in chronic HCV patients, yet recent studies by (Longman and others 2005), report the converse.

In addition to envelope proteins, HCV core has also been implicated in inhibiting DC and macrophage function (Sarobe and others 2003). Addition of extracellular core or expression of core/E1 in mice by recombinant adenovirus demonstrated an effect on DC maturation and T cell responses upon allogenic stimulation. A molecular mechanism(s) has not fully elucidated to explain this inhibition.

Dysregulation of T Cell Function Mediated by HCV Core Protein

Evidence suggests that the 21kD HCV core protein, the first to be synthesized during infection, contains multiple functions that have numerous downstream effects on immune related cellular responses. This immunomodulatory effect has been demonstrated by the suppression of anti-viral CTL activity (Large and others 1999b) and diminished T cell responses to HBV envelope proteins in mice immunized with HCV-core/HBV chimeric constructs (Geissler and others 1998). HCV core has also been found circulating in the bloodstream of infected patients (Maillard and others 2001) suggesting that the effects of core may extend beyond the specific cells that are infected with HCV. This is important because in relative numbers, HCV infects few peripheral blood mononuclear cells (PBMCs) (*e.g.* lymphocytes). Furthermore, extracellular HCV core has been shown to inhibit the T lymphocyte responses that are crucial for viral clearance (Kittlesen and others 2000; Yao and others 2001a), thus implying a role for core in establishing chronicity. It is well known that, in general, an early and sustained virus-specific T-cell response is critical for viral clearance (Missale

and others 1996; Cooper and others 1999; Gerlach and others 1999; Gruner and others 2000; Takaki and others 2000; Thimme and others 2001). However, in patients who are chronically infected with HCV, the frequency of virus specific T-cells is relatively low. Furthermore, the effector functions of HCV specific CD4⁺/CD8⁺ cells as well as the production of Th1-type cytokines (e.g. IL-2 and interferon gamma) are severely decreased (Rehermann and others 1996; Chang and others 1997; Lechmann and others 1999; Wedemeyer and others 2002; Sugimoto and others 2003). It is clear that HCV will establish a chronic infection in most persons who are infected. The available data strongly support the thought that HCV, through some action(s), suppresses the human immune system, thus establishing chronicity. The available data also strongly suggest that the HCV core protein has a significant, yet not fully understood role, in the immune suppression.

Studies have implicated that gC1qR (the glycoprotein portion of the C1q cellular receptor) is the cellular target of core protein (Kittlesen and others 2000). The natural ligand for gC1qR is C1q. C1q is an integral part of the C1q complex that is well established as the first component in the classical pathway of complement activation, thus playing a critical role in the early defense against foreign antigens (Ghebrehiwet and others 2001). The 33kD gC1qR and the 60kD cCqR bind the globular “head” and collagen-like “stalk” domains of C1q, respectively, and form a heterodimer that is expressed in most cell types, including T cells (Ghebrehiwet and others 2001). Interestingly, treatment of PBMCs with extracellular HCV core leads to suppression of T-cell proliferation in a manner

similar to that observed when T-cells are exposed to C1q (Chen and others 1994; Kittlesen and others 2000). This suppressive effect can be abrogated by the addition of anti-gC1qR suggesting that core is indeed mediating suppression through gC1qR. Therefore, the interaction between HCV core and gC1qR may provide the virus with a means to affect the human host immune response.

gC1qR has also been reported to interact with several other viral and bacterial proteins potentially providing other micro-organisms with a “shared” mechanism of immune evasion (Luo and others 1994; Bruni and Roizman 1996; Wang and others 1997; Matthews and Russell 1998; Braun and others 2000; Nguyen and others 2000). These data suggest that numerous human pathogens exploit a similar strategy to subvert the host immune response. The underlying mechanisms for the role of gC1qR in the interference of the immune system have yet to be elucidated with regards to HCV infection as well as the other pathogens that may use this pathway. It is, however, clear that the ability of HCV core to alter the immune response is not as simple as it binding to gC1qR. *In vitro* studies show that HCV core from genotype 1a will dampen the host immune response (Yao and others 2006b), while other *in vivo* studies using HCV core from genotype 1b conducted in transgenic mice report no such effect (Liu and others 2002). This suggests that the immunosuppressive effects may be genotype specific, and, indeed, this has been reported (Sugimoto and others 2005). Another *in vitro* study conducted in HepG2 cells reported that HCV core genotype 1a will significantly suppress nuclear factor kappa-B (NF- κ B) when compared to genotype 1b (Ray and others 2002). NF- κ B is a well-known

transcription factor that is important in many cellular immune responses, as many of its target genes; such as interferon beta, TNF alpha, IL-2, IL-6, and IL-8 are immunologically relevant (Pellegrini and Schindler 1993). Yet, patients who are infected with HCV genotype 1b still become chronically infected, further suggesting host factor(s) impact whether a newly infected person clears the virus or becomes chronically infected. This is supported by studies conducted in chimpanzees that showed the level of innate expression of gC1qR will affect core's ability to suppress the immune system. Chimpanzees that had lower levels of gC1qR expression were more likely to resolve the HCV virus challenge versus those chimpanzees with higher levels of innate gC1qR expression (Yao and others 2006a).

To date and with regard to HCV core-gC1qR binding and any subsequent immune suppression, most of the data produced have come from work in lymphocytes; lymphocytes obtained from either cell lines, such as Jurkats, or primary lymphocytes isolated from human volunteers. One notable exception was a set of experiments conducted in normal human lung fibroblasts (NHLF) cell line. The impetus for this study was reported links of HCV infection to declines in pulmonary function in patients with underlying pulmonary diseases such as asthma and chronic obstructive pulmonary disease (Kanazawa and others 2003; Kanazawa and Yoshikawa 2004). The study investigators focused on IL-8 stimulation given its known role in mediating inflammatory pulmonary processes and general pulmonary pathology (Kaplanski and others 1997; Polyak and others 2001; Mukaida 2003). They found that when HCV core is added to

cultures of NHLFs in an extracellular manner, IL-8 mRNA and protein are stimulated in a dose and time dependent manner (Moorman and others 2005b). They also reported that this up-regulation could be blocked by the addition of anti-gC1qR that blocks the core binding site on gC1qR. While more studies need to be conducted in this area, this study offers an intriguing glimpse toward other (non-PBMC) possible ramifications of interactions between core and gC1qR.

The early lymphocyte work investigating the consequences of core-gC1qR binding employed transfection methods in order to introduce core to the cell and focused on core's ability to induce or up-regulate apoptosis of T-cells. These studies found that transient expression of core appeared to up-regulate apoptosis mediated by interaction with members of the tumor necrosis family of receptors (TNFR) (Hahn and others 2000; Zhu and others 2001; Moorman and others 2003). It was found that expression of core sensitized Jurkat cells to FasL induced apoptosis (Hahn and others 2000) mediated by the cytoplasmic domain of Fas. Additional studies also indicate that core can induce ligand independent apoptosis of Jurkats by activating caspase 3 and 8. This same study also reported that intracellular expression of core can also induce Fas multimerization at the cellular level as a means of activating the apoptotic pathway (Moorman and others 2003). While the evidence indicating that expression of HCV core will induce apoptosis in a T-cell cell line is strong, it is worth remembering that, as noted earlier, HCV infects a relatively low number of PBMCs. Yet, in spite of HCV tropism for lymphocytes, the immune system can still produce HCV

specific T cells and HCV antibody, neither of which have significant efficacy given the high rate of chronicity.

While inducement of apoptosis in T-cells may have a role in HCV establishing a chronic infection, it is likely that T-cell inhibition or suppression plays a larger role. The search for a molecular basis for HCV core's ability to suppress the immune system has identified several possibilities, all mediated by core's ability to bind to gC1qR. Core was first found to inhibit proliferation in activated T-cells by blocking the phosphorylation of extracellular signal-regulated kinase (ERK) and mitogen-activated ERK kinase (MEK) (Yao and others 2001a) in activated T-cells. HCV core-induced impairment of ERK/MEK mitogen-activated protein kinase resulted in the inhibition of IL-2 and IL-2R gene transcription that led to the inhibition of IL-2 production and high-affinity IL-2R alpha expression. Importantly, the ability of anti-gC1qR Ab treatment to reverse HCV core-induced inhibition of ERK/MEK phosphorylation reveals that the interaction between HCV core and gC1qR is linked to the interference of ERK/MEK mitogen-activated protein kinase activation. HCV core has also been shown to inhibit cell cycle progression from G₁ to S phase by stabilizing p27^{Kip1}, which is a negative regulator of Cdk4/cyclin D and Cdk2/cyclin E (Yao and others 2003) complexes. These cyclin dependent kinases (Cdk) and their cyclin partners serve to phosphorylate proteins that push cells from G₁ to S phase. Therefore, stabilization of proteins that inhibit these Cdk-cyclin complexes will in turn lead to inhibition of cell cycle progression.

Further studies have reported additional instances of core interfering with cellular signaling. As previously mentioned, core will bind to the complement receptor gC1qR whose natural ligand is C1q and that C1q also delivers inhibitory signals to T-cells. Recent studies have shown that the affinity of core for gC1qR is greater than that of C1q, and core appears to deliver stronger inhibitory signals than does C1q (Yao and others 2004). These same studies also reported that CD8⁺ T-cells are more susceptible to core mediated inhibition than CD4⁺ cells most likely due to CD8⁺ cells having higher expression of gC1qR. Based on previously reported data that indicated core had an inhibitory effect on ERK/MEK signaling in T-cells (Yao and others 2001a) the investigators searched for other inhibitory effects on signaling. They focused on early signaling in the early stages of T-cell activation and found that core treated T-cells show a decrease in the expression of Src kinases, Lck, and ZAP-70 that may impede cell cycle progression (Yao and others 2004). Some of the latest data regarding signaling inhibition was conducted using recombinant core from patients who had a chronic infection versus patients who had spontaneously resolved their infection. It was found that core from the chronic patients had a greater inhibitory effect possibly mediated by diminishing the phosphorylation of STAT1/3 that, in turn, up regulates the expression of SOCS1/3 (Yao and others 2006b), which are negative regulators of T-cell proliferation. This finding is strengthened when siRNAs, designed against SOCS1/3, abrogated the core mediated T-cell inhibition.

Programmed Cell Death 1 (PD-1).

In the early 1990s Honjo and colleagues began to search for gene products that contributed to apoptotic cell death that was dependent on *de novo* synthesis of RNA and protein *e.g.*, the classical type programmed cell death. They began their work in four cell lines consisting of T-cells, B-cells, and a lymphoid progenitor line. In 1992 they reported that in two of the four cell lines *de novo* synthesis of RNA was necessary for apoptosis to occur. Further work determined that a novel member of the immunoglobulin gene superfamily, termed program cell death 1 (PD-1), was linked to the observed apoptosis (Ishida and others 1992).

By 2002 it had been reported that, *in vivo*, PD-1 was expressed predominately on activated T-cells (Iwai and others 2002). Further studies indicated that cross-linking of PD-1 with its ligand would induce phosphorylation of its immunoreceptor tyrosine-based switch motif that would then recruit SHP-2 with its subsequent negative regulatory signal (Okazaki and others 2001). The ligands were then identified as PD-L1 (B7-H1) and PD-L2 (B7-DC) (Dong and others 1999; Freeman and others 2000; Latchman and others 2001; Tseng and others 2001) with PD-L1 shown to be expressed in numerous nonlymphoid tissue (Dong and others 1999; Freeman and others 2000). The receptor-ligand distribution pattern, PD-1 expressed in activated T-cells and PD-L1 expressed in peripheral tissue, led to the hypothesis that PD-1/PD-L1 interaction might occur at the effector phase of the immune response when activated T-cells migrate to sites of inflammation or infection. Beginning with known liver involvement in the

immune response, a set of experiments was designed around liver nonparenchymal cells (LNPCs). It was found that PD-L1 was constitutively expressed on LNPCs and that this expression was essential to inhibit proliferation of activated T-cells in the liver (Iwai and others 2003), suggesting that the PD-1/PD-L1 interaction may play important roles in the negative regulation of immune responses in the liver.

The pathways that memory CD8⁺ T-cells take after an acute viral infection, versus a chronic infection, are distinct (Wherry and others 2003; Wherry and others 2004; Klenerman and Hill 2005). Memory CD8⁺ T-cells generated from an acute viral infection are, in general, highly functional and make up an important component of protective immunity. On the other hand, memory CD8 T-cells generated from a chronic viral infection are often characterized by varying degrees of functional impairment that is the most likely reason the virus is able to establish a chronic infection. However, even in viral infections that eventually become chronic, initial T effector cells are generated. Yet, they soon lose function over the course of a chronic infection and become 'exhausted'. This exhaustion effect was first reported in mice with a chronic LCMV infection (Gallimore and others 1998; Zajac and others 1998) but was quickly shown to be a factor in humans with chronic viral infections; particularly those persons with human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) (Letvin and Walker 2003; Pantaleo and Koup 2004; Rehermann and Nascimbeni 2005).

The next step taken was to determine gene expression in exhausted T-cells versus functional T-cells in the mouse LCMV model. PD-1 mRNA and protein was found to be significantly up-regulated in the exhausted T-cells (Barber and others 2006) when compared to the functional T-cells. Further investigation by found that the LCMV strain that consistently causes an acute infection in mice had only transient up-regulation of PD-1 mRNA and protein, whereas the strain that consistently causes a chronic infection had higher and sustained levels of PD-1 mRNA and protein. They then determined that by blocking the PD-1/PD-L1 interaction they could restore function, e.g. the exhausted T-cell where induced to clonally expand, to CD8⁺ cells even in the absence of CD4⁺ cells.

Hypothesis

Based on the available data the following hypothesis is considered:

Persistence of HCV infection is mediated by the dysregulation of the PD-1 pathway in T-cells via HCV core-gC1qR interaction.

CHAPTER 2

T cell Dysfunction by HCV Core Protein Involves PD-1/PDL-1 Signaling

Abstract

Recent reports show that a negative T cell co-stimulatory pathway mediated by PD-1 and PDL-1 is associated with T cell exhaustion and persistent viral infection. Persistent hepatitis C virus (HCV) infection in humans is also characterized by impaired T lymphocyte function, but the role of the PD-1 and PDL-1 pathway in HCV infection is unknown. Here we report that T cells isolated from chronically infected HCV patients express significantly higher levels of PD-1 when compared to healthy donors. In addition, PD-1 and PDL-1 expression is up-regulated on T cells from healthy donors exposed to HCV core, a nucleocapsid protein that is immunosuppressive; up-regulation of PD-1 is mediated through interaction of HCV core with the complement receptor, gC1qR. Importantly, T cell functions that are dysregulated by HCV core, including T cell activation, proliferation, and apoptosis, can be restored by blocking PD-1 or PDL-1 engagement. Our results indicate that HCV core can up-regulate a key negative T cell signaling pathway associated with viral persistence and highly expressed on the T cells of persistently infected individuals. This up-regulation of the PD-1 and PDL-1 pathway in humans represents a novel and perhaps

common mechanism by which a virus usurps host machinery to facilitate persistence.

Introduction

T cell dysfunction is a common feature of many persistent viral infections, although the underlining mechanism(s) remains poorly understood. Hepatitis C virus (HCV) infection provides an excellent model to study the mechanisms of persistent viral infection as this virus is remarkable at evading host immune surveillance, resulting in chronic infection in the majority of infected individuals (Moorman and others 2001a). Chronic HCV infection is associated with liver cirrhosis and hepatocellular carcinoma and has become a leading cause of liver transplantation in the United States (Lauer and Walker 2001). Numerous studies have reported that effective T cell responses are crucial for viral clearance, and impaired viral specific CD4⁺ and CD8⁺ T cell functions are associated with chronic HCV infection (Gerlach and others 1999; Gruner and others 2000; Lechner and others 2000; Takaki and others 2000; Thimme and others 2001; Wedemeyer and others 2002; Shoukry and others 2003). Other studies have suggested the possibility of a more non-specific immune dysregulation in the setting of HCV infection, perhaps supported by the high frequency of autoimmune disease in individuals with chronic HCV infection (Yao and others 2001a; Yao and others 2001b; Lucas and others 2004; Graham and others 2005; Par and others 2006). Despite extensive investigations into the mechanisms of

virus-mediated T cell dysfunction, it still remains unclear why a small percentage of HCV patients exhibit effective T cell responses and clear the virus following acute infection, while the vast majority fail to do so and progress to chronic infection.

It is likely that a gene product(s) encoded by HCV directly affects T cell functions that are crucial for limiting virus replication, and thus facilitates persistent infection. Several HCV-derived proteins, including the nucleocapsid core protein, may play a role in the impairment of host immunity either directly or through interaction with host molecules (Ray and Ray 2001; Eisen-Vandervelde and others 2004). It has been previously demonstrated that HCV core protein is necessary and sufficient to suppress host immune responses in a murine model (Large and others 1999a). The molecular mechanism of HCV core-mediated immunomodulation was subsequently determined by identifying a host-binding partner, C1q complement receptor-gC1qR, on human T lymphocytes (Kittlesen and others 2000). C1q, the natural ligand for gC1qR, is the first molecule to be activated in the classical complement cascade and plays a critical role in modulating both innate and adaptive immunity (Ghebrehiwet and others 2001). Binding of C1q to gC1qR on T lymphocytes leads to suppression of T cell responsiveness (Chen and others 1994); similarly, HCV core can inhibit T cell responses through interaction with gC1qR (Yao and others 2001a; Yao and others 2003; Yao and others 2004). Thus, the engagement of circulating HCV core protein with gC1qR displayed on the surface of T lymphocytes may provide the virus with a direct means of affecting host immunity (Yao and others 2001b).

In light of the observations that free core particles circulate in the bloodstream of HCV-infected patients (Masalova and others 1998; Maillard and others 2001), our findings may be particularly salient to the pathogenesis of HCV. It is notable that in addition to HCV core, gC1qR has been shown to interact with several viral and bacterial proteins, potentially providing these organisms with a “shared” mechanism of immune modulation (Ghebrehiwet and others 2001; Yao and others 2001b). While the early events following the HCV core/gC1qR interaction have yet to be elucidated, T cell receptor (TCR) signaling pathways--and in particular other negative regulatory pathways--would be prime targets for viral immunomodulation.

The recently described PD-1 and PD-L inhibitory pathway represents just such a negative regulatory mechanism to maintain the intricate balance between positive and negative costimulatory signals delivered to T cells after antigenic encounter (Nishimura and Honjo 2001; Khoury and Sayegh 2004). PD-1 (programmed death-1) is an immunoinhibitory receptor predominantly expressed on activated T cells; its natural ligands include PDL-1 (also known as B7-H1, expressed on both haematopoietic and parenchymal cells) and PDL-2 (B7-DC, primarily expressed on monocytes). Activation of the PD-1 pathway induces immunoreceptor tyrosine phosphorylation and recruitment of tyrosine phosphatases, including src-homology proteins (SHP-1/SHP-2), to deliver a negative signal to TCR activation pathways. Evidence is emerging for the involvement of PD-1 inhibitory pathway in normal immune tolerance, autoimmune responses, and antitumor and antiviral immune evasion (Nishimura and Honjo

2001; Iwai and others 2003; Khoury and Sayegh 2004). Perhaps most interesting, PD-1 was recently found to be selectively up-regulated on “exhausted” CD8⁺ T cells during chronic viral infection in mice (Barber and others 2006). In addition, very recent reports now show similar findings in the setting of HIV infection (Day and others 2006; Freeman and others 2006; Petrovas and others 2006; Trautmann and others 2006b).

To further characterize underlying mechanism(s) of HCV-induced T cell dysfunction, we examined the role of PD-1 and PDL-1 expression on human T cells. We found that individuals with chronic HCV infection exhibit up-regulation of both PD-1 and PDL-1 on CD4⁺ and CD8⁺ T cell populations compared to healthy control populations. To explore potential mechanisms for this up-regulation, we exposed naïve healthy T cells to HCV core and found that expression of both PD-1 and PDL-1 are up-regulated, and this core-induced PD-1 and PDL-1 induction is gC1qR-dependent. Importantly, blocking PD-1 and PDL-1 signaling pathway can restore HCV core/gC1qR-mediated T cell function, suggesting that the PD-1 pathway may be employed during HCV core/gC1qR interaction as a means of dysregulating T cell functions.

Materials and Methods

Subjects.

An IRB-approved protocol at ETSU has contributed to a database for the storage of blood samples from HCV-infected individuals. Blood from healthy donors serves as a normal control. Peripheral blood mononuclear cells (PBMC) were isolated from these subjects by Ficoll density centrifugation with lympholyte-H (Cedarlane Labs, Ontario, Canada) and then cryopreserved in freezing medium (10% DMSO, 20% FBS in RPMI 1640) in liquid nitrogen until examined. Twelve chronically HCV-infected patients and seven healthy donors were selected, and their PBMC were thawed, washed, and counted for the following studies. One subject was co-infected with HIV but was a long-term nonprogressor with repeatedly negative HIV viral load. All subjects had HCV infection detected by ELISA and confirmed by HCV RNA testing and none were considered to be acute infections.

Reagents.

Recombinant HCV core protein, NS3, and control β -galactosidase proteins were obtained from ViroGen (Watertown, MA). These proteins have been used extensively in immunologic studies of HCV antigens and have been documented to be free of LPS or RNA. Anti-CD3/CD28, anti-CD45RA/RO antibodies, and

FITC-conjugated anti-CD4 and CD8 antibodies were obtained from BD Pharmingen (San Diego, CA). Anti-PD-1 and anti-PDL-1 were obtained from eBioScience.

Flow Cytometry.

To determine PD-1 or PDL-1 expression on the surface of T lymphocytes, 1×10^6 PBMC from HCV patients or healthy donors were stimulated with or without anti-CD3/CD28 (1 $\mu\text{g}/\text{ml}$) in the presence of HCV core or a control protein, β -gal (2 $\mu\text{g}/\text{ml}$). After 24 h treatment, the cells were washed in fluorescence-activated cell sorting (FACS) medium (RPMI 1640 supplemented with 10% FBS and 1% NaN_3) at 200 x g for 5 min at 4 °C. The cells were counted and resuspended in 100 μl of FACS medium containing 20 μl PE-anti-human PD-1 and PDL-1 conjugate (eBioScience) and 20 μl of FITC-anti-human CD4 and CD8 conjugate (BD Pharmingen) at 4 °C for 1 h in the dark. The cells were then washed three times and fixed with 1% paraformaldehyde in PBS before analysis by flow cytometry (Becton Dickinson, San Jose, CA). The primary isotype controls were used to determine the level of background staining. 20,000 events were collected after gating on lymphocyte populations and using consistent instrument settings to avoid bias.

To determine the role of PD-1 and PDL-1 in HCV core-induced T cell inactivation, PBMC were anti-CD3/CD28-stimulated and HCV core-treated as described above in the presence of anti-human PD-1 or PDL-1 or isotype control

antibodies (Santa Cruz) for 24 h, and CD69 cell surface expression was detected by incubating cells with PE-anti-human CD69 conjugate (BD Pharmingen) followed by FACS analysis (Yao and others 2003).

RT-PCR.

Purified PBMCs (2×10^6) were treated with or without HCV core protein (2 $\mu\text{g/ml}$; ViroGen) for various time points (6, 12, 24, 48 h), and total RNA was isolated from these cells by the TRIzol method (Life Technologies). A total of 1 μg of RNA was treated with DNase to digest genomic DNA and 0.27 μg of RNA was then reverse transcribed using MuLV reverse transcriptase under conditions of 10 min at room temperature, 20 min at 42 °C, 5 min at 99 °C, and 5 min at 4 °C. 1 μl of 1:10 serially diluted cDNA generated in the RT reaction was added to the PCR reaction. PCR was carried out using the following primer pairs: PD-1 sense 5'-GCT CAG GGT GAC AGA GAG AAG-3'; antisense 5'-CAC CAA CCA CCA GGG TTT G-3'; β -actin sense 5'-CGA GCG GGA AAT CGT GCG TGA CAT-3'; antisense 5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT-3' for 35 cycles of 95 °C for 15 s, 58 °C for 15 s, 72 °C for 15 s, followed by a single 10 min extension at 72 °C. To control for genomic DNA contamination, equal amount of cDNA from each sample were PCR amplified without RT. The resulting PCR products were separated on a 2% BioGel (Bio 101, Carlsbad, CA) and viewed by a multimeter. To examine whether gC1qR mediates the HCV core-induced induction of PD-1, a 1:10-diluted anti-gC1qR antibody or pre-

bleeding control serum (kindly provided by Dr. Hahn) was coincubated with cells treated with core protein, and PD-1 mRNA expression was assessed as described above.

T Cell Proliferation.

The ability of anti-PD-1 or anti-PDL-1 to reverse the HCV core-induced T cell inhibition was evaluated by using Quick Cell Proliferation Assay (BioVision, Mountain View, CA). Briefly, 1×10^5 of PBMC were stimulated with or without anti-CD3/CD28 in the presence of HCV core or β -gal protein. Anti-PD-1 or anti-PDL-1 or isotype control antibody (2 μ g/ml, Santa Cruz) was added simultaneously and the cells were cultured for 24 h. T cell proliferation was performed according to manufacturers' instructions. The absorbance of the treated and untreated samples was measured using a microtiter plate reader at 420-480 nm with a reference wavelength 650 nm. The experiments were set up as triplicates of each treatment and the data shown as mean \pm SD of the optical density (OD) values.

Cellular Apoptosis.

Blocking PD-1 and PDL-1 engagement to reverse the HCV core-mediated lymphocyte apoptosis was assessed by Annexin V (AV)/Propidium iodide (PI) staining (BD Pharmingen). Briefly, 1×10^6 PBMC were treated with HCV core or

β -gal (2 μ g/ml; ViroGen) in the presence or absence of anti-PD-1 or anti-PDL-1 or isotype control antibody (Santa Cruz) for 24 h. Cell apoptosis was examined by FACS using an AV/PI staining kit according to manufacturer's instructions. The primary isotype controls were used to determine the level of background staining. Twenty thousand events were collected after gating on lymphocyte populations.

Statistical Analysis.

Data were shown as mean \pm SD and level of significance was determined using ANOVA as a program of Stata/SE 8.0 software. $P < 0.05$ was considered significant.

Results

Increased Expression of PD-1 and PDL-1 on CD4⁺ and CD8⁺ T Cells of Individuals with Chronic HCV Infection.

It has been reported that T cells adapt to persistent antigen exposure by down-regulating their responsiveness to TCR signal transduction. Furthermore, recent data suggest a key role for PD-1 in decreasing TCR signaling as a means of promoting viral persistence. To determine if PD-1 might be playing a role during HCV infection in humans, we examined PD-1 expression on T cells from

chronically HCV-infected individuals (n=12) and compared these with T cells from healthy donors (n=5). To this end, peripheral blood mononuclear cells (PBMC) isolated from HCV patients and healthy donors were stimulated with or without anti-CD3/CD28 antibodies, mimicking the activation of the TCR/CD28 pathway by MHC/peptide/B7 complex on T cells. The cells were harvested at 24 hr after the stimulation and the expression of PD-1 on the surface of T cells was determined by flow cytometric analysis. As shown in fig. 2.1A, PD-1 expression on the surface of unstimulated (39.02 ± 7.28) as well as anti-CD3/CD28-stimulated PBMCs (54.01 ± 8.14) isolated from HCV patients is significantly higher than the expression observed on the cells of healthy donors (17.88 ± 3.72 , $P=0.002$; 36.70 ± 9.17 , $P=0.005$; respectively). Consistent with the aggregate PBMC population data, the expression of PD-1 on the surface of distinct subpopulations of $CD4^+$ and $CD8^+$ T cells from HCV patients is also higher than that found on healthy donors, with representative dot blots shown in fig. 2.1B. The expression of PDL-1 on PBMCs from HCV infected individuals was similarly increased, with representative histograms shown in fig. 2.1C. Treatment with other viral proteins, including HCV NS3 as well as HIV Tat and gp120, revealed no effect on PD-1 expression levels in PBMC (data not shown).

It is possible that we started with different lymphocyte phenotype subpopulations and that the increases in expression of PD-1 and PDL-1 are related to different baseline phenotypes. Examination of PBMC from two HIV-infected subjects, however, revealed similar $CD45RO^+$ and $CD45RA^+$ subpopulations and increases in expression in PD-1 and PDL-1 upon TCR-

stimulation (fig. 2.1D) This is consistent with our hypothesis that HCV infection leads to up-regulation of PD-1 and PDL-1 expression via an interaction of HCV core with gC1qR that is expressed on multiple T cell phenotypic subpopulations, and that there is both HCV-specific and generalized T cell dysfunction in the setting of chronic HCV infection.

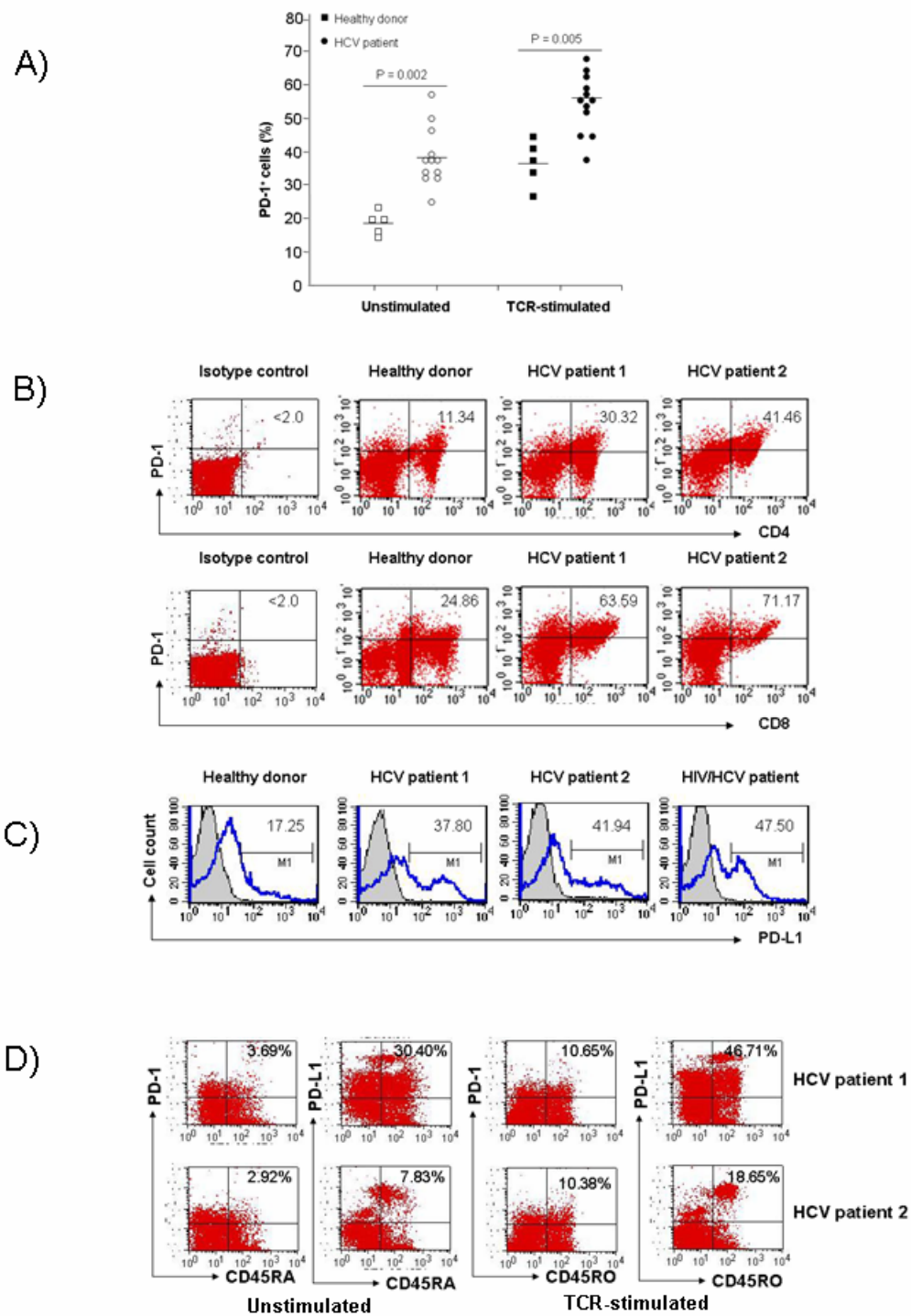


Figure 2.1 PD-1 expression on PBMCs from chronically HCV-infected individuals is increased compared to healthy donors

gC1qR-dependent PD-1 and PDL-1 Induction on Healthy T Cells by HCV Core Protein.

The above data suggested that PD-1 and PDL-1 are up-regulated in response in the setting of chronic HCV infection; how this occurs is unclear, but it seems likely that a viral product induces this up-regulation. Because we have previously shown that the HCV core nucleocapsid protein is both necessary and sufficient to inhibit T cell responses against viral infection (Yao and others 2001a; Yao and others 2001b; Yao and others 2003; Yao and others 2004), we examined expression of PD-1 and its ligand, PDL-1, on healthy T cells exposed to the HCV core protein. Peripheral blood mononuclear cells (PBMC) isolated from healthy donors were stimulated with or without anti-CD3/CD28 antibodies in the presence or absence of HCV core protein. To confirm that the complement pathway is indeed involved in up-regulation of PD-1 signaling, we also treated PBMC with C1q protein. The cells were harvested at 24 hr after treatment and the expression of PD-1 and PDL-1 was determined by flow cytometric analysis. As shown in fig. 2.2A, treatment of PBMC with C1q leads to an increase in PD-1 expression compared to control β -gal treatment in unstimulated PBMC; PDL-1 expression was also increased in response to C1q treatment (data not shown). Both PD-1 and PDL-1 expression can be up-regulated on unstimulated lymphocytes exposed to the HCV core protein (from 8% to 12%; and from 27% to 69%, respectively). Notably, PD-1 and PDL-1 expression are up-regulated on lymphocytes stimulated with anti-CD3/CD28 (from 8% to 20%; and from 27% to

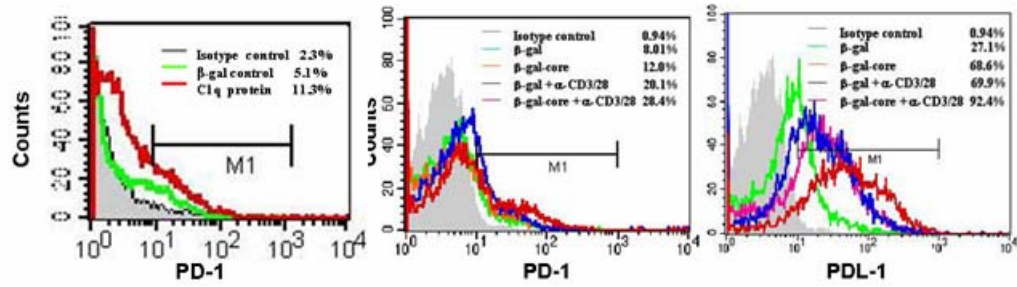
70%, respectively), and their expressions can be further up-regulated with HCV core treatment (from 20% to 28%; and from 70% to 92%, respectively). These data revealed statistically significant differences. A titration analysis with HCV core protein revealed a dose-dependent increase in PD-1 expression, with minimal increases seen at 0.125 µg and saturation by 2 ug/ml of protein (data not shown). Notably, while C1q treatment does appear to up-regulate the PD-1 pathway, HCV core appears to be more efficient at doing so. HCV core has, however, been shown to have a higher affinity for gC1qR than C1q in previous binding studies.

We next wanted to characterize the expression of PD-1 and PDL-1 on specific human T cell subpopulations. PBMCs were treated as above, followed by double staining with FITC-anti-human CD4 and PE-anti-human PD-1 and PDL-1 conjugates. As expected based on the data in the aggregate lymphocyte population, the expression of PD-1 and PDL-1 are up-regulated by HCV core protein on both unstimulated and TCR-activated CD4⁺ T lymphocytes (fig. 2.2B). CD8⁺ T cells revealed similar trends (data not shown).

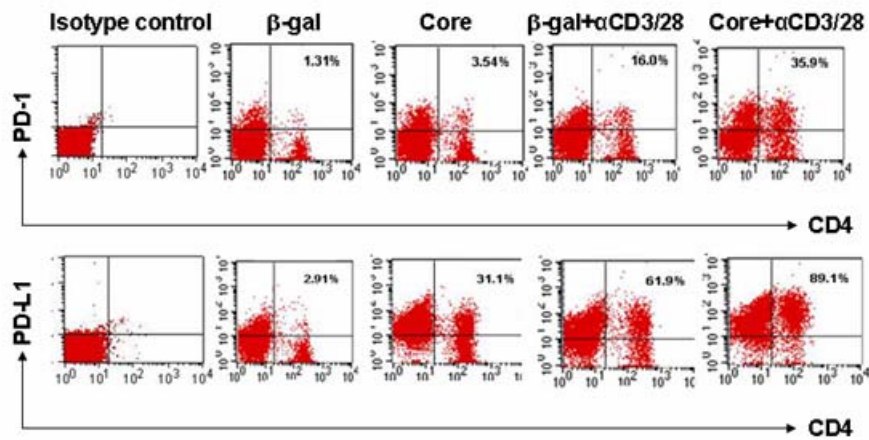
To determine the relationship between HCV core and PD-1 expression, we next detected PD-1 mRNA expression in PBMCs stimulated with anti-CD3/CD28 in the presence or absence of HCV core protein for various times after treatment using semi-quantitative RT-PCR. As shown in fig. 2.2C, PD-1 mRNA in human cells is consistently up-regulated, starting as early as 6 hrs and peaking at 24 hrs after treatment with HCV core protein. Because we have shown that HCV core protein impairs T cell functions through an interaction with

the complement receptor, gC1qR, we employed anti-gC1qR antibodies in the above experiments and found inhibition of HCV core-induced PD-1 up-regulation. This reversal effect can be also achieved by addition of anti-HCV core antibody, which interferes with the engagement of core protein with cell surface gC1qR, but not by a control serum. To support these findings, healthy, TCR-stimulated PBMC were treated with HCV core along with anti-gC1qR or control antibodies and PD-1 expression was detected on the surface of healthy CD8+ T cells by flow cytometry. PD-1 expression was diminished in cells treated with antibodies to gC1qR, consistent with our PCR data in PBMC. Notably, treatment of PBMC with anti-gC1qR antibodies alone has no effect on either PD-1 expression levels as measured by flow cytometry or lymphocyte apoptosis as measured by AV staining (data not shown). These results suggest that HCV core induces PD-1 expression directly through the gC1qR displayed on the surface of lymphocytes.

A)



B)



C)

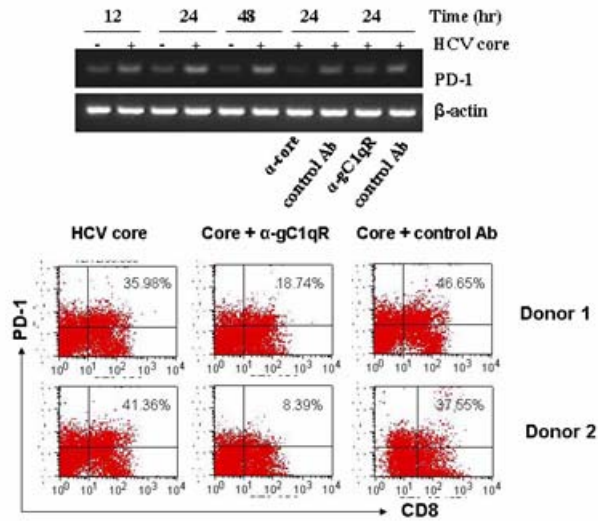


Figure 2.2 PD-1 induction on healthy human T cells by HCV core protein

Blocking PD-1 and PDL-1 Engagement Restores HCV Core/gC1qR-Mediated T Cell Dysfunction.

To explore the role of PD-1 and PDL-1 in the HCV core-induced T cell dysfunction, we next addressed the possibility of restoring HCV core-mediated inhibition of T cell function by blocking PD-1 and PDL-1 engagement. We have previously shown that HCV core modulates T cell activation status, proliferative capacity, and apoptosis (Yao and others 2001a; Moorman and others 2003; Yao and others 2003; Yao and others 2004). To this end, we incubated TCR-stimulated healthy PBMCs with HCV core protein in the presence of anti-PD-1 or anti-PDL-1 or isotype control antibodies and assayed the above measures of T cell function. We found that treatment of PBMC with anti-PD-1 or anti-PDL-1 antibodies alone had no effect on any of these measures and found no increase in the ability to activate T cells despite blocking PD-1 and PDL-1 signaling (data not shown). We first detected the expression of the T cell activation marker, CD69, on the surface of T cells. As shown in fig. 2.3A, CD69 expression on CD4⁺ and CD8⁺ T cells was suppressed by exposure to HCV core protein, and this suppression was almost totally reversed by the treatment of anti-PD-1 or anti-PDL-1 antibody. CD69 expression on T cells inhibited by HCV core was not affected by adding control antibody.

We next examined T cell proliferation in response to HCV core in the presence of antibodies to PD-1 and PDL-1. We incubated TCR-stimulated PBMC with HCV core protein in the presence of anti-PD-1 or anti-PDL-1 or

control antibody, and T cell proliferation was evaluated as described in Methods. As shown in fig. 2.3B, T cell proliferation was inhibited by HCV core protein, and the proliferative ability of T cells inhibited by HCV core could be partially restored by simultaneously adding either anti-PD-1 or anti-PDL-1 antibody into the culture. These data suggest that the PD-1 pathway plays a role in HCV core-induced T cell suppression.

The induction of cellular apoptosis is one manifestation of HCV core-mediated T cell dysfunction. To determine whether blocking PD-1 and/or PDL-1 engagement can reverse HCV core-induced cell apoptosis, we incubated human PBMC with HCV core or control proteins in the presence of anti-PD-1, anti-PDL-1, or a control antibody for 24 hrs, and apoptosis was evaluated by AV/PI staining. As shown in fig. 2.3C (top), HCV core induces apoptosis, as represented by increasing percentages of AV/PI positive cells, when compared with those treated with a control protein, β -gal; this core-induced cell apoptosis can be successfully blocked by the treatment with either anti-PD-1 or anti-PDL-1 antibody. These data are reproducible in multiple independent experiments (bottom).

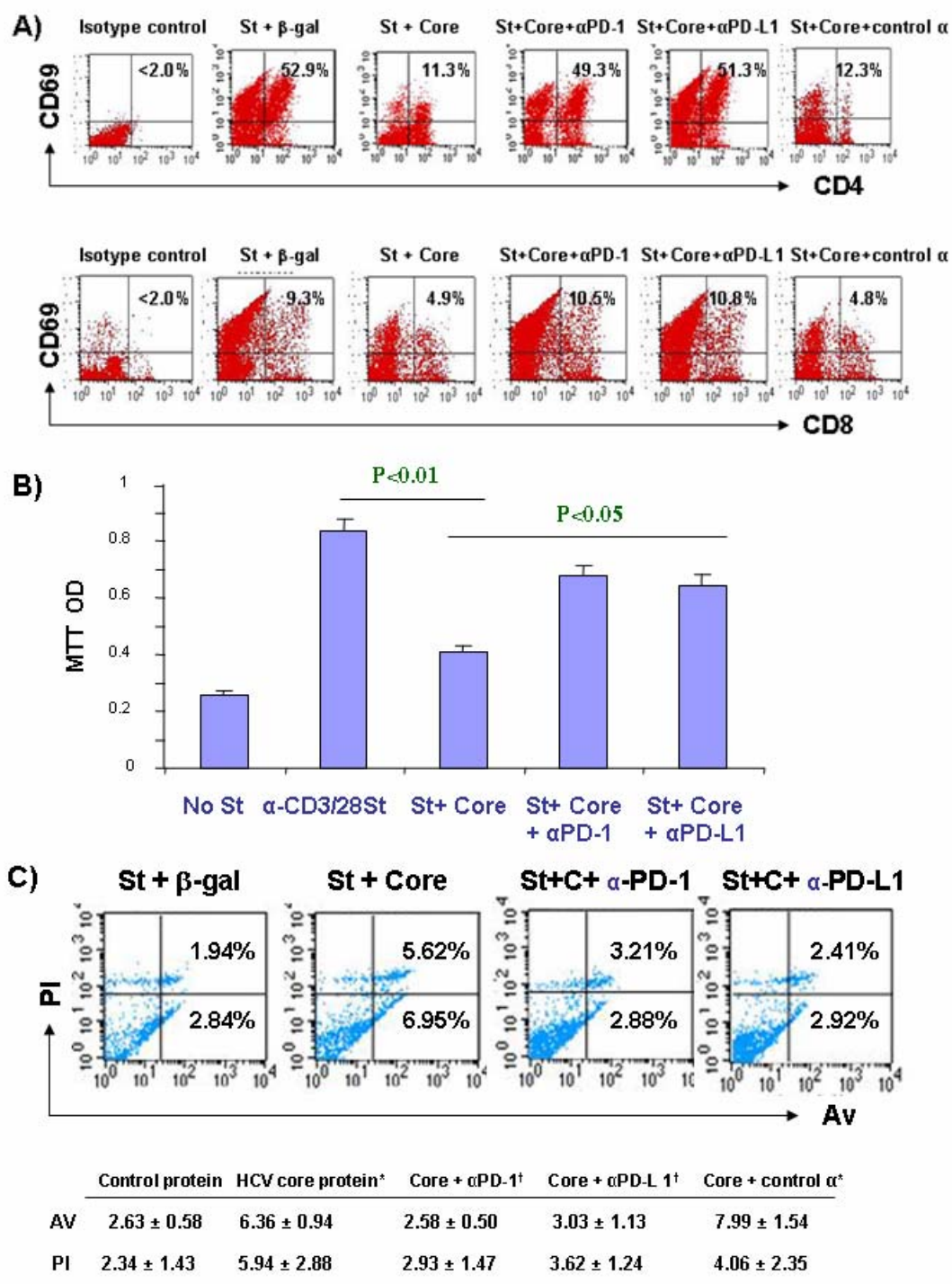


Figure 2.3 Blocking PD-1 and PDL-1 engagement inhibits HCV core/gC1qR-mediated T cell dysfunction

Discussion

The most enigmatic facet of HCV infection is the fact that the majority of individuals infected with this virus exhibit impaired T cell responses and develop persistent infection, while a limited group are able to successfully control their infections and experience spontaneous virus eradication. It is generally agreed that both viral and host factors play a role in determining the outcome of viral infection. Our previous studies (Yao and others 2001a; Moorman and others 2003; Yao and others 2003; Yao and others 2004) and current data would suggest that HCV core protein may be a major viral antigen involved in immunomodulating host responses. Intriguingly, HCV core is secreted from infected cells and circulates in the bloodstream of infected individuals (Masalova and others 1998; Maillard and others 2001) at levels consistent to those used in our experiments. In addition, the amount of free core protein or core protein expressed on the surface of infected cells is greater in the micro-environment of the liver, where virus replication occurs quite vigorously in early infection when chronicity is either avoided or established. Thus, HCV core protein appears to be available in the setting of clinical infection and based on our studies could be contributing to establishment of persistent HCV disease.

While it seems clear that HCV evades relatively weak host immune responses, the issue of the level and specificity of immunosuppression in individuals with HCV infection remains debatable. Several reports suggest that selective dysfunction of HCV-specific T cells occurs in HCV-infected patients,

with preservation of immune responses to unrelated viruses and/or antigens (Gruner and others 2000; Takaki and others 2000; Thimme and others 2001; Wedemeyer and others 2002). Other studies, however, have found abnormalities with T cell responses to unrelated or nonspecific antigens in the setting of HCV infection. For example, CMV-specific CD8⁺ maturation markers were dramatically lost in an HCV-infected cohort despite the fact that inducible responses to antigen were preserved (Lucas and others 2004). This study suggested that HCV infection had a pervasive effect on numerous CD8⁺ T cell populations and not merely HCV-specific T cells. Interestingly, a recent study in an HIV/HCV co-infected cohort found that IFN γ secretion to recall antigens including *Candida* (as well as HCV antigens) correlated with the degree of hepatic fibrosis, with the data suggesting that impaired cellular responses were a qualitative defect rather than a function of CD4 cell count (Graham and others 2005). It is by no means impossible that both HCV-specific and non-specific immunodysregulatory mechanisms are occurring during acute and chronic HCV infection, perhaps supported by the strong clinical evidence of autoimmune disease associated with HCV infection (Manns and Rambusch 1999; Vogel and others 2002).

Our data would support nonspecific immunomodulation of both CD4⁺ and CD8⁺ T cell functions by HCV core antigen. We have in this and previous studies consistently noted effects on T cell proliferation, activation, and apoptosis in response to HCV core and dependent upon gC1qR engagement (Yao and others 2001a; Moorman and others 2003; Yao and others 2003; Yao and others 2004).

This does not rule out the presence of HCV-specific T cell dysfunction by HCV core or other viral gene products, and certainly further studies in this area are warranted. It is also feasible that HCV core expression in the peripheral blood of infected patients, while perhaps not sufficient to induce the severe, generalized immunosuppression seen with HIV, may be high enough to reach a threshold locally in the liver to facilitate persistent HCV infection.

The novel role of PD-1 and PDL-1 in HCV described herein is exciting and may ultimately provide a marker and perhaps a mechanism by which such viruses establish persistent infection. The recent studies of LCMV infection in a murine model and HIV in humans clearly demonstrate a key role for PD-1 up-regulation in CD8⁺ T cell exhaustion and control of viral infection (Barber and others 2006; Day and others 2006; Freeman and others 2006; Petrovas and others 2006; Trautmann and others 2006b). Our data in human T cells suggest that that HCV might function in a similar model, with up-regulation of PD-1 being associated with and perhaps contributing to chronicity. While it is certainly possible that this up-regulation of PD-1 in chronically HCV-infected individuals is more a marker of chronicity, there is little doubt that signaling via this pathway in general leads to immunomodulation (Nishimura and Honjo 2001). We expect that other chronic viruses may be associated with PD-1 up-regulation as well.

Once again, our data would suggest that up-regulation of the PD-1 and PDL-1 pathway occurs in the general T cell population, but it would clearly be worth pursuing an analysis of PD-1 expression in HCV-specific CD8⁺ and CD4⁺ T cell populations from chronically infected individuals using tetramer studies and

antibodies to PD-1 and PDL-1. Complicating these studies is the very real technical challenge of analyzing virus-specific T cells that are often absent or present at very low frequencies in individuals with chronic HCV infection (He and others 1999); studies in this area are in progress but reversal of virus-specific T cell dysfunction thus far has not been accomplished. It is possible that core-induced up-regulation of PD-1 and PDL-1 might be occurring on antigen presenting cells or hepatic tissues and promoting T cell dysfunction through cell-cell interaction.

Our data demonstrated the ability to reverse T cell dysfunction in healthy donor PBMCs exposed to HCV core by blocking PD-1 engagement, supporting a link between HCV core, gC1qR, PD-1, and T cell functions. Whether this reversal can occur in chronically infected HCV-infected individuals is no doubt the most pressing question, but has thus far been technically challenging for us to determine in infected individuals in the setting of years of chronic antigen exposure and T cell anergy. The intracellular mechanism(s) linking these pathways are also as yet unclear, but possibilities include SHP-1/2 or the SOCS family that have been linked to negative regulation of T cell signaling pathways (Balasubramanian and others 2003; Bode and others 2003). Studies addressing these questions are ongoing.

We have thus found up-regulation of the PD-1 pathway in chronically infected individuals with HCV that may be mediated through HCV core protein, a known immunomodulatory viral antigen that circulates in the serum of infected individuals. This study raises the possibility of targeting this inhibitory pathway

for the treatment of persistent viral infections. Blocking either the gC1qR or PD-1 inhibitory pathways might be of clinical benefit during the acute phase of viral infection when persistence is established in the majority of hosts. Restoring impaired T cell function during chronic infection, albeit challenging, may be the more desirable goal. This might be achievable based on limited evidence of occasional resolution of HCV infection with increasing CD4⁺ T cell numbers and function following HAART for HIV infection. Furthermore, the most common reason for failure of therapeutic vaccine in eliminating chronic diseases is limited T cell proliferative potential, and as such a better understanding of this novel mechanism by which viruses impair T cell responses may open new avenues for immunotherapy by inhibition of fundamental, negative regulatory pathways.

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Figure Legends

Figure 2.1 PD-1 Expression on PBMCs from Chronically HCV-infected Individuals is Increased Compared to Healthy Donors.

A) PMBC were isolated from 12 chronically HCV-infected patients (circles) and 5 healthy donors (squares). The cells were stimulated with (filled circles or squares) or without anti-CD3/CD28 (anti-TCR stimulation, open circles or squares) for 24 h, and PD-1 expression on lymphocytes was examined as described in Materials and Methods. Percentage of PD-1⁺ cells in the gated lymphocyte population was shown. Statistical comparison (p values) amongst different groups, including PD-1 expression on T cells from chronic HCV patients versus healthy donors as well as unstimulated cells versus TCR-stimulated cells, are all < 0.01. Data represent cumulative results of three independent experiments. B) Representative dot blots from subjects showing percentages of PD-1⁺ cells in the TCR-stimulated CD4⁺ and CD8⁺ T lymphocyte populations. Lymphocytes were double-stained and gating was performed on distinct populations of either CD4⁺ or CD8⁺ cells. Isotype controls for these experiments are shown. C) Representative histograms of PDL-1 expression on unstimulated lymphocytes from HCV-infected or healthy subjects. D) PD-1 and PDL-1 expression on different PBMC phenotype populations. PBMC from 2 chronic HCV patients were subjected to mock stimulation or TCR-activation and PD-1 and PDL-1 expression on CD45RA⁺ and CD45RO⁺ populations were detected by

flow cytometric analysis. Percentages of PD-1 and PDL-1 on CD45RA⁺ and CD45RO⁺ lymphocytes are shown on the upper right corner.

Figure 2.2 PD-1 Induction on Healthy Human T Cells by HCV Core Protein.

A) PD-1 induction on lymphocytes by C1q protein and HCV core protein. Left, unstimulated PBMC from healthy donors were exposed to C1q or a control protein β -gal for 24 hr, and PD-1 expression on the surface of the treated lymphocytes was examined as described in Methods. The percentages of PD-1 expression on lymphocytes are shown and the data are representative of experiments from two separate donors. Middle and right, unstimulated or TCR-stimulated PBMC from healthy donors were exposed to HCV core or a control protein β -gal for 24 h, and PD-1 and PDL-1 expressions on the surface of the treated lymphocytes were examined as described in Methods. The percentages of PD-1 or PDL-1 expression on PBMCs are shown and are representative of three independent experiments. Cumulative analysis of these experiments revealed statistical significance between β -gal- and β -gal core-treated unstimulated cells and stimulated cells (unstimulated PD-1: 10.09 ± 2.7 vs 16.5 ± 5.03 , $p < 0.05$; stimulated PD-1: 18.05 ± 2.9 vs 32.15 ± 5.3 , $p < 0.05$; unstimulated PDL-1: 31.27 ± 19.98 vs 58.60 ± 16.03 , $p < 0.05$; stimulated PDL-1: 65.9 ± 5.6 vs 90.75 ± 2.3 , $p < 0.02$). B) PD-1 and PDL-1 induction on the CD4⁺ T cell subpopulation by HCV core protein. Unstimulated or TCR-stimulated PBMC

from healthy donors were exposed to HCV core or control protein β -gal for 24 h, and PD-1 and PDL-1 expression on the surface of the treated CD4⁺ T lymphocytes were examined as described in Methods. Cells were double-stained and gating was performed on a distinct CD4⁺ population; data shown are representative dot blots from three independent experiments. The percentages of PD-1 or PDL-1 expression on CD4⁺ T lymphocytes are shown. C) Time- and gC1qR-dependent PD-1 mRNA induction by HCV core protein. Above, TCR-activated PBMC were treated with or without HCV core in the presence or absence of anti-HCV core, anti-gC1qR or a control antibody for various time points and PD-1 expression was detected by semi-quantitative RT-PCR as described in the Methods. β -actin serves as a control. Results are representative of two independent experiments. Below, flow cytometric analysis of gC1qR-dependent PD-1 induction on CD8⁺ T cells by HCV core protein. PBMC from 2 healthy donors were TCR-stimulated and treated with HCV core in the presence or absence of anti-gC1qR or a control antibody for 24 hr, and PD-1 expression on CD8⁺ T cells was analyzed by flow cytometry. Percentage of PD-1⁺ cells in the CD8⁺ populations is shown on the upper right corner.

Figure 2.3 Blocking PD-1 and PDL-1 Engagement Inhibits HCV Core/gC1qR-mediated T Cell Dysfunction.

A) Blocking PD-1 pathway signaling restores the expression of the early T cell activation marker CD69 that is inhibited by HCV core. PBMC were

stimulated with anti-CD3/CD28 in the presence of HCV core or control protein β -gal for 24 h. Anti-PD-1 or anti-PDL-1 antibody was added to the TCR-stimulated and core-treated cells simultaneously. T cell activation was assessed by detecting CD69 expression on the surface of CD4⁺ and CD8⁺ T lymphocytes as described in Methods. Isotype controls for staining are shown. Results are representative of two independent experiments with separate donors. In the CD4⁺ subpopulation, statistical significance was observed between β -gal and β -gal-core-treated cells (55.6 ± 3.7 vs 13.2 ± 2.7 , $p < 0.01$), between β -gal-core-treated cells and β -gal-core-treated cells with antibody to PD-1 (41.5 ± 11.1 vs 13.2 ± 2.7 , $p < 0.04$), and between β -gal-core-treated cells and β -gal-core-treated cells with antibody to PDL-1 (57 ± 8.1 vs 13.2 ± 2.7 , $p < 0.01$). B) Blocking PD-1 pathway prevents HCV core-induced inhibition of T cell proliferation. PBMC were stimulated with or without anti-CD3/CD28 in the presence or absence of HCV core protein for 24 h. Anti-PD-1 or anti-PDL-1 antibody was added to the TCR-stimulated and core-treated cells simultaneously. T cell proliferation was assessed by Quick Cell proliferation assay. Statistical significance is shown and results are derived from three independent experiments with separate donors. C) Blocking PD-1 pathway prevents HCV core-induced T cell apoptosis. PBMC were TCR-stimulated in the presence or absence of HCV core protein for 24 h. Anti-PD-1 or anti-PDL-1 antibody was added to the TCR-stimulated and core-treated cells simultaneously. T cell apoptosis was assessed by AV/PI staining as described in Methods. Above, percentages of cells in the early apoptotic stage (only AV positive) and in death (AV/PI double positive) after the treatment are

shown. Below, the data were shown as percentage of cells positive for AV or PI (mean \pm SD) on the gated T cell populations from six independent experiments with separate donors. * denotes statistical significance when compared to control treatment ($p < 0.01$). † denotes statistical significance when compared to HCV core treatment ($p < 0.01$).

CHAPTER 3

Hepatitis C: the Complications of Immune Dysfunction

Summary

Hepatitis C virus (HCV) infection has been linked to numerous diseases of immune dysfunction, including but not limited to essential mixed cryoglobulinemia and non-Hodgkin's lymphoma. Clinical studies support these associations and treatment of the underlying HCV infection has been variably successful. Recent studies focusing on the role of HCV gene products have discovered evidence of dysregulated responses in multiple aspects of host immunity that may be contributing to the genesis of these diseases. Novel treatments that target these areas of dysregulation offer hope for improved therapy for the diseases associated with immunodysregulation by HCV.

Introduction

HCV was identified as the agent of transfusion related non-A, non-B hepatitis in 1989 (Choo and others 1989). The Centers for Disease Control and Prevention estimate 2.7 million Americans have chronic hepatitis C infection with an annual monetary cost (medical and work loss) of >600 million dollars. The majority of acute cases occur in adults 30-49 years old and HCV infection is the leading indicator for liver transplant in the U.S. and Europe (Alter and others

1997; Hoofnagle 1997). Persons who have chronic hepatitis C infection are at significant risk of developing cirrhosis, hepatocellular carcinoma and notably diseases of immune dysfunction, including essential mixed cryoglobulinemia and lymphoproliferative disorders (Hoofnagle 1997), upon which this review will focus. Disease progression varies greatly from person to person, making it not only difficult to predict who will develop chronic disease but also which of the chronically infected will go on to develop significant sequelae. To further compound the issue, it appears that the six major genotypes described may have differing disease patterns and responses to treatment (Simmonds and others 1993b).

Hepatitis C is a 9.5-kb, positive strand, RNA virus of the *Flaviviridae* family. The genome consists of ~9500 nucleotides encoding a single polyprotein of ~3000 amino acids that is cleaved into structural and non-structural proteins. It encodes three structural proteins (core, E1 and E2) and at least six non-structural proteins (Figure 3.1).

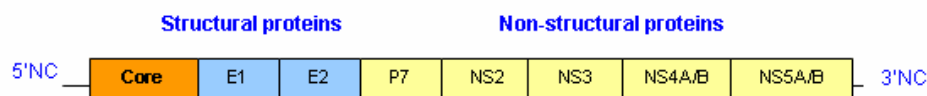


Figure 3.1 HCV genomic structure

HCV is classified into 6 genotypes with over 30 subtypes based on sequence variation in the core and E1 genes, additionally, there are significant

geographical variations (Simmonds and others 1993b). Persons with long-term infections tend to have greater levels of genetic diversity within their genotypes (quasispecies) (Farci and others 1997), most likely due to the poor fidelity of the HCV RNA polymerase. The phenomena of high levels of quasispecies is associated with poor clinical prognosis, which may be linked to the poor cytotoxic lymphocyte (CTL) response in chronically infected persons.

In the U.S., HCV genotypes 1a and 1b are most prevalent and the most resistant to the currently available therapy, pegylated interferon- α (IFN- α) and ribavirin (Hoofnagle 2003). This combination treatment will induce a sustained virologic response (SVR) in 42-48% of persons infected with genotype 1a or 1b but up to 76-88% of persons infected with genotypes 2 or 3 (Fried and others 2002). Side-effects, however, are common and can be serious. For example, IFN- α is associated with significant depression and ribavirin can induce anemia and neutropenia and is teratogenic.

The study of HCV is made difficult by two key factors. HCV will only reliably infect humans and chimpanzees, thus limiting animal models, and *in vitro* replication is very poor (although some replicon systems have been functional in hepatocytes.) Since newly infected persons are often asymptomatic and therefore are rarely clinically recognized as infected, it has also been difficult to determine the initial immune responses that correlate with viral clearance. Finally, because diseases associated with immune dysfunction occur frequently in individuals without HCV infection, it has been difficult to confirm strong associations between HCV and a given disease. This review will describe the

common diseases of immune dysfunction associated with HCV infection and discuss potential mechanisms underlying these diseases.

Potential Mechanisms of HCV-mediated Immune Dysfunction

While the precise mechanisms for how the disruption of the host immune response by HCV can result in various immune disorders have yet to be defined, it is apparent that HCV modulates host immunity on several different levels (Moorman and others 2001b). Over the past decade, there have been numerous studies examining different facets of HCV-associated immune dysfunction which have included B-cell, T-cell, NK cell, and dendritic cell dysregulation (Figure 3.2). The significance of such immunomodulatory potential appears to be twofold: evasion of the host immune response leading to viral chronicity, and the potential for development of lymphoproliferative disorders.

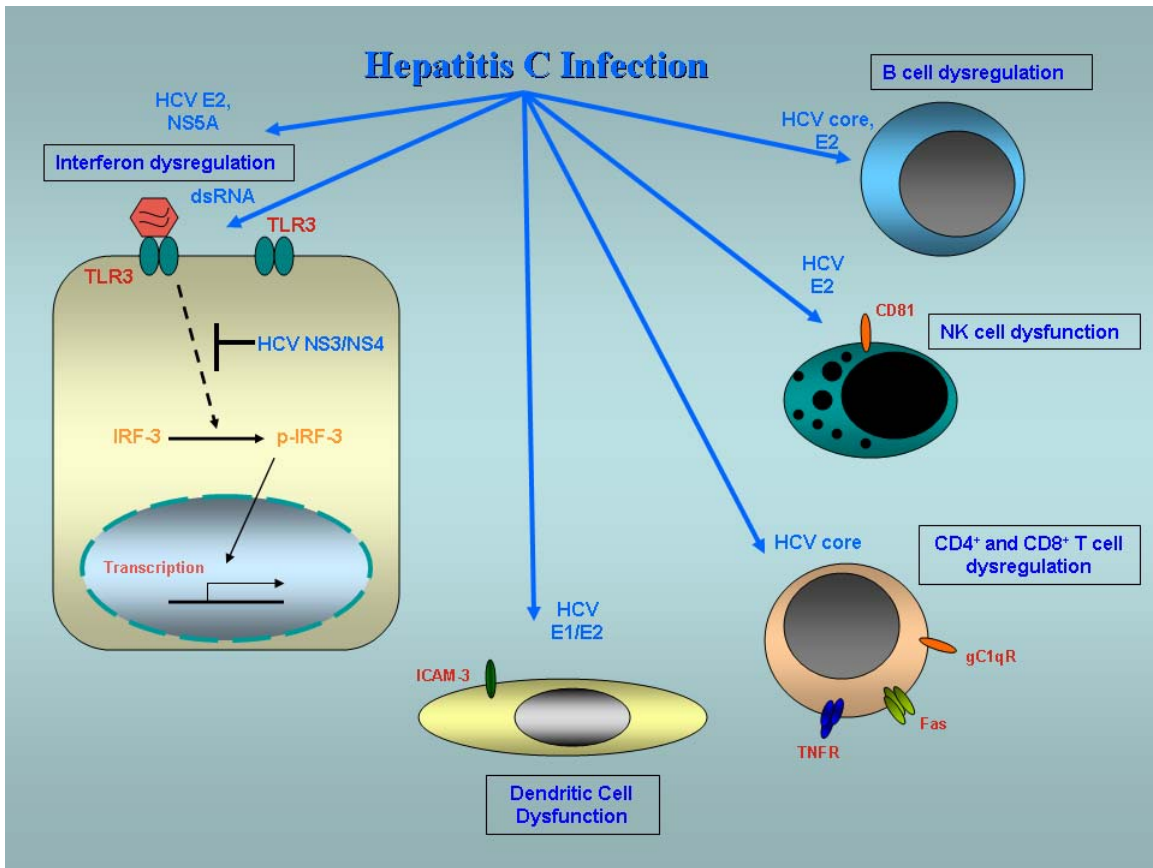


Figure 3.2 Proposed model for immune dysregulation by HCV infection

It is noteworthy that HCV clearly infects multiple extrahepatic cell types, including peripheral blood mononuclear cells, and this is observed both *in vivo* and *in vitro* (Shimizu and others 1992; Shimizu and others 1997; Lerat and others 1998; Radkowski and others 2000; Radkowski and others 2002; Nowicki and others 2005). It is therefore possible that the immune dysfunction observed in HCV is a function of its ability to infect such cells, although the frequency of this infection appears low. HCV sequences observed in different cellular compartments and in particular peripheral blood mononuclear cells may vary, suggesting viral tropism for specific cells that may be in part affected by the HCV diversity that occurs as quasispecies develop (Lerat and others 1998). The role

of a given genotype in targeting hematopoietic cells remains controversial (Kao and others 1997; Lerat and others 1998).

Dysregulation of B Cell Function Mediated by HCV

Since the association between chronic HCV infection and both mixed cryoglobulinemia and B-cell lymphoma was first recognized, there have been an increasing number of studies examining B cell dysfunction in the context of chronic HCV infection. While it has clearly been shown that clonal populations of B lymphocytes are disproportionately present in increased numbers in patients with chronic HCV infection, there are also numerous data detailing specific mechanisms behind B cell clonality. Moreover, such mechanisms offer insight into the presence of immune dysfunction in patients with chronic HCV infection. Most notably, B cells have become a target of drug development for the treatment of certain HCV-associated immune diseases (Kazkaz and Isenberg 2004).

Franzin et al. were the first to report the presence of clonal B cell expansion in the peripheral blood of 38 HCV-infected patients (Franzin and others 1995). Subset analyses in their study revealed the presence of clonal immunoglobulin gene rearrangements in 100% of HCV-positive patients with type II mixed cryoglobulinemia (MC) as opposed to only 24% of HCV-positive patients without mixed cryoglobulinemia. The reversibility of B cell clonality in chronic HCV infection was similarly assessed in a cohort of 20 patients with HCV-associated MC undergoing interferon- α therapy (Mazzaro and others 1996).

While 80% of these patients (all of whom were noted to have clonal populations of B cells) achieved complete or partial clinical responses, 15% reverted to polyclonal B cell populations following therapy. Recent studies also suggest upregulation of B-lymphocyte stimulator (BLyS) in the setting of MC (Fabris and others 2006; Sene and others 2006), further supporting a significant relationship between clonal expansion of B lymphocytes and HCV-associated mixed cryoglobulinemia. Zignego et al. found an increased frequency of *bcl-2* gene rearrangement(t(14;18)) in a prospective study of individuals with HCV and mixed cryoglobulinemia (Zignego and others 2002), described in detail below. While Toubi et al. observed enhanced B cell apoptosis in B cells from chronically infected HCV patients, there was relative resistance to apoptosis seen in the CD95/Fas+ B cell subpopulation, suggesting a potential role for this subpopulation in B cell proliferative disorders (Toubi and others 2004).

As the propensity of HCV to induce lymphoproliferation in patients with HCV-associated mixed cryoglobulinemia has been further elucidated, there has been some effort to define the underlying mechanisms. Machida et al. reported that an HCV E2-CD81 interaction modulates host B cell responses by enhancing activation-induced cytidine deaminase (AID) and hypermutating V(H) immunoglobulin genes in B cells, suggesting a mechanism for HCV-associated B cell lymphoproliferative disorders (Machida and others 2005). HCV E2 was also shown to activate the JNK pathway leading to preferential proliferation of CD27⁺ B cells (Rosa and others 2005).

The effect of HCV core protein on molecular profiling in human B-

lymphocytes was recently examined by Wu et al., who found dramatic evidence of inhibition of B lymphocyte apoptosis by HCV core in several steps of the apoptotic cascade (Wu and others 2006). The investigators expressed HCV core in an adenoviral vector in a healthy population of human B lymphocytes and observed downregulation of MHC class II molecules, and caspase-1 and -4, which are proapoptotic proteins; as well as upregulation of nuclear factor of κ light peptide inhibitor gene and TATA box protein, both of which are associated with B cell lymphoma. Similarly, we have very recently found a differential response of B cells and T cells to HCV core protein, with upregulation of B cell functions (immunoglobulin expression and cell proliferation) and inhibition of B cell apoptosis in the setting of downregulated T cell responses (personal communication, JPM). These findings underscore the potential of HCV to disrupt antigen presentation and apoptosis and may indicate yet another mechanism for HCV-related autoimmunity and lymphoproliferation.

Oncogenes have also been a focus of investigation in patients with MC. In a prospective study of 37 patients with HCV-associated mixed cryoglobulinemia, Zignego et al. found that 75.7% had *bcl-2* rearrangement in peripheral blood mononuclear cells, as opposed to 37.6% of patients with chronic HCV infection without mixed cryoglobulinemia (Zignego and others 2002). Galli-Stampino et al. further expanded on the relationship between oncogenesis and clonal B cell expansion in patients with HCV-associated mixed cryoglobulinemia by demonstrating the presence of restricted V(H)I gene sequences in peripheral blood, hepatic, and lymph node B lymphocytes in three of four patients (Galli-

Stampino and others 2003). These gene sequences are usually associated with B-cell non-Hodgkin's lymphoma and their presence in patients with chronic HCV patients may indicate a mechanism of developing NHL through non-malignant, clonally expanded B-cell populations.

Type I Interferon Dysregulation

Interferon- α (IFN- α) stimulation is a critical and non-specific response to viral infection. Its two main effects are induction of an anti-viral state in infected cells and interferon receptor ligation which results in activation of Janus kinase (Jak) and signal transducer and activator of transcription (STAT). Jak/STAT signaling in turn activates numerous interferon-response genes including 2'-5' oligoadenylate synthetase (OAS), Mx proteins and the double-stranded RNA dependent protein kinase (PKR) (Katze 2002). PKR is induced by double stranded viral RNA and in-turn, phosphorylates eukaryotic initiation factor 2 (eIF-2), resulting in inhibition of cellular protein syntheses and viral replication. Two HCV proteins, the envelope glycoprotein E2 and the nonstructural protein NS5A, have been reported as potential inhibitors of the IFN response (Taylor 2000; Pflugheber and others 2002).

Phosphorylated interferon factor 3 (pIRF-3) is the key transcription factor for type I interferon (Foy and others 2003). Type I interferon in turn, upregulates the expression of PKR. Recently, the HCV protein NS3/4A (a serine protease) was shown to interrupt the IFN signaling pathway; thus, IRF-3 is never phosphorylated and IFN production is not induced (Foy and others 2003). The

importance of IRF-3 was also demonstrated with mutations resulting in dominant negative or constitutively active IRF-3 leading to enhanced or restrained HCV replication, respectively.

Dysregulation of the NK Response

Natural killer (NK) cells play a critical role in innate immunity. NK cells mediate lysis of target cells by releasing cytotoxic granules which contain perforin and granzymes or by binding apoptosis inducing receptors on the target cells. NK cells also secrete numerous cytokines including IFN- γ and TNF- α during inflammation (Poccia and others 2001). Recent reports have implicated the HCV E2 protein in suppression of NK activity (Crotta and others 2002; Tseng and Klimpel 2002).

The HCV E2 protein binds CD81 which is expressed on the surface of host cells. When NK cells are exposed to immobilized E2 or anti-CD81 (*in vitro*), their function is impaired. The impairment ranges from suppressing of cytotoxicity and IL-2 induced proliferation to suppressing IL-2, IL-12 and IL-15 induction of IFN- γ (Crotta and others 2002; Tseng and Klimpel 2002). Additionally, ligation of CD81 inhibits CD16-mediated activation of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) in NK cells (Crotta and others 2002). These mechanisms, if employed by HCV, could facilitate HCV persistence. However this possibility needs further research as NK cells do not play a role in all viral infections.

Dysregulation of Dendritic Cells by HCV Envelope Glycoproteins

One possible cause of an impaired CD8⁺ response to HCV infection may be abnormal dendritic cell (DC) function preventing appropriate antigen processing and presentation. Interestingly, HCV E1 and E2 glycoproteins interact with DC-specific intercellular adhesion molecule 3 (ICAM-3), grabbing nonintegrin (DC-SIGN) and its receptor, DC-SIGNR in immature DCs (Lozach and others 2003; Pohlmann and others 2003). The role of HCV interactions with DC remains controversial. Numerous studies demonstrate abnormal DC activity in chronic HCV patients, yet recent studies by Longman et al. report the converse (Longman and others 2005).

In addition to envelope proteins, HCV core has also been implicated in inhibiting DC and macrophage function (Sarobe and others 2003). Addition of extracellular core or expression of core/E1 in mice by recombinant adenovirus demonstrated an effect on DC maturation and T cell responses upon allogenic stimulation. A molecular mechanism(s) has not fully elucidated to explain this inhibition.

Dysregulation of T Cell Function Mediated by HCV Core Protein

Patients who have a strong and broad cytotoxic lymphocyte (CTL) response are more likely to spontaneously resolve a HCV infection when compared to those with a weaker response (Lechner and others 2000). The CTL response is suppressed during all phases of HCV infection, although the suppression mechanism(s) are not well understood. One hypothesis for weaker

CTL responses is that the HCV proteins interact either directly or indirectly against CTLs. Data have been published indicating that HCV core antagonizes host T cell responses, thus potentially contributing to disease progression (Large and others 1999b; Ray and Ray 2001).

HCV core appears to interact with at least two major cellular signaling pathways: intracellular core interacts with tumor necrosis factor receptor (TNFR) and Fas, and extracellular core interacts with the complement receptor gC1qR (Kittlesen and others 2000). Interactions between core and TNFR or Fas are known to induce apoptosis in both hepatocytes and lymphocytes, suggesting a possible mechanism for HCV-associated liver pathology (Hahn and others 2000; Zhu and others 2001; Moorman and others 2003). However, whether the liver pathology results from core-induced apoptotic hepatocytes or is a secondary necrosis induced by infiltration of apoptotic lymphocytes remains to be determined.

Intracellular core appears to localize to the cytoplasm, ER and nucleus, while extracellular core is found in transfected cell lines and in the serum of infected patients (Sabile and others 1999; Maillard and others 2004). This same extracellular core has also been found to interact with the complement receptor gC1qR, which results in inhibition of T cell activity (Kittlesen and others 2000). This inhibition appears to take place early in infection and targets T cell proliferation and IFN- γ production (Yao and others 2001a). C1q, the natural ligand for gC1qR, is part of the C1 complex and is the first component in the classical complement pathway. C1q, like HCV core, will also inhibit T cell

proliferation, suggesting that core may usurp this pathway to its advantage. Other viruses such as Epstein Barr and HIV appear to exhibit similar mechanisms to suppress the host immune response by interactions with the complement system (Hilleman 2004).

While HCV core appears to be a useful target as an antigenic stimulus because of its highly conserved nature, this protein has also been associated with the development of auto-antibodies such as anti-LKM, anti-p450, and anti-GOR. Interestingly, alteration of core antigen to remove the molecular mimic sites responsible for induction of these auto-antibodies was recently used to elicit strong antigen-specific CTL responses with less self-recognition (Liu and others 2006).

HCV and the Associated Clinical Diseases of Immune Dysfunction

At least 36 extrahepatic disease manifestations have been associated with HCV infection, with disorders of immune function frequently described (Table 3.1). With the exception of mixed cryoglobulinemia, acceptance of other disease associations with HCV infection is not universal (Agnello 1997), but many have fairly strong anecdotal support in the literature. While the data linking HCV infection with non-Hodgkins lymphoma (NHL) is strong, the mechanism(s) underlying the association remain poorly understood (Gisbert and others 2003).

Table 3.1 Extrahepatic disease manifestations associated with HCV infection

Antiphospholipid syndrome	MALToma
Aplastic anemia	Membranoproliferative glomerulonephritis*
Autoimmune hemolytic anemia	Membranous glomerulonephritis
Autoimmune thyroiditis	Mixed cryoglobulinemia*
Chronic fatigue syndrome	Moore corneal ulcers
Behcet's syndrome	Multiple myeloma
Carotid atherosclerosis	Non-Hodgkin's lymphoma (NHL)*
CREST syndrome	Neurocognitive impairment
Dermatomyositis	Pancreatitis
Diabetes mellitus	Polyarteritis nodosa
Fibromyalgia	Polymyositis
Guillain-Barre syndrome	Porphyria cutanea tarda
Hypertrophic cardiomyopathy	Rheumatoid arthritis
Hypocholesterolemia	Sialadenitis
Idiopathic pulmonary fibrosis	Sjogren's syndrome
Idiopathic thrombocytopenia purpura	Systemic lupus erythematosus
IgA deficiency	Uveitis
Lichen planus	Waldenstrom's macroglobulinemia

*Presented in greater detail in the text.

CREST: calcinosis Cutis, Raynaud's phenomenon, esophageal dysfunction, sclerodactyly and telangiectasia; HCV: Hepatitis C infection; MALT: Mucosa-associated lymphoid tissue

Mixed Cryoglobulinemia (Type II Cryoglobulinemia)

Disease.

Cryoglobulinemia is defined as the presence of circulating immunoglobulins (Ig) that reversibly precipitate at $\leq 37^{\circ}\text{C}$. The term “mixed cryoglobulins” was coined to differentiate types II and III, which is characterized by a mixture of 2 different kinds of antibodies, from type I, characterized by just a single monoclonal antibody. Cryoglobulins may precipitate *in vivo* in small blood vessels (venules, capillaries, arterioles), leading to vasculitis. The evidence linking HCV and mixed cryoglobulinemia (MC) is strong and is supported by epidemiological, molecular, and virological studies.

Prevalence.

In the early 1990s, multiple investigators reported the presence of HCV infection in 50-90% of MC patients (Ferri and others 1991; Agnello 1997). The frequency of dual status exhibits geographic variation, with southern Europe having a higher rate than northern Europe or North America (Cacoub and others 2000). The most likely explanation for this difference is differing laboratory techniques used to identify cryoglobulins. This hypothesis is supported by the fact that regions that reported a higher frequency of HCV with MC generally used

more sensitive laboratory methods to identify MC and consistent use of these more sensitive methods has revealed increasing prevalence rates over time.

One alternative to using the more sensitive methods for detecting cryoglobulins is to test for rheumatoid factor (RF). RF displays a high avidity for IgG and thus easily forms immune complexes. Common tests for RF are generally much more sensitive than tests for cryoglobulins. This was demonstrated when data from 408 patients infected with HCV revealed that 40% were positive for cryoglobulins, yet 100% were positive for RF (Karlsberg and others 1995). HCV is now linked to approximately 73% of all forms of cryoglobulinemia (Trejo and others 2001). Notably, the presence of cryoglobulinemia is highly associated not only with extrahepatic disease, as described below, but also with the development of cirrhosis (Kayali and others 2002).

Clinical Manifestations.

Traditionally, the presence of purpura, arthralgias, and weakness were used to diagnose MC; this is no longer adequate as other symptoms are now known to be associated with MC as well (Table 3.2) (Mayo 2003). Many persons with detectable cryoglobulins display no obvious symptoms. When present, the classic skin rash is a palpable purpura that may rarely progress to a necrotizing lesion or systemic vasculitis. Arthralgias are a common complaint of persons with MC and most often affect the proximal interphalangeal joints,

metacarpophalangeal joints, and knees. In some cases, exposure to cold temperatures will increase the pain and stiffness in these areas. Changes in renal function and peripheral neuropathies are also more common than previously thought.

Table 3.2 Symptoms of cryoglobulinemia

Symptom	Incidence
Skin disease	22%
Joint disease	16%
Weakness	65%
Renal disease	25-30%
Raynaud phenomenon	3-50%
Neuropathy	8-25%
Sicca syndrome	20%
Lymphadenopathy	3%
Fever	3%

Adapted from: (Dickson 1997; Hoofnagle 1997; Lunel and Musset 2001; Schott and others 2001; Raanani and Ben-Bassat 2002; Mohammed and Rehman 2003; Nocente and others 2003; Han 2004; Ramos-Casals and Font 2005; Vassilopoulos and Calabrese 2005; Vigani and others 2005)

Notable differences are observed when HCV-associated MC (HCV-MC) patients are compared to MC patients without HCV infection. Both groups display the same frequency of cutaneous involvement, renal involvement and peripheral neuropathy, but the HCV-MC patients are less likely to manifest fever, lymphadenopathy, articular involvement, or Raynaud's phenomenon. ANA and

RF are common in both groups, but MC patients without HCV are more likely to have ANA, whereas HCV-MC patients are more likely to have RF (Trejo and others 2001). Circulating immune complexes will activate complement and therefore low levels of C3, C4, and CH50 are seen in all patients with MC. However, the levels of C4 and CH50 are significantly lower in HCV-MC versus non-HCV-MC patients (Trejo and others 2001).

Pathogenesis.

The symptoms of MC are due the vascular deposition of the cryoprecipitate, which contains HCV-RNA, LDL, VLDL, IgG and monoclonal IgM that has RF activity; these deposits can then lead to vasculitis. The importance of HCV infection in this process is demonstrated by the increased levels of virus and RF antibody (up to 1000 fold higher) at the site of vascular lesions when compared to serum levels (Agnello and others 1992). Persons with HCV-MC can also present with abnormal B cell proliferation, as described in detail above, which appears to be an antigen driven process that can ultimately result in a dominant B cell clone. This may be the result of genetic mutation; for example, 88% of patients who are HCV-MC demonstrate a translocation of the *bcl-2* gene from chromosome 18 to chromosome 14, compared to 8% of patients with HCV alone and 2% of patients with other liver diseases (Zignego and others 2002). However, translocation has not been shown to be a definitive cause, since *bcl-2* overexpression does not always accompany t(14;18) translocation

(Papakonstantinou and others 2001). B cells with the t(14:18) translocation without *bcl-2* overexpression and mRF B cells that over express *bcl-2* without the t(14:18) translocation may be present in the blood. The two populations cannot be discerned without isolation studies that thus far have not been performed.

Management

Prior to the recognition that HCV is associated with MC, plasmapheresis in conjunction with high-dose corticosteroids and immunosuppressive therapy was employed to treat symptomatic exacerbations of the disease; there is a paucity of evidence showing long-term benefit from such an approach (Ballare and others 1995). Plasmapheresis remains the cornerstone of therapy in the setting of MC-associated renal failure, limb ischemia attributable to cryoglobulinemic vasculitis, and progressive motor neuropathy. Observational data suggests that plasmapheresis can halt the progression of acute renal failure in 55% to 87% of patients, although there is no clear mortality benefit (Madore and others 1996). Corticosteroids are also generally advocated in patients with progressive renal failure or vasculitis, although there are data suggesting that they may increase HCV RNA viremia (Dammacco and others 1994).

Antiviral therapy directed towards chronic HCV infection has been shown to be highly effective in HCV-associated MC. Interferon- α or pegylated interferon- α in combination with ribavirin have been used successfully in this manner (Table 3.3) (Zuckerman and others 2000; Mazzaro and others 2003;

Cacoub and others 2005; D'Amico and others 2005; Mazzaro and others 2005). Cacoub et al. reported complete clinical responses in eight of nine patients with HCV-MC at 18 month follow-up after treatment with pegylated interferon- α plus ribavirin for a mean of 13.5 months (Cacoub and others 2005). Mazzaro et al. also reported favorable responses in eighteen patients with HCV-MC treated with pegylated interferon- α plus ribavirin, although 44% (8 patients) relapsed both virologically and clinically within a few weeks after cessation of therapy (Mazzaro and others 2005).

Table 3.3: Summary of trials examining interferon- α (IFN- α) or pegylated IFN- α with ribavirin in the setting of HCV-associated mixed cryoglobulinemia

Year	Authors	No. of patients	Treatment	Complete Virologic Response* (%)	Complete Immunologic Response** (%)	Complete Clinical Response (%)	Sustained Response (Clinical or Immunologic) (%)
2005	D'Amico et al	25	IFN- α plus ribavirin (6 – 12 months)	100	96	ND	ND
2005	Cacoub et al.	9	Pegylated IFN- α plus rivavirin (10 – 26 months)	78	56	89	89-100
2005	Mazzaro et al.	18	Pegylated IFN- α plus rivavirin (48 weeks)	83	56-100	89	44
2003	Mazzaro et al.	27	IFN- α plus rivavirin (1 year)	18	33	85	30
2000	Zuckerman et al.	9	IFN- α plus rivavirin (6 months)	22	78	100	ND

ND- no data

*Defined in all studies as achievement of undetectable viral load

**Reduction in cryocrit or rheumatoid factor concentration

Adapted from: (Zuckerman and others 2000; Mazzaro and others 2003; Cacoub and others 2005; D'Amico and others 2005; Mazzaro and others 2005)

Rituximab, which is a chimeric monoclonal antibody directed towards CD20, is a novel therapy to be tried in HCV-associated MC and appears to be useful based on limited but numerous anecdotal studies (Zaja and others 1999;

Basse and others 2005; Bruchfeld and others 2006; Quartuccio and others 2006a; Scheinfeld 2006; Seror and others 2006).

Non-Hodgkin's Lymphoma

Disease.

Each year, 54,000 people in the United States are diagnosed with non-Hodgkin's lymphoma (NHL). NHL is a set of malignant diseases arising from lymphoid tissues (B or T lymphocytes). Given the multiplicity of subsets of NHL, epidemiological studies regarding causative factors have not been satisfactorily conducted in comparison to other malignant diseases. However, several possible causative factors have been identified, including pesticides, blood transfusion, immunodeficiency, radiation, smoking and several types of diet, as summarized previously (Matsuo and others 2001).

Prevalence.

The data linking HCV infection to NHL are significant but show considerable variation (0-42%) (Kashyap and others 1998; Germanidis and others 1999). Epidemiologic studies support a role for HCV in the development of NHL as well as multiple myeloma (Ohsawa and others 1999; Duberg and others 2005), but others have failed to show significance (Rabkin and others

2002). An interesting parallel is data showing that random bone marrow biopsies of HCV positive persons reveal a lymphoproliferative disorder 25-40% of the time (Ferri and others 1994; Rasul and others 1999). One case control study from Italy found that HCV infection increased the risk for NHL 50-fold in the liver or salivary glands--greater than the relative risk for hepatocellular carcinoma. The same study found a 4-fold increase of lymphoma in other sites (De Vita and others 1998).

Clinical Manifestations

Numerous NHL tumor types associated with HCV infection have been reported (Silvestri and others 1998), with follicular and lymphoplasmacytoid lesions being the most common. Extranodal involvement is common (found in 65% of individuals with HCV), with liver and salivary glands being over-represented (De Vita and others 1997; Zuckerman and others 1997). Individuals with NHL present with symptoms related to enlargement of lymph nodes that may be located throughout the lymphatic system. Visceral involvement with NHL may remain occult for prolonged periods prior to diagnosis. A subset of individuals will develop symptoms such as fever, night sweats, and weight loss that may bring them to medical attention.

Pathogenesis.

While it is clear that individuals with chronic HCV infection and hepatitis leading to cirrhosis often develop hepatocellular carcinomas, the direct oncogenic potential of HCV is controversial. Such a role cannot be completely ruled out, since, e.g., HCV core protein has been reported to be involved in transformation of cells to malignant phenotypes (Ray and others 1996). HCV does show some lymphotropic specificity but only in relatively small numbers of peripheral blood mononuclear cells (PBMC) (Zignego and Brechot 1999), and perhaps only under abnormal conditions such as immunosuppression (Muratori and others 1996; Laskus and others 2002) or cell transformation (Sung and others 2003). The preponderance of data suggests that HCV-associated lymphomas arise from abnormal B cell proliferation, with the mechanisms outlined previously in this review.

Management.

As the potential of HCV to clonally expand B-lymphocytes and promote lymphomagenesis has been further elucidated, there have been several small trials examining the response of HCV-associated B-cell lymphoma to antiviral therapy. Zuckerman et al. reported complete hematologic responses and regression of B-cell clonality in thirteen of fifteen patients with chronic HCV infection and t(14:18) translocation or IgH rearrangement, treated with either

interferon- α or interferon- α and ribavirin for 6 to 12 months (Zuckerman and others 2001). Vallisa et al. also reported complete or partial hematologic responses in nine of twelve patients with HCV-associated, low grade B-cell lymphoma treated with pegylated interferon- α and ribavirin for 6 to 12 months, although molecular remission was not achieved as in the Zuckerman trial (Vallisa and others 2005). Whether antiviral therapy for HCV-associated B-cell lymphoma should supplant or be added to conventional chemotherapy, including rituximab, is unclear as there have been no supporting clinical studies.

Membranoproliferative Glomerulonephritis (MPGN)

Disease.

MPGN is a disease that affects the glomeruli of the kidney. It is characterized by endocapillary proliferation, monocytic infiltration, double contour membranes, large eosinophilic, PAS-positive intraluminal deposits and vasculitis of the small and medium sized renal arteries (Fornasieri and D'Amico 1996). Electron microscopy indicates subendothelial deposits that may have structure similarities to cryoglobulins (Cordonnier and others 1975). MPGN appears to occur predominantly with type II cryoglobulins (Pucillo and Agnello 1994; Fornasieri and D'Amico 1996). mRF appears to be a critical element in deposition of cryoglobulins in the glomeruli (Fornasieri and D'Amico 1996) and since HCV infection produces relatively high concentrations of mRF (Pucillo and

Agnello 1994), this may be a reason for the predominance of HCV in cryoglobulinemic glomerulonephritis.

Prevalence.

Reports linking HCV-related mixed cryoglobulinemia to MPGN find this association in 33% (Agnello 2000) to 81% (Misiiani and others 1992) of cases. Data linking HCV infection as a whole to MPGN are more controversial. Reports have been published supporting and opposing this premise. Neither argument is convincing since studies to detect and characterize RF in MPGN lesions in the absence of cryoglobulinemia and B cell clonal analysis that can detect mRF B cells in the absence of cryoglobulinemia have not been performed.

Clinical Manifestations

The average patient with renal disease associated with HCV infection is recognized when diagnosed with nephritic syndrome (71%). Most will have serum albumin <3 g/dl with mild renal insufficiency (72%). Over time, a relatively small number may progress to dialysis (Fornasieri and D'Amico 1996). Most of these patients will have detectable cryoglobulins (59% initially – 85% overall) but only 44% will present with extra-renal manifestations of cryoglobulinemia. Clinical presentation of liver involvement is not a given. Up to 82% will show no clinical manifestations of liver disease. However, up to 88% will have an

abnormal liver biopsy consistent with chronic HCV infection (Fornasieri and D'Amico 1996). As in primary MC disease, complement levels will also be depressed in most of these patients (Misiani and others 1992).

Pathogenesis

Renal biopsy will generally reveal endocapillary proliferation, monocytic infiltration, and double contour membranes; all morphological features consistent with immune complex disease. Detection of HCV RNA in the affected glomeruli is inconsistent due to the high levels of RF-induced artifact. Notable is the fact that RF from patients with type II MC shows a high affinity for cellular fibronectin, which is highly concentrated in the glomerular mesangium. This has led to speculation that RF induces deposition of Ig in the glomerulus irrespective of HCV infection (Fornasieri and D'Amico 1996).

Management.

Antiviral therapy aimed at chronic HCV infection remains the most effective treatment for HCV-associated MPGN, although recent studies using rituximab are quite encouraging. Interferon- α and pegylated interferon- α both in combination with ribavirin have been used successfully in this regard and have been shown to halt the progression of renal failure in such patients (Giannico and others 2000; Loustaud-Ratti and others 2002; Sabry and others 2002). While

there is concern over the use of ribavirin in patients with renal insufficiency whose threshold for hemolytic anemia is lowered, its combination with interferon is advocated in these patients as long as its dose is titrated to account for a lower creatinine clearance (Orlent and others 2005). As noted, very recent studies suggest a role for rituximab in treating HCV-related glomerulonephritis (Roccatello and others 2004; Garini and others 2005; Quartuccio and others 2006b).

Other Diseases

Evidence has been published linking a number of diseases to HCV infection. In general, more research needs to be done to prove or disprove these links. For example, HCV has been repeatedly linked to progressive declines in pulmonary function in patients with underlying lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) (Kanazawa and others 2003; Kanazawa and Yoshikawa 2004; Moorman and others 2005a). In patients who already had a diagnosis of COPD, chronic HCV infection led to a more rapid decline in forced expiratory volume (FEV1) and diffusing capacity for carbon monoxide (DLCO), findings that were abrogated in those treated with interferon (Kanazawa and others 2003). In a recent 6-year prospective trial, asthmatic patients with chronic HCV who did not respond to interferon had greater impaired reversibility to bronchodilators when compared to either HCV-negative controls or to HCV-positive individuals who responded to interferon (Kanazawa and

Yoshikawa 2004). Some data suggests that HCV infection may alter acetylcholine-mediated airway tone (Kanazawa and Yoshikawa 2004). We have shown that HCV core protein can alter IL-8 cytokine expression in pulmonary fibroblasts, mediated by p38 MAPK (Moorman and others 2005b). Other smaller studies also suggest a role for HCV infection in various pulmonary diseases, including idiopathic pulmonary fibrosis (Manganelli and others 1996; Idilman and others 2002).

Expert Commentary

While HCV infection has in the past been primarily considered a disease of the liver, it is now clear that this systemic infection leads to serious health issues beyond just the propensity to lead to chronic hepatitis and cirrhosis. The majority of the diseases outlined in this review are at their core disorders of immune signaling. HCV gene products such as envelope and core proteins and NS5A appear to have significant effects on host immune responses, and these may ultimately also facilitate the establishment of viral chronicity following acute infection. The lack of latency with this particular virus supports the existence of viral mechanisms by which host immune responses are either usurped or avoided in an effort to establish chronicity.

For the majority of individuals who do go on to chronic HCV infection, the consequences on the immune system appear to persist. While there are multiple lines of evidence suggesting that HCV can be an immunosuppressive virus, the

authors firmly believe that it is more accurate to state that HCV is *immundysregulatory*. We and others have consistently found differential effects on B and T lymphocyte function in the setting of both clinical disease and in *in vitro* studies, with B cells exhibiting profiles consistent with activation and CD4⁺ and CD8⁺ T cells exhibiting altered or impaired cytokine and receptor expressions, proliferation, and cell survival. The immune-associated disease states that are observed in the setting of chronic HCV likely represent the results of a complex interplay between these dysregulated immune responses (Figure 3.3).

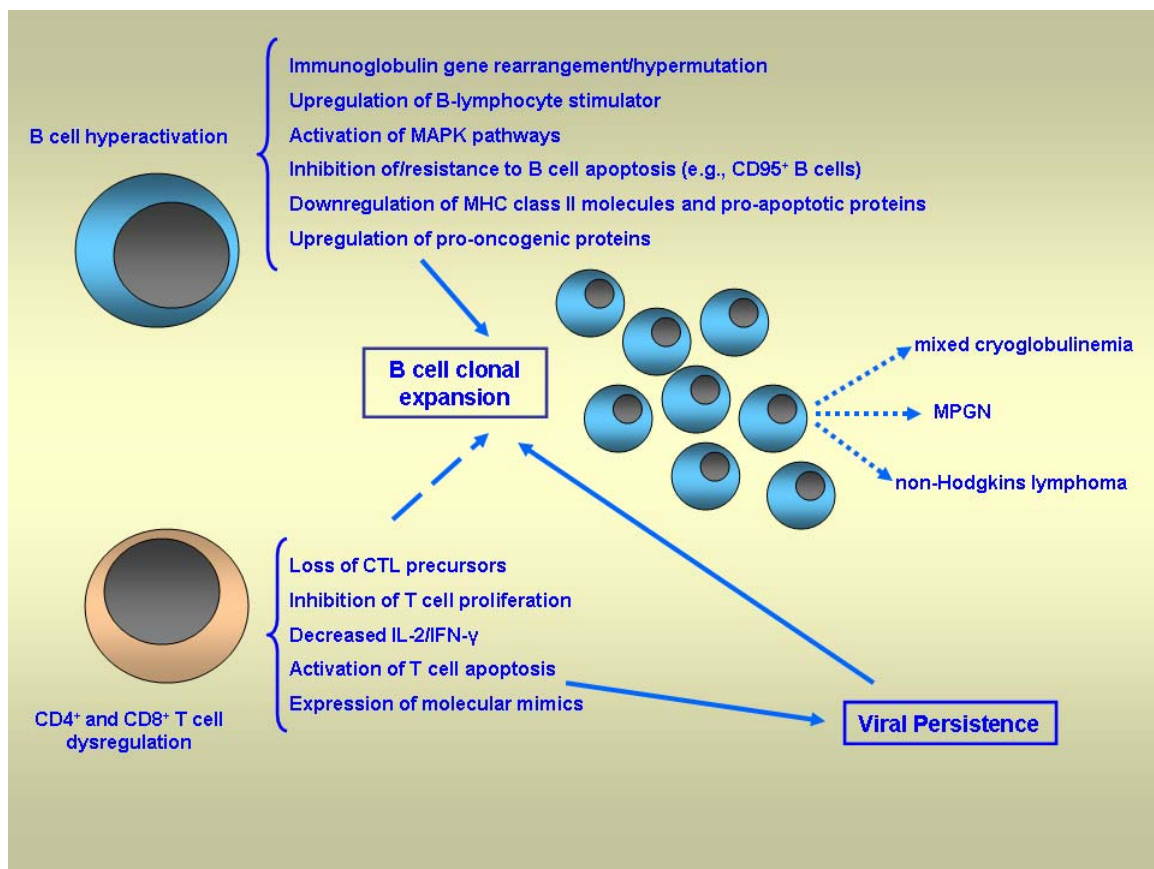


Figure 3.3 Immunodysregulatory aspects of chronic HCV infection based on published studies.
 CTL: Cytotoxic T lymphocyte; IFN: interferon; IL: Interleukin; MAPK: Mitogen-activated protein kinase;
 MHC: Major histocompatibility complex; MPGN: Membranoproliferative glomerulonephritis

In contrast to HIV infection, characterized by a very dramatic loss of CD4⁺ T cells and extreme immunosuppression, chronic HCV infection leads to dysregulated immune responses that are generally more subtle and certainly more variable within a given infected individual. Not every infected patient will develop mixed cryoglobulinemia, for example, but it would not be surprising to us if the majority of HCV-infected patients exhibit altered B cell responses to some degree when assayed in detail. We believe that translational studies focusing on individuals with HCV-associated immune conditions will be the most fruitful approach to determining how HCV and its gene products are altering the specific arms of host immunity.

Unfortunately, much of the data regarding HCV-associated immune dysfunction is derived from small populations and the data are often anecdotal. The difficulty in accruing large numbers of affected individuals could be offset by a multicenter approach to studying these diseases. Such an approach would be excellent for facilitating clinical trials and translational studies and would better define the specific immune-related issues that occur in the setting of HCV infection.

In terms of therapy, there are a fair amount of clinical data suggesting that combination therapy with interferon- α and ribavirin will ameliorate many of the HCV-associated conditions to some extent, particularly in interferon responders. In addition, it is of course desirable to completely eradicate this potentially curable disease if at all possible to avoid not only immunodysregulatory diseases but chronic hepatitis and all of its sequelae. All individuals with HCV infection

should *ideally* be evaluated for treatment and offered combination therapy if appropriate, although in practice this has not been the rule for a multitude of reasons ranging from cost to toxicity of treatment to social barriers to care. These patients need close clinical follow up, immunizations, and teaching regarding lifestyle alterations including alcohol cessation. Treatment by either a hepatologist or infectious diseases specialist is recommended because current treatment options are quite toxic and require intense monitoring.

Therapy with rituximab is perhaps the most exciting modality that has emerged for individuals with immunodysregulatory diseases associated with HCV. Although still primarily anecdotal, the limited data do suggest that targeting of B cells may be relatively effective for several of the conditions outlined herein. The authors believe that individuals with HCV infection who are either not eligible for or do not respond to combination interferon- α and ribavirin therapy, or who have persistent immune disease despite therapy, should be considered for treatment with rituximab and ideally should be enrolled in ongoing clinical trials with this drug.

Five-year View

It is likely that within 5 years there will be much more clinical data confirming the efficacy of drugs such as rituximab in treating HCV-associated diseases. Anecdotal studies throughout the literature are quite encouraging for this treatment, which targets B cells in particular and has comparatively few side

effects. Clinical trials with rituximab are enrolling for the treatment of cryoglobulinemic vasculitis, NHL, and Sjogren's syndrome, but other HCV-associated conditions will assuredly be targeted and clinical data are forthcoming.

Although treatment of the underlying HCV infection does lead to improvement in some of these diseases, our current regimens of pegylated interferon- α and ribavirin are fraught with side effects and cure less than half of our infected patients, depending on their HCV genotype. A large number of new treatments, however, are now in early phase clinical trials and promise improved sustained virologic responses. These include novel protease, RNA polymerase, and RNA helicase inhibitors. The ability to clear infection in the majority of individuals may be a reality for clinicians in the very near future.

Key Issues

- Hepatitis C virus (HCV) can dysregulate host immune responses through interactions with host proteins and perturbation of signal transduction cascades; this immunodysregulation may afford the virus the opportunity to persist and to facilitate the development of immune disorders.
- Mixed cryoglobulinemia is strongly associated with HCV, and renal disease and lymphoma manifest at least some overlap with cryoglobulinemia.
- The interaction of the HCV gene products with key aspects of the host

immune system seems to be important in the pathophysiology of several of the extrahepatic manifestations of HCV.

- Antiviral therapy and/or treatment targeted at B cell populations are efficacious for certain immune-related manifestations of HCV infection.

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CHAPTER 4

DISCUSSION

Despite the advances made in the area of HCV research we are still left with many unanswered questions and few good options. There is no vaccine available for HCV; there is no treatment that is consistently effective from patient to patient and viral genotype to genotype; and finally, there is no conclusive evidence that explains how HCV evades the human immune system and establishes a chronic infection. In these studies we attempt to identify one possible pathway that the virus may exploit to establish a chronic infection.

It has been established that extracellular hepatitis C core protein will bind to the complement receptor gC1qR (Kittlesen and others 2000) and subsequently suppress T-cell activity *in vitro*. This suppressive ability mimics C1q, which is the natural ligand for gC1qR (Chen and others 1994), and can be blocked by the addition of anti-gC1qR. Given that a strong T-cell response is necessary to eliminate viral infections any perturbations of the T-cell response suggests a possible mechanism for establishing a chronic viral infection.

Very recent data have shown that the expression of PD-1/PDL-1 to be related to whether an acute viral challenge will be cleared by the host or develop into a chronic infection. These data show that PD-1/PDL-1 is upregulated in a chronic infection versus an acute infection and may play a role in T-cell anergy.

Of most importance, these studies have shown that anergic T-cells can be rescued by blocking the ligation of PDL-1 to PD-1.

We began these studies by determining the PD-1 expression levels on PBMCs isolated from healthy donors versus PBMCs isolated from persons chronically infected with HCV. Upon activation of these PBMCs by anti-CD3/CD28 or no activation, we were able to demonstrate that in fact PD-1 expression is upregulated on PBMCs from the chronically infected donors when compared to the healthy donors. We were further able to show that PD-1 is also upregulated on the CD4⁺ and CD8⁺ subpopulations. We were also able to demonstrate that the ligand for PD-1, PDL-1, is also upregulated on chronically infected persons. Moreover, when isolated PBMCs were treated with other viral proteins, such as HCV NS3, HIV Tat, and gp120, no change in PD-1 expression was noted. Finally, to rule out the possibility that we had started with different lymphocyte phenotype subpopulations, we compared the PD-1/PDL-1 expression levels of CD45RA and CD45RO on T-cells isolated from chronically infected persons. In both a stimulated and unstimulated setting we show no difference in the levels of PD-1/PDL-1 expression.

Our data suggest that PD-1/PDL-1 are upregulated in response to the setting of a chronic HCV infection; how this occurs is unknown but seems likely related to a hepatitis C viral product. Because previous data have shown that the HCV core protein has suppressive effects on T-cells, we investigated whether HCV core protein would have an effect on the expressed levels of PD-1 and PDL-1. We demonstrated that HCV core will increase the expression of PD-1 in

a dose dependent manner and that C1q will also increase the expression of PD-1 when compared to a control protein. This upregulation of expression, as induced by HCV core, was also shown to be present on CD4⁺ and CD8⁺ T-cells.

Next, to determine if the upregulation of PD-1 is mediated through HCV core binding to gC1qR and to further support the link between core and PD-1 expression, we measured the levels of PD-1 mRNA in T-cells exposed to HCV core. We found consistent upregulation of the message, beginning as early as 6 hours, after treatment with the core protein. We also employed anti-gC1qR in these experiments and found that the antibody inhibited core's ability to increase the level of PD-1 message. To support the gC1qR findings we again measured the expression level of PD-1, this time in healthy donor cells treated with core alone, core plus a non-specific antibody, or core plus anti-gC1qR. Again, we found that anti-gC1qR inhibited core's ability to effect PD-1 changes, *e.g.* PD-1 upregulation.

Our last set of experiments were designed to determine if blocking the PD-1/PDL-1 interaction in the presence of core protein would restore T-cell function. We found that CD4⁺ and CD8⁺ cells in the presence of core protein show a suppression of CD69 (a T-cell activation marker); yet, these same cells demonstrated an increase of CD69 when treated with anti-PD-1 or anti-PDL-1. We were also able to show that blocking with anti-PD1 or anti-PDL-1 will partially restore T-cell proliferation (in the presence of core protein) and will decrease the amount of apoptosis of T-cells (in the presence of core protein).

HCV core is a known immunomodulatory viral antigen that circulates in the plasma of infected individuals. Whether core is the cause or a cause of chronic infection remains to be determined. Regardless of the role core is eventually discovered to have, it seems clear that levels of PD-1 and PDL-1 do have a role in establishing a chronic infection.

Data supporting the importance of PD-1 and its role in the immune response are many; evidence suggests that PD-1/PDL-1 involvement is normal for proper immune tolerance, autoimmune responses, and antitumor or antiviral immune evasion (Nishimura and Honjo 2001; Dong and others 2002; Iwai and others 2003; Chen 2004; Khoury and Sayegh 2004); the recent discovery that PD-1 is upregulated on exhausted T-cells during a chronic viral infection in mice (Barber and others 2006) and their subsequent reactivation by blocking the interaction between PD-1 and PDL-1. Interestingly, this exhaustion of host T-cells as a means of immune evasion may also be a mechanism employed by other persistent viruses in humans, such as HIV (Day and others 2006; Freeman and others 2006; Petrovas and others 2006; Trautmann and others 2006a). The data so far make a strong suggestion for the importance of PD-1 in general and its potential role in the establishment of viral chronicity. If this is indeed the case then either blocking the PD-1/PDL-1 interaction or blocking the HCV core protein/gC1qR interaction would offer potential new treatment options for management of hepatitis C.

Our lab has also recently shown that suppressor of cytokine signaling-1 (SOCS-1) is also upregulated in T-cells in the presence of HCV core. SOCS-1,

like PD-1, is a negative regulator of T-cells. With this knowledge that two known negative regulators of T-cells are upregulated by HCV core protein the next step will be to examine the role of PD-1 and SOCS-1 in regulating T-cell signaling during HCV infection as described in Figure 4.1.

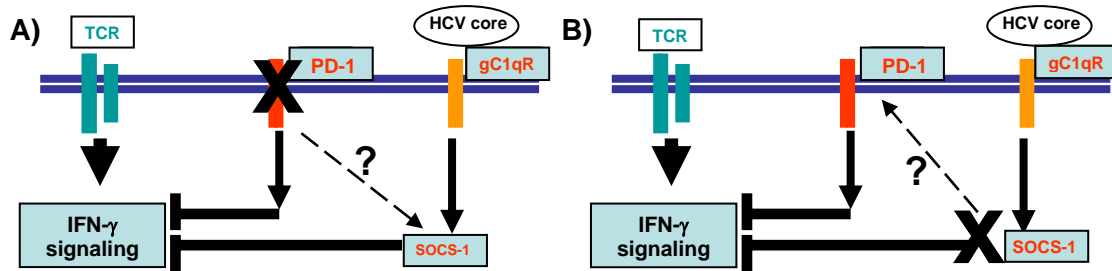


Figure 4.1 Schematic representation of experiments designed to delineate signaling by negative modulators PD-1 (A) or SOCS-1 (B) during HCV core/gC1qR-mediated T cell inhibition.

CHAPTER 5

ADDITIONAL INFORMATION

In addition to the data presented and discussed in chapters one through three, we also attempted to further characterize the hepatitis C core protein. Our hypothesis was that HCV core protein inhibited specific T-cell signaling pathways mediated by its ability to bind to gC1qR and this inhibition subsequently inhibited the T-cell response. Our overall strategy was to create and express mutations of the HCV core protein and compare the mutated core effects on T-cell responses to the wild type core effects on T-cell responses.

We employed a scanning alanine mutagenesis technique to generate the core mutants. We were able to create 10 mutations (as confirmed by sequencing) in the area of HCV core deemed most likely to contain the binding site to gC1qR. We were then able to clone these mutants into an *E. coli* expression system that would express the now mutant proteins with a 6 histidine tag. We then attempted expression and were able to express the mutant proteins and confirm the expression via western blot and coomassie gel. We were also able to purify the proteins by using Ni⁺ columns. We were less successful in our attempts to concentrate the protein. Concentration attempts seemed to cause the core protein to 'disappear' as we could see no signal on post-concentration analysis. Efforts to block the concentration system using unrelated proteins had no positive effect.

We then compared T-cell responses using proteins that we were able to express and found no differences. Whether this was due to small concentrations of the mutant proteins or other reasons is unknown. We ultimately abandoned this avenue of research but feel that with adequate funding and time that needed progress could be accomplished in this area.

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