CHARACTERIZING B CELL PHENOTYPE DURING CHRONIC HCV INFECTION

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DEDICATION

I would like to dedicate this to my mother, who has been my inspiration and will always be my spiritual guide.

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I would like to thank, first of all, my mentor Dr. Nitin J. Karandikar. Dr. Karandikar has been a solid supporter for my continued education. He was gracious enough to provide a lab for me to continue my scientific education when there was no where else to turn, and for that I am thankful. Nitin has a great teaching style which allows him to easily convey the fundamentals of research and he is a vital source of pertinent information, scientific or otherwise. Dr. Karandikar has given me the confidence to pursue my intellectual journey, even when I believed that my capabilities have run their course. Nitin has personal and professional qualities where many others fail and that is something that is inherent in him. I have become a better scientist, a better friend, and a better person because of him.

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Chronic hepatitis C virus (HCV) infection is characterized by an attenuation of virus-specific T cell responses. The mechanisms leading to T cell attenuation are still not well understood, and likely involve several integrating correlates. We hypothesized that dysfunctions of antigen presenting cells (APC) may contribute to the immunosuppresed phenotype. We also reasoned that direct viral interactions of HCV with immune cells may be responsible for such dysfunction. We employed a strand-specific real time RT-PCR assay and found that virus is frequently associated with B cells (predominantly positive strand was detected). Interestingly, we also found that ex-vivo derived B cells from chronic HCV individuals were better inducers of allogeneic T cell proliferation and this

ability correlated with the presence of HCV RNA in those B cells. During such enhanced allostimulation, we also found an increase in the proportion of CD4+CD25+FoxP3+ T cells, which correlated with an increased suppressive capacity thereby demonstrating a paradoxical link between hyperactive B cells and the generation of suppressive T cells. Furthermore, ex-vivo derived chronic HCV B cells had an attenuated response to mitogenic stimulation with associated apoptosis. In an effort to determine direct HCV involvement in immune cell dysfunction, we evaluated the possibility of culture adapted JFH-1 virus to infect PBMC populations. While we found no evidence of viral replication in PBMC, exposure to JFH-1 resulted in vigorous activation of B cells. Interestingly, the B cell activation did not require viable virions, but was dependent upon CD81 availability and the presence of monocytes. We also determined that upon viral exposure, these B cells replicated the hyper-activation of MLR responses found in ex-vivo derived B cells from chronic HCV individuals. In all, our results suggest a novel model wherein HCV-B cell interaction leads to B cell hyper-activation and consequent paradoxical T cell suppression.

Keywords: B cells, T regulatory cells, HCV, immune dysfunction, lymphotropism

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LIST OF ABBREVIATIONS

HCV – Hepatitis C Virus

MLR – Mixed Lymphocyte Reaction

UTR – Untranslated Region

mG – Methylated glutamine

RdRP – RNA-dependant RNA polymerase

INF – Interferon

Huh – Human hepatoma cell line

hVAP - Human VAMP-associated protein

PAMP -Pathogen-associated molecular pattern

dsRNA-double stranded ribonucleic acid

PKR – Protein kinase R (Eukaryotic translation initiation factor 2-alpha kinase 2)

FACS –fluorescence assisted cell sorting

NCR – Non-coding region

FACS –fluorescence assisted cell sorting

rTth – recombinant Thermus thermophilus DNA polymerase

EMC – essential mixed cryoglobulinaemia

NHL – non-Hodgkin's lymphoma

LPD – lymphoproliferative disorder

JFH1 –Japanese fulminate hepatitis virus

CHAPTER ONE
Introduction

HISTORY AND INCIDENCE OF HEPATITIS C INFECTION

History of Viral Hepatitis

Viral hepatitis has been documented since the onset of recorded history. It is currently a worldwide infectious disease that kills approximately 1.5 million people every year (Patlak and Blumberg, NAS 2003). The causative agents of viral hepatitis were not elucidated until a providential moment in Australia when two doctors converged on the finding that many recipients of blood transfusions had antibodies that reacted to a foreign protein. In fact, upon its discovery, researchers were trying to determine genetic differences of ethnic susceptibility to a disparate disease. At the time, inherent ethnic variations were thought to present in the profile of blood proteins. In one instance of an immunodiffusion experiment, researchers Baruch Blumberg and Harvey Alter discovered that serum from a hemophilia patient in New York reacted with that of an aboriginal mans serum. Further experimentation with the hemophiliac found that only 1 in 1000 non-hemophilia sera samples reacted with the patient while 1 in 10 sera samples from leukemia patients reacted. The antigen found in the blood of the aboriginal man and frequently found in leukemia patients was termed Australian antigen (Aa). A study of the blood of children with down syndrome eventually lead to the hypothesis that the Aa found in individuals whom have had multiple

transfusions correlated with hepatitis, and eventually was determined to be an antigen associated with Hepatitis B virus (HBV) infection. A retrospective study on the incidence of HBV infection shows that approximately 2 billion people have been infected worldwide. More that 350 million people are chronically infected and are at an increased risk of liver failure, cirrhosis, and hepatocellular carcinoma (Lavanchy 2004). In 1973, another hepatitis causing agent, Hepatitis A virus, was identified. HAV is a common cause of acute hepatitis, and is ubiquitously found around the world. Improvements in the serological detection of viral hepatitis associated antigen has reduced the worldwide burden of infection, yet in many endemic parts of the world, the prevalence of HBV and HAV infection maintains a stronghold on health and prosperity.

Defining the causative agents of viral hepatitis established that HBV and the subsequently identified Hepatitis A virus (HAV) were transmitted, presumably, in the blood. A massive effort was undertaken to screen donor blood for both types of virus. The screening process reduced the incidence of HBV and HAV infection, yet many post-transfusion patients were still contracting hepatitis (Tateda, Kikuchi et al. 1979). The causative agent of 'non-A, non-B Hepatitis' (NANBH) was not established until a cDNA clone derived from NANBH infected chimpanzee serum hybridized exclusively to NANBH infected chimpanzee liver extracts (Choo, Kuo et al. 1989). The resultant viral RNA sequence was termed Hepatitis C virus, and it was quickly determined that

approximately 80-90% of archived NANBH infected blood samples were positive for HCV (Alter, Purcell et al. 1989). The impact of the incidence of Hepatitis C infection was not appreciated until physicians observed steady increases in serum ALT levels and liver disease. The self limiting hepatic injury common of HAV and HBV infection was in sharp contrast to the hepato-pathogenic effects of HCV infection. Early epidemiological studies did not provide an accurate picture of HCV prevalence in the general population because most participants were volunteer blood donors who had already been screened for infectious disease, or were cross sectional cohorts of patients needing blood transfusions. Currently, it is estimated that 3% of the world population is infected with HCV. In the United States, HCV is the most common blood born infection, and has affected over 4 million people (Armstrong, Wasley et al. 2006). HCV has a propensity to maintain chronic infection, which increases the risk for secondary sequelae, including hepatocellular carcinoma and end stage liver disease.

Transmission of Hepatitis C Virus

There are several risk factors that can account for the majority of HCV transmissions worldwide. The most frequently cited factors for infection are blood transfusions from unscreened donors and injection drug use (Wasley and Alter 2000). In the developed world, transfusion related incidence is down from 13% prior to blood screening practices to 0.6% (Takano, Nakamura et al. 1996).

Blood screening in the developing world remains a pertinent problem based on the inadequate support systems and financial burden associated with the screening process. A study of the HCV incidence from a blood bank in Brazil estimates that from 1991-2001 there was a significant drop in transfusion acquired HCV, yet the incidence was still 10 times higher than that of developed countries (Sy and Jamal 2006).

The implementation of blood screening practices in the United States has drastically reduced the transmission of HCV from transfusion of blood, and blood products. Injection drug use has long since been the primary risk factor associated with newly acquired HCV infections. A surveillance program implemented by the CDC over a seven-year period reports that acute HCV infections has fallen among the blood transfusion recipients, yet has doubled in cohorts of people who have reported injection drug use (Alter, Hadler et al. 1990). In fact, it is estimated that between 70-90% of IDU's are positive for HCV (Donahue, Nelson et al. 1991; Alter and Moyer 1998). Based on these findings, one has to consider the impact IDU creates on the burden of HCV infection. When considering the propensity for chronic infection in 75% of untreated patients and the epidemic of injection drugs in the United States alone, it is paramount to realize the potential for increased transmission rates among this group.

The strongest correlate of HCV risk factors revolves around long term or repeated exposure to blood and blood products. Aside from transfusions and injection drug use, this parameter includes hemophiliacs treated with clotting factor, recipients of organs from HCV positive persons, hospital workers, and hemodialysis.

There are several cohorts of high incidence populations that do not follow the canonical risk factors associated with infection. In many instances, war veterans have an increased risk of exposure to HCV. In fact, a study of the Veterans Health Administration (VHA) determined that approximately 6% of Vietnam veterans had antibodies to HCV (Roselle, Danko et al. 2002). There are usually no strong correlations of any risk factors in war veteran studies, except for a subgroup of homeless veterans that had increased usage of injection drugs (Cheung, Hanson et al. 2002). There is even an increased rate of HCV seroprevalence amongst mentally ill patients, where there is an 11 fold increase in the HCV infection rate (Rosenberg, Goodman et al. 2001).

Prior to the early 90's, the prevalence of HCV infection among injection drug users can also be divided based on the cumulative exposure to blood products. Prior to the mandated screening of donor blood for HIV, HBV, and HCV

BIOLOGY OF THE HEPATITIS C VIRUS

HCV Classification

Hepatitis C Virus was not recognized as an infectious particle related to post transfusion hepatitis until 1989 when researchers at Chiron isolated a cDNA clone related to NANBH (Choo, Kuo et al. 1989). The reverse transcription PCR (RT-PCR) method of isolation gave an important indication as to the nature of viral nucleic acid organization. It was also realized that HCV had no sequence similarities to either HAV or HBV even though they are all hepatotropic viruses. HCV is a positive sense, single stranded RNA virus and is classified in the *Flaviviridae* family of RNA viruses, which includes flaviviruses and pestiviruses. Common flaviviruses include Dengue virus and yellow fever virus. Well known pestiviruses include bovine viral diarrhea virus and swine fever virus. HCV became the first and only member of hepacivirus genus of the *Flaviviridae* family. A common feature of RNA viruses is the genetic variability found in clinical isolates. The error prone nature of HCV RNA polymerase (discussed in detail in *HCV Structural Organization*) results in a heterogeneous accumulation of daughter virus RNA sequences, termed quasispecies (Kato, Hijikata et al. 1990). The high degree of variability amongst isolated HCV virions can be subdivided into 2 subtypes (1 and 2) which can be further divided into 6

subgroups or genotypes. Despite the diversity in HCV sequences, all genotypes share identical genes with consistent inter-relationship between variants.

Structural Organization of HCV

The hepatitis C Virus (HCV) is a positive sense single-stranded RNA virus that belongs to the flaviviridae family of viruses (Di Bisceglie 1998). The HCV genome is approximately 9600 nucleotides long and encodes for a single polyprotein of about 3000 amino acids (Major and Feinstone 1997). The genome is structured to have untranslated regions (UTR) at both the 5' and 3' flanks (figure 1). The 5' UTR is approximately 340 nucleotides long, and contains a ribosomal entry site (IRES) which initiates HCV protein translation. The advantage of the IRES is dictated by mG cap independence. Therefore, HCV translation bypasses the need for a proper eukaryotic preinitiation complex (Spahn, Kieft et al. 2001). Translation of the HCV open reading frame produces a single polypeptide that is subsequently cleaved into 10 structural and non-structural proteins. Core, glycoprotiens E1/E2, and integral membrane protein p7 make up the structural components, and NS2, NS3, NS4A, NS4B, NS5A and NS5B are the non-structural cluster. The structural proteins are initially cleaved by cellular signal peptidases that reside in the lumen of the endoplasmic reticulum (Rice et al 1996: Fields Virology). The remainder of the HCV polypeptide is cleaved by two virally encoded enzymes; NS2 autoprotease and the NS3-4A

serine protease. Proper coordination of the virus life cycle involves intricate functional properties of all viral proteins. Core protein is a component of the viral nucleocapsid and is suspected to bind both viral and cellular nucleic acid. Combined with its promoter regulating properties, it is likely core protein is also a transcription factor (Kim, Suzuki et al. 1994). E1 and E2 are glycosylated, membrane anchored, and function as a heterodimer (Dubuisson, Hsu et al. 1994). A cDNA library screen also has shown E1 and E2 to interact with cellular receptor CD81 as a proposed mechanism of virus binding (Pileri, Uematsu et al. 1998). The p7 protein is hydrophobic in nature and is thought to function as an ion channel (Pavlovic, Neville et al. 2003). NS2, the first of non-structural proteins, works in concert with downstream NS3 as a NS2/3 protease; cleaving the NS2/3 peptide junctions in cis (Hijikata, Mizushima et al. 1993). NS3 has many other functions, including C-terminal helicase/NTPase activity and capacities to unwind RNA/RNA, RNA/DNA, and DNA/DNA complexes (Tai, Chi et al. 1996). NS3 also interacts with NS4A to fully utilize the NS3 serine protease activity, which is necessary to cleave the polypeptide at the NS3/4A, 4A/4B, 4B/5A, and 5A/5B junctions (Bartenschlager, Ahlborn-Laake et al. 1993; Eckart, Selby et al. 1993). NS4A, as mentioned, is an essential cofactor, stabilizing and enhancing NS3/4 and NS4B/5A cleavage events (Reed and Rice 1998). NS4B has an undefined function, yet is implicated in virus replication (Blight 2007). NS5A is a heavily phosphorylated protein and is involved in both

viral replication and pathogenesis. NS5A was also implicated in the alteration of intracellular antiviral response pathways when interferon sensitivity of the virus was mapped to a residue change in the NS5A protein, termed the interferon sensitivity determining region (ISDR) (Enomoto, Sakuma et al. 1995). The exact role NS5A and most HCV proteins may play on immune response phenotypes are complex. The final protein in the HCV polypeptide sequence is NS5B, which encodes the RNA-dependant RNA polymerase (RdRP) (Behrens, Tomei et al. 1996).

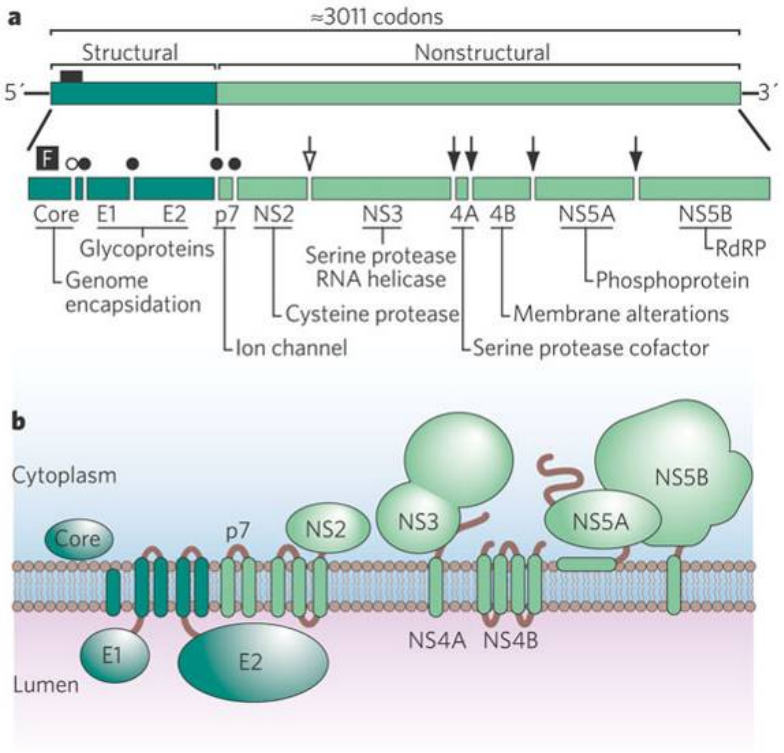

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Figure 1. **HCV genes and gene products. A**. The structure of the viral genome, including the long open reading frame encoding structural and nonstructural genes, and 5' and 3' NCRs. The polyprotein processing scheme is shown below. Closed circles refer to signal peptidase cleavage sites; the open circle refers to the signal peptide peptidase cleavage site. All other terms are defined in the text. **B**. The topology of HCV proteins with respect to intracellular membrane localization.

HCV Replication

Advancement in the understanding of the HCV life cycle has greatly benefited from infectious clone development both for in vivo and in vitro analysis. Upon complete genome sequencing, a functional complimentary DNA (cDNA) clone was constructed and shown to transfer hepatic disease following injection into chimpanzees (Kolykhalov, Agapov et al. 1997; Yanagi, Purcell et al. 1997). The cDNA clones are valuable in defining both viral and host factors involved in infectious HCV replication. In fact, it was shown that all four HCV encoded enzymes; NS2/3 protease, NS3/4 serine protease, NS3 helicase, and the NS5B polymerase, along with a highly conserved 3' UTR 98 base single stranded region are essential for productive HCV infection (Kolykhalov, Agapov et al. 1997). Although the HCV cDNA full length clones are invaluable in dissection of important viral components of infection, these clones can not replicate efficiently in cell culture. To this end, a subgenomic HCV replicon was developed and reported to replicate in the human hepatoma cell line, Huh-7 (Lohmann, Overton et al. 1999). The HCV replicon consisted of a consensus genotype 1b sequence with a 5' neomycin resistance gene in place of the structural genes followed by a encephalomyocarditis virus IRES to translate the nonstructural genes. The development of an in vitro HCV replicon system allows a deeper analysis of the HCV replication cycle and subsequent developments of antiviral targets. It was shown that adaptive mutations in the NS4A, NS4B, and NS5A genes enhanced

HCV replication, and mutations in the NS3 gene produced the opposite effect (Bartenschlager, Frese et al. 2004). Interestingly, mutations which increase replication efficiency correlate with a decrease in NS5A hyperphosphorylation (Blight, Kolykhalov et al. 2000) (Blight, McKeating et al. 2003). In opposition to decreased hyperphosphorylation of NS5A, it was determined that an increase in the phosphorylation levels reduced the replication efficiency and reduced the capacity of NS5A to interact with host protein, hVAP-A (Evans, Rice et al. 2004). The HCV protein NS4B has also been shown to be an integral constituent of the replication process. Adaptive mutations in the NS4B correlate with the capacity to produce cellular 'membranous webs' that are presumed to be the sites of active replication (Einav, Elazar et al. 2004). In light of HCV association with cellular membranes, it was subsequently found that fatty acid composition dictates the level of HCV RNA replication (Kapadia and Chisari 2005). Interestingly, the 5' and distal 3' UTR was shown to also be essential in HCV replication, exemplified by the base pairing of a conserved NS5B epitope and the 3' X domain (Friebe and Bartenschlager 2002; Friebe, Boudet et al. 2005).

HCV structural proteins, although not essential for RNA replication, are found in the replication complex. Expression of core protein reveals its association with the cytoplasmic side of cellular ER membranes, while E1 and E2 glycoproteins are sequestered inside the ER (Bartenschlager, Frese et al. 2004). The E2 glycoprotein has been shown to bind CD81, scavenger receptor class-B I,

L-SIGN, and DC-SIGN, which suggests their importance in binding and entry of the HCV virion into host cells. Interestingly, experiments using retrovirus pseudoparticles (HCVpp) show that infection of primary hepatocytes can be neutralized with antibodies to the E2 glycoprotein and require CD81 (Cormier, Tsamis et al. 2004). Despite advances the mechanisms of HCV replication, the replicon system is unable to produce progeny virions. Also, adaptive mutations in nonstructural proteins, while advantageous for RNA replication efficiency, did not allow virion formation. This observation leads one to speculate of the duality in the HCV life cycle. HCV protein translation and subsequent function may impart divergent advantages exclusive of progeny virus production during the viral adaptation to its cellular environment.

IMMUNE RESPONSE AND MECHANISMS OF HCV PERSISTENCE

HCV and modulation of the Innate Intracellular Immune Response

HCV employs many unique protein functions advantageous to its sequestration and evasion of the host innate antiviral immune response. The advent of in-vitro HCV infectious clones has given detailed insight on the functional capacity of the virus in thwarting intracellular antiviral responses. There are many pathogen related signals of infection, termed pathogen-associated molecular patterns (PAMPs), which alert the cell/cellular environment of foreign

invasion. HCV encodes several PAMPs, including dsRNA and a polyU tract, that are detected intracellularly by nucleic acid specific receptors (Sen 2001; Iwasaki and Medzhitov 2004). RIG-I and TLR3 comprise the two major intracellular pathways activated in response to dsRNA detection (Yoneyama, Kikuchi et al. 2004; Sumpter, Loo et al. 2005). RIG-I was discovered as a viral PAMP receptor using complementation studies in cells permissive to HCV subgenomic replication (Sumpter, Loo et al. 2005). The detection of HCV dsRNA results in the rapid induction of downstream antiviral genes including Interferon regulatory factor (IRF)-3 and nuclear factor kB (NF-kB) (Au, Moore et al. 1995). The end transcriptional response to viral dsRNA recognition is production and secretion of IFNβ (Sen 2001). IFNβ production results in autocrine and paracrine activation of interferon-stimulated genes (ISG), including Interferon regulatory factor (IRF)-7 (Der, Zhou et al. 1998). IRF-7, in turn, promotes IFNα production, which induces the activation of immune cells and the potentiation of pro-inflammatory cytokines by the resident tissue cells (Sen 2001).

HCV has a unique ability to combat many of steps of the intracellular antiviral response pathway. HCV proteins can also directly interfere with the function of some IFN-stimulated genes (ISGs) representing important antiviral effector proteins, such as PKR, 2', 5' OAS, ISG p56, IFNβ promoter stimulator 1, and virus-induced signaling adaptor (VISA) (Kawai, Takahashi et al. 2005; Seth,

Sun et al. 2005; Xu, Wang et al. 2005)(Summarized in Figure 2). The HCV NS3/4A protease activity cleaves several upstream IFNβ signaling components, TRIF and RIG-1, by cleavage of the TRIF adapter protein thereby disrupting the host antiviral response pathway at the level of RIG-I induced IFNβ activation (Foy, Li et al. 2005). NS3/4A also cleaves Consequently, this breaks the IFN signal amplification loop and reduces the overall effectiveness of all aspects of the IFN related antiviral response pathway. The expression and presentation capacity of MHC molecules are also components of the antiviral response pathway and are also affected by a reduction in IFN production (Der, Zhou et al. 1998). IRF-3 has been shown to be involved in tumor suppression and mediation of viral induced cellular apoptosis (Heylbroeck, Balachandran et al. 2000; Duguay, Mercier et al. 2002). HCV NS3/4A protease down regulation of IRF-3 activity consequently may be linked to hepatocellular carcinoma commonly found in chronic infection. NS5A protein expression rescues the replication of INF sensitive virus in cell culture, likely through the actions NS5A induced IL-8 production (Macdonald and Harris 2004).

Both NS5A and E2 have been shown to inhibit PKR activity, a kinase involved in the amplification cycle of INF (Gale, Korth et al. 1998). Interestingly, the extent of HCVs ability to thwart PKR dependant IFN response is dependant upon the ISDR sequence of NS5A (Taguchi, Nagano-Fujii et al.

2004). The ISDR is not the only instance of viral genetic polymorphism associated with viral evasion. IFN production also induces activity of the RNase L/ 2', 5'-oligoadenlyatesynthetase (OAS) pathway. RNase L has been shown to cleave dsRNA at single stranded UA and UU dinucleotide sites (Dougherty, Samanta et al. 1981). There are fewer UA and UU dinucleotide sites in genotype 1 when compared to genotypes 2 and 3 (Han, Wroblewski et al. 2004). Again, variation in HCV sequence contributes to the viral fitness and resistance to the innate antiviral response. This bias is also translated clinically, as antiviral therapy is less successful against genotype 1 infection.

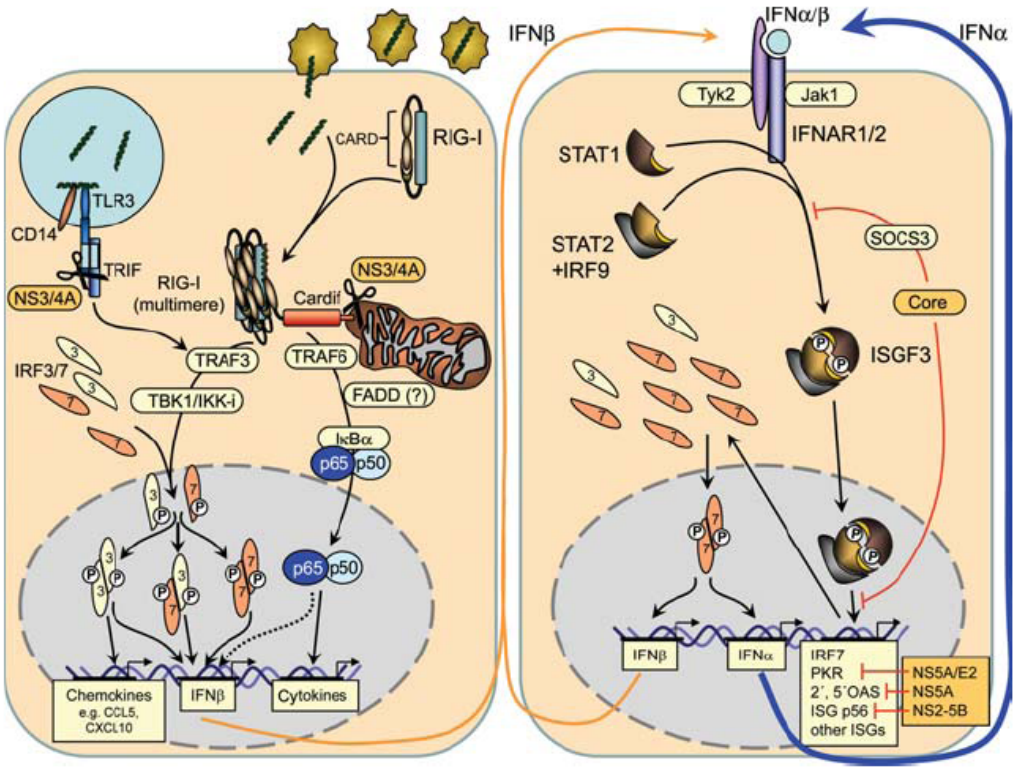


Figure 2. Schematic summary of the interference of HCV proteins with the signaling pathways elicited by the TLR3, RIG-I and type I IFNs. Cytosolic viral replication of HCV is recognized by RIG-I. Downstream signaling results in the formation of a mitochondria-associated signaling complex, leading to IRF3/7 activation. IRF3/7 translocates into the nucleus and mediates type I IFN production. Ligand binding of type I IFN results in activation of Stat1 and Stat2 which results in interferon-stimulated gene (ISG) induction. HCV proteins interfere with the intracellular antiviral response pathway at both RIG-I mediated IFN production and ISG production and activity.

HCV Modulation of the Adaptive Immune Response

The resolution of HCV infection correlates with immune response and function. In accordance with this observation, acute hepatitis C infection is rarely resolved without clinical therapy (Wasley and Alter 2000). Initial observations into the immune phenotype of resolution versus chronicity were complicated by a lack of unique clinical manifestations. Therefore, individuals would not seek therapeutic intervention until chronic infection was well established. The confounding relationship between viral persistence and adaptive immune function is, therefore, the subject of intense examination, and successful intervention of chronic infection will benefit immensely from a complete definition of successful immune features. Unintentional nosacomial exposure of health care workers and experiments with infected chimpanzees established that control of acute viral replication relies on a vigorous response of virus specific CD4 and CD8 T cells during the initial phases of infection (Bowen and Walker 2005). Details of these clinical findings reveal a peak of viral RNA in serum 10-12 weeks post infection (Beach, Meeks et al. 1992). Control of viraemia is often transient, and becomes a predictive factor for the onset of chronic infection (Thimme, Oldach et al. 2001). Often, there is a delay in the onset of detectable CD4+ and CD8+ T cell responses, which results in failure to control viraemia. This ambiguity in correlation of T cell function during the acute phase of infection and resolution predictability requires, still, significant definition of features associated with

infection outcome. The CD8 T cell response has been characterized extensively during the initial phases of acute infection. A successful response is marked by multiple MHC class I restricted HCV specific epitopes, with a high clonal frequency (Lechner, Wong et al. 2000). The vigor of CD4 T cell responses in successful resolution mirrors that of the CD8 T cell response. There is a strong, sustained proliferative response to a multitude of structural and nonstructural HCV epitopes (Missale, Bertoni et al. 1996). By contrast, persistent infection is marked by low frequencies of HCV specific cytotoxic T cells (CTL) that target a narrow range of viral epitopes. Also, antigen specific proliferative responses of CD4 T cells are weak or absent.

Mechanisms of T cell attenuation during chronic HCV infection

The mechanisms involved in the failure of T cells to mount a robust immune response are still unclear. One possible mechanism involves the deletion or anergy of antigen specific cells. There was some precedence from a model of chronic LCMV infection, where robust CD8 T cell activation results in disappearance of the antigen specific repertoire (Moskophidis, Lechner et al. 1993). There is no evidence for this phenomenon in HCV infection, but there is evidence of CD8 T cell anergy from functional and phenotypical studies. Antigen specific CD8 T cells were found, in one study, at similar or higher frequencies during the chronic phase of infection, but these cells were reduced in their

proliferation and INFg production in response to antigen specific stimulation (Wedemeyer, He et al. 2002). Anergy provides a clear mechanism that would explain the characteristic paucity of CD8 responses found in chronic infection, but there are several studies showing impairment even after viral resolution and phenotypical alterations of T cells from a non related virus (Lucas, Vargas-Cuero et al. 2004). Similar results were found in the CD4 population as well. In one study, MHC class II tetramer staining from chronic viraemic patients did not detect HCV specific populations (Day, Seth et al. 2003). In this experiment MHC class II tetramer staining was a novel approach used to identify small populations of antigen specific cells amongst a milieu of non-specific types. Although this served as a useful approach, it was still inconsistent in its ability to tease out populations where there was a low precursor frequency. In an effort to further define and evaluate HCV specific cell populations, our lab has adapted a novel CFSE based proliferation assay and analyzed the response of CD4 and CD8 T cells against overlapping 18mer HCV peptide pools. Previous data from the Karandikar lab shows a highly sensitive response against a multitude of HCV specific peptides that was previously unappreciated. What's more, the data shows that the advent of successful antiviral therapy increases the proliferative response of both CD4 and CD8 compartments (Pillai, Lee et al. 2007). The results demonstrate the transient nature of defective T cell responses during the chronic phases of HCV infection.

The large viral load during infection has been postulated as a mechanism of T cell unresponsiveness. There is no direct examination of this phenomenon during chronic HCV infection, aside from the observation that T cell proliferation in both CD4 and CD8 T cell is increased during and after successful therapy (Barnes, Lauer et al. 2002; Kamal, Fehr et al. 2002). There are also studies indicating that viral factors can directly alter T cell responsiveness. In-vitro studies of HCV core protein expression in lymphocytes show an interaction with proteins of the apoptotic pathway (Matsumoto, Hsieh et al. 1997; Zhu, Ware et al. 2001). Also, core protein interacts with complement receptor gC1qR and inhibits INFg production and cell proliferation (Yao, Nguyen et al. 2001). Interestingly, the inhibition of INF secretion from CD8 T cells exposed to core protein could be reversed with the exogenous addition of IL-2 (Accapezzato, Francavilla et al. 2004).

Functional analysis of T cell lines derived from liver infiltrating CD8 T cells revealed that there was a subset of specific MHC class I restricted cells that produced IL-10 in response to antigenic stimulation (Koziel, Dudley et al. 1995). IL-10 is an anti-inflammatory cytokine and works by inhibiting the synthesis of IL-2, TNFα and INFα. Interestingly, his was one of the first observations showing examples of immune suppressing function of a virus specific T cell during chronic HCV infection. This observation supported by the finding that

intrahepatic CD8 T cells suppressed proliferation of HCV specific responses and this was dependant on IL-10 (Accapezzato, Francavilla et al. 2004).

A major mechanism of inducing T cell attenuation involves regulatory T cells. Regulatory T cells function to mediate the extent of the immune response by suppressing the activation to self proteins and control of the vigor to antigenic insults. T regulatory cells are either naturally occurring through thymic development, or are stimulated in the periphery. T regulatory cells are generally defined by CD25 and Foxp3 expression. In the context of chronic HCV infection, there is in-vitro assays demonstrating that depletion of CD4+25+ regulatory T cells increases the functionality and proliferation capacity of HCV-specific CD8 T cells (Cabrera, Tu et al. 2004). Aside from intrahepatic regulatory T cells, there is also evidence of an increase in peripheral circulating CD4+25+ regulatory T cells compared with healthy individuals (Boettler, Spangenberg et al. 2005). Unfortunately, the suppression extends to CD8 responses against CMV and EBV antigens, obscuring the antigen specific nature of T cell regulation (Rushbrook, Ward et al. 2005).

Dendritic cell dysfunction during chronic HCV infection

A potent adaptive immune response to viral infection relies on the ability of antigen presenting cells (APC) to properly prime and activate appropriate downstream functions. In light of this, it would be potentially revealing to

evaluate the extent of APC function as a mediator of T cell attenuation during chronic HCV infection. There are numerous examples of viruses altering the functional state of antigen presenting cells. HIV, Measles virus, CMV, HSV-1, and Dengue virus all have been reported to induce functional and phenotypic disparity, primarily induced by direct infection of dendritic cells (Fugier-Vivier, Servet-Delprat et al. 1997; Grosjean, Caux et al. 1997; Ho, Wang et al. 2001; Moutaftsi, Mehl et al. 2002). The extent of APC dysfunction is commonly measured by several standardized assays, including the allogeneic mixed lymphocyte reaction (MLR). The MLR functions as a surrogate for the antigen presenting capacity of the APC, culminating as a measure of allogeneic T cell proliferation. In many instances of chronic viral infection, DC function in an MLR is attenuated. In cases of chronic HCV infection, DC function is unclear. For example, Bain et al found a reduced capacity of monocyte-derived DC from chronic HCV patients to stimulate an allogeneic T cell response. On the contrary, Piccioli et al found no significant differences in allostimulation, cytokine secretion, or maturation in the DC population between healthy controls and HCV patients. One can speculate that the nature of the disparity in experimental findings is a result of the divergence in author specific DC definitions. The use of in vitro matured differentiated monocytes versus bulk PBMC or protocol defined isolated DC makes it difficult to delineate unique functional clarification. To this end, the Karandikar lab has shown that ex-vivo isolated HCV patient Dendritic

cells of myeloid lineage, defined as CD11c+, CD1c (BDCA-1)+, and CD303 (BDCA-2)- have a distinct attenuated ability to stimulate an allogeneic response. The Karandikar lab has also found that ex-vivo isolated plasmacytoid Dendritic cells, defined as CD304 (BDCA-4)+, CD45RA+, and CD1c-; are deficient in Interferon production and T cell allostimulation (Averill, Lee et al. 2007). Although some find little to no functional diminution in DC during chronic HCV infection, our data (as well as others) suggest that intricate lineage definition provides insightful analytical value. The phenotype of DC in chronic infection provides further evidence of declining or attenuated adaptive immune responses in HCV infection that correlates with an initiation of the proper T cell response.

B cell abnormalities during chronic HCV infection

A great number of extra-hepatic manifestations during chronic HCV infection are associated with B cell lymphoproliferative disorders (LPD) (Agnello and De Rosa 2004; Zignego, Ferri et al. 2007). The most documented manifestation during chronic HCV infection is mixed cryoglobulinaemia (MC) (Lunel and Musset 1998). MC is characterized by autoantibody complexes that can be precipitated out of blood at cold temperatures. There are three types of cryoglobulins; type (I) monoclonal rheumatoid factor against monoclonal autoantibodies, type (II) (the most prevalent) monoclonal rheumatoid factor against polyclonal antibodies, and type (III) polyclonal rheumatoid factor against

polyclonal autoantibodies. Chronic HCV infection also correlates with a high incidence of B-cell non-Hodgkin's lymphoma (NHL) (Pozzato, Mazzaro et al. 1994; Zignego, Ferri et al. 1997). Both of these HCV-related manifestations are based on B cell lymphoproliferation. Much of the research profiling these B cell malignancies during chronic HCV infection is taken from liver infiltrating cells (Dammacco, Sansonno et al. 2000). There is a high preponderance of intra-tumoral clonaltypic B cell expansion, which suggests that B cell expansion has an antigen driven component (Racanelli, Sansonno et al. 2001). Interestingly, there is no correlation between HCV infection and non-B cell LPD. Whatever the mechanisms may be, there is a strong indication that HCV may mediated the emergence of B cell LPD.

Immune cell tropism and rationale for research

The mounting evidence for HCV utilization of extrahepatic reservoirs was initially suggested when transplanted livers became infected very rapidly post transplantation (Araya, Rakela et al. 1997). This observation is supported with evidence of HCV receptor usage. A human T-cell cDNA library screened against recombinant HCV E2 protein revealed that CD81 had high affinity for this HCV glycoprotein (Pileri, Uematsu et al. 1998). Subsequent in-vitro experiments show that CD81 expression is integral in HCV infectivity of a hepatocyte cell line (Lindenbach, Evans et al. 2005). Interestingly, a scan of cell types that express

CD81 reveals that it is ubiquitously expressed, including cells of the immune system. It would be reasonable, therefore, to predict that HCV may infect immune cells. In fact, several lines of evidence show HCV RNA detection in PBMC (Bouffard, Hayashi et al. 1992; Wang, Sheu et al. 1992; Muller, Pfaff et al. 1993; Schmidt, Klinzman et al. 1995). Also, HCV genomic analysis reveals divergent sequence profiles between liver, blood, and PBMC based nucleic acid (Navas, Martin et al. 1998; Roque Afonso, Jiang et al. 1999). This apparent divergence in HCV sequences suggests unique sites of HCV quasispecies replication. Although there are numerous examples of HCV RNA detection in non-hepatic tissue, there has been very little evidence of replication. One hypothesis suggests that the low relative abundance of negative strand RNA, compared to positive strand, makes it difficult to detect, experimentally. The advent of a thermostable rTth DNA polymerase assay has provided the sensitivity and fidelity necessary to detect down to 10 copies of viral RNA (Lanford, Chavez et al. 1995). Never the less, there is still a lack of experimental evidence detecting HCV negative strand RNA in PBMC. The paucity of negative strand detection suggests that HCV may not replicate in immune cells, however it still would be reasonable to predict that direct interaction or HCV specific protein production may alter cell function. In this study, we focus on the phenotype of B cells during chronic HCV infection. We hypothesize that direct associations between HCV and immune cells could be a factor which manipulate cell function and contribute

to the attenuated adaptive immune response observed during chronic HCV infection. We adapted the strand-specific RT-PCR assay to evaluate and report on the functional and phenotypic status of ex-vivo derived B cells in the course of chronic HCV infection. We propose that HCV has direct involvement in the modulation of immune cell function.

CHAPTER TWO

RESEARCH METHODS AND MATERIALS

Donor Recruitment

Patient and donor material was obtained as per a protocol approved by the UT Southwestern Medical Center institutional review board (IRB). All HCV patients gave written consent, and healthy donor blood was obtained from Carter blood care donation center. All healthy donor blood tested serologically negative for HCV, HIV, and Hepatitis B virus, and those with known infection or other serious immune suppressing diseases were excluded from analysis. All HCV patients tested negative serologically for HIV. Cross sectional analysis was performed on 50 treatment-naïve patients and 23 healthy subjects. Detailed information and summary of baseline patient characteristics is provided in Table 1.

TABLE 1. PATIENT CHARACTERISTICS

Characteristics	*HCV Patients N=50*	*Healthy Subjects N=23*
Age (year); mean (range)	46 (24-61)	45 (20-60)
Sex (M/F)	**34/26**	**10/13**
AST (IU/L)	62 (14-177)	N/A
ALT (IU/L)	**80 (9-259)**	**N/A**
HCV viral load (KIU/ml) mean (range)	1989 (50-15,100)	N/A
Histology: Grade/Stage; per Batts and Ludwig; range	**I-IV**	**N/A**

Isolation and storage of donor lymphocyte subsets

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation method using Ficoll-Hypaque Plus (GE Healthcare, Uppsala, Sweden) ,according to manufactures protocol, and were cryopreserved at 10 x 10^6/ml in 10% DMSO and 90% FBS (Hyclone, Utah). On day of experiment, thawed PBMC cell subsets were isolated using isolation kits from Miltenyi Biotech, according to manufactures instructions (Auburn, CA). B cell isolation used a CD19 (+) selection kit or (-) selection kit for 'untouched' B cells; Monocyte isolation used CD14 (+) selection; T cells were isolated into several sub groups based on experimental parameters including: CD3 (+), CD3-depleted, CD8 (+), total CD4 (+) or CD4 (+) CD25 (-) (by CD25 depletion). Separation was considered successful when purity was $\geq$ 85% (in most cases $\geq$ 95%). Fractionated and total PBMC populations were either used same day in experiment or cryopreserved in 10% DMSO and 90% FBS for future use.

Cell culture conditions

Experiments involving isolated PBMCs and PBMC subsets were cultured in RPMI 1640 media (Mediatech, VA) supplemented with 5% heat-inactivated Human AB serum (Gemini Bioproducts, West Sacramento, CA). In some instances, 10% heat-inactivated fetal calf serum was used instead of human AB

serum. All time sensitive incubations were performed in a tissue culture incubator at 37^0C/ 5% CO_2 (Cornelius, OR).

In-vitro translation of synthetic HCV RNA

For strand specific HCV RNA PCR, synthetic RNA standards are first produced for internal control. The synthetic RNA was produced from the pSP73/72 transcriptional vectors (Promega) containing an HCV insert of nucleotides 1-582, spanning the proximal 5' untranslated region (UTR). The insertion was cloned in both orientations, such that T7 can amplify both positive and negative strands. Prior to in-vitro translation (IVT) the plasmids are linearized with the restriction enzyme *Nde*I (Invitrogen) then purified using phenol/chloroform extraction and ethanol precipitation. The purified plasmid(s) were stored in water at a 1mg/ml concentration. Synthetic HCV RNA was synthesized from 1ug of linear plasmid DNA using the T7 Megascript IVT kit (Ambion, Austin, TX), following the manufacturers instructions. Purification of amplified RNA product used the Megaclear (Ambion, Austin, TX) RNA cleanup kit, following the manufactures instructions.

Strand-specific rTth RT-PCR

Up to 100 K cells were evaluated for HCV positive or negative strand RNA. This assay procedure was also used in defining the limitations of

HCV RNA detection (discussed previously). Importantly, prior to lysis and RNA extraction, cells were subjected to 1ml of 0.5% Trypsin EDTA (Invitrogen) for 30' at 37^0C and stored in 50ul of RNA*later* (Qiagen) at -80^0C until use. Total RNA was extracted from frozen samples using the Qiagen RNeasy kit, according to manufactures instructions. Total RNA was eluted in 40ul of nuclease free water (Qiagen), of which 10ul will be used for each of the strand specific reactions (20ul in total). The remainder of isolated RNA was stored at -80^oC until use (typically in an alternative downstream cDNA generation protocol, to be discussed later). Detection of HCV positive and/or negative strand RNA used 10ul (per strand analysis) of the isolated total RNA in the following strand specific procedure: first round cDNA synthesis was performed on an Eppendorf master cycler (20pM of forward primer [CACTCCCCTGRGAGGAAC] for negative strand and 20pM of reverse primer [TGCACGGTCTACGAGACCTC] for positive strand) using 5U of the thermostable enzyme Tth DNA polymerase (applied biosystems), 1x RT buffer, 1mM MnCl2, and 200um of each deoxynucleoside triphosphate. After 20 minutes at 70oC, the reaction was chelated with 10X chelating buffer, 2.2mM MgCl2 and the reaction volume adjusted to 100ul with nuclease free water. The cDNA has a primary amplification step as follows: 20 cycles of 70^0C for20', (Eppendorf master cycler).

Quantitative real-time PCR

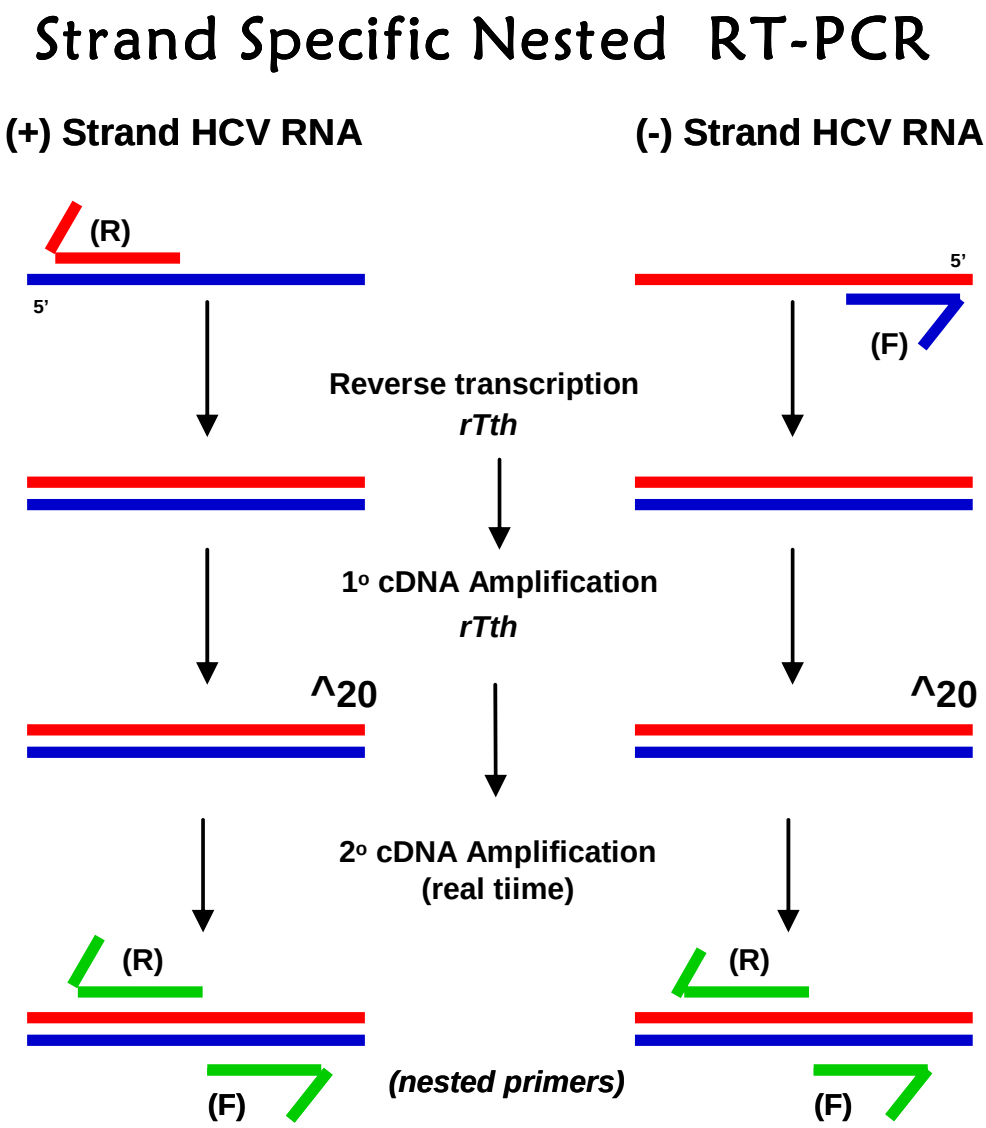
A second, nested, PCR (nested forward primer: [ACTGTCTTCACGCAGAAAGCGTC]; Reverse nested primer: [CAAGCACCCTATCAGGCAGTACC]), analysis was performed on 10ul of the strand specific products using a real time thermocylcer (Mx3000P, Stratagene) as follows: 1 cycle at 95oC for 10’’; 30 cycles of 95oC for 30’’, 57oC for 60’’, 72oC for 30’’; 1 cycle of 95oC for 60’’; and 1 melting curve cycle of 55oC for 30’’, 95oC for 30’’.

Real-time RT-PCR assay validation

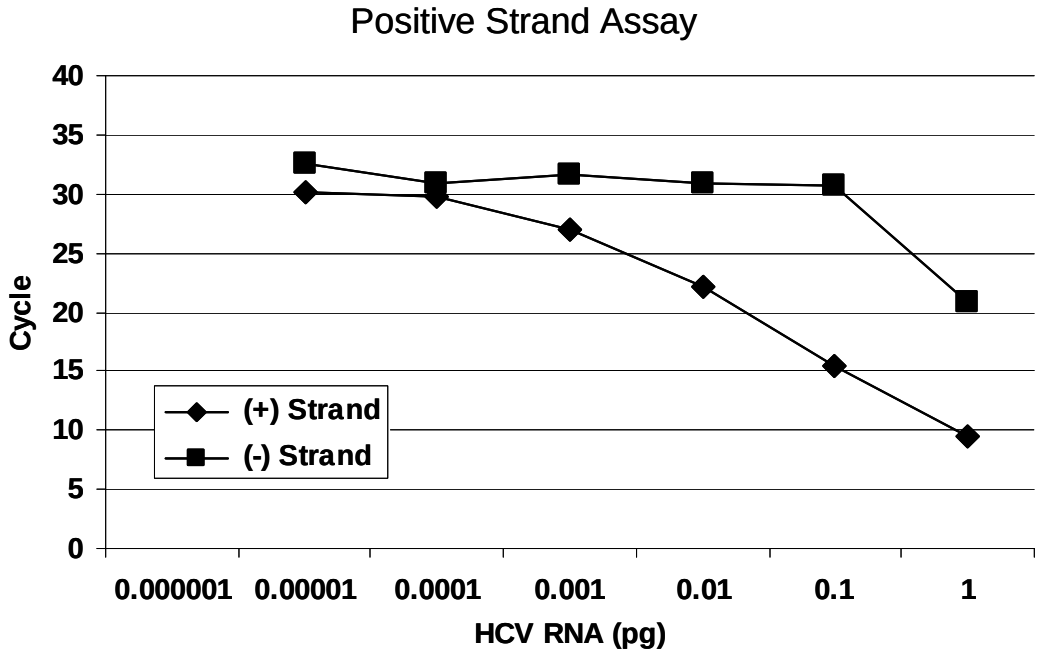
In order to verify the utility of our unique assay design, it was important to define its specific empirical limitations. To this end we evaluated both the specificity and sensitivity of our real-time based RT-PCR assay. We used synthetic, plasmid derived, in vitro translation (IVT) amplified RNA templates representing both the HCV positive and negative strand from the proximal end of the 5’ un-translated region (UTR). The RNA templates were mirror copies of one another from an insert that was integrated in both directions on separate plasmids (Lanford, Sureau et al. 1994). Purity of the product was evaluated by direct PCR on the RNA IVT templates to assess any contaminating plasmid DNA carryover, and was considered pure when no real time PCR product was detected from 30

cycles of amplification. Once pure HCV RNA template was achieved, titration assays were implemented in a design that encompasses the limitations of our assay conditions. Assay details are illustrated in figure (4A), showing two stages of PCR amplification. It was conventionally gratifying that the recombinant rTth enzyme has both RT and polymerase function, as this reduced the probability of contaminating nucleic acid because it involved fewer steps and less manipulation. The sensitivity of positive strand detection was determined to amplify down to 1.0fg of RNA template, which is the genomic equivalent to ~250 viral copies (figure 4B). The specificity of positive strand detection was determined to uniquely amplify positive strand in 1000 fold excess of the negative strand. For the negative strand, it was determined that sensitivity is down to 10fg of RNA template (~2000 viral copies), and specificity had a 100 fold differential in detecting negative strand over positive strand. Although negative strand assay conditions were not as specific as its counterpart, we were confident in its predictability. Active viral replication need only occur in approximately 500 to 1500 total cells in order to confidently detect negative strand. Our experimental setup evaluates up to 100,000 cells at a time, which means that less than 1% of cells need to harbor actively replicating virus in order to detect it.

A.)


Strand Specific Nested RT-PCR
(+) Strand HCV RNA
(-) Strand HCV RNA
(R)
5'
5'
(F)
Reverse transcription
rTth
1º cDNA Amplification
rTth
^20
^20
2º cDNA Amplification
(real tiime)
(R)
(R)
(F)
(nested primers)
(F)

B.


Positive Strand Assay
40
35
30
25
20
15
10
5
0
Cycle
(+) Strand
(-) Strand
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
HCV RNA (pg)

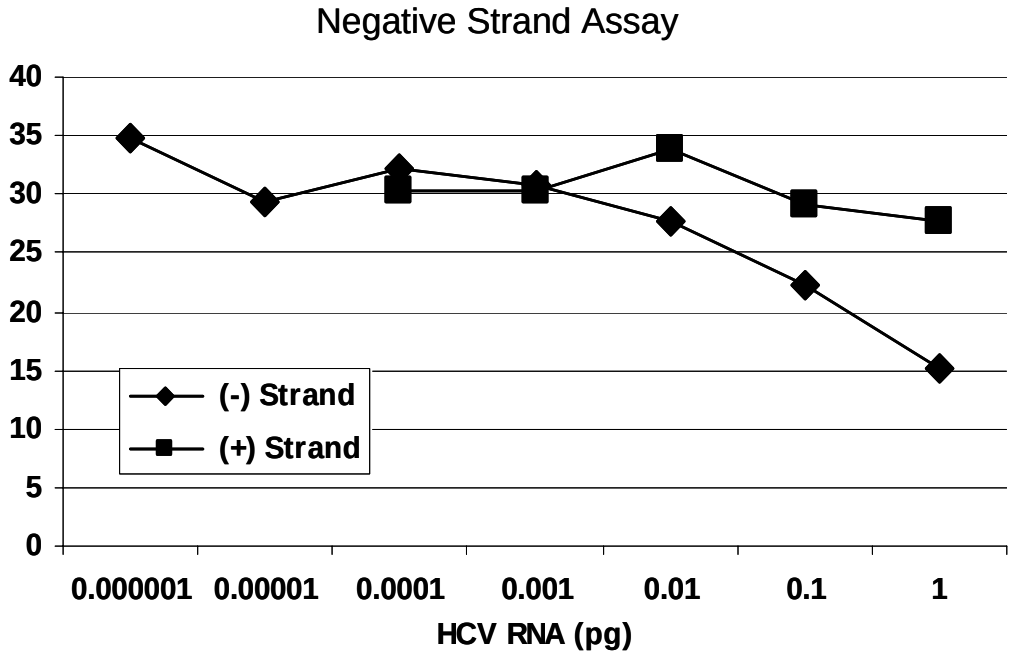

Negative Strand Assay
40
35
30
25
20
15
10
5
0
(-) Strand
(+) Strand
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
HCV RNA (pg)

Figure 3. Nested real-time RT-PCR assay design. A.) Schematic diagram of the strand specific real-time RT-PCR protocol. Purified total cellular RNA was subjected to an initial reverse transcription reaction using 1^0-primers complementary to the 5' UTR of either genomic or replicative intermediate strand HCV RNA, each in an isolated reaction. Subsequent cDNA amplification was performed with the addition of the opposing primer and Mg^+ to hinder RT function and facilitate polymerase activity. A fraction of primary product was then amplified in a secondary PCR reaction using nested primers directed toward sites proximal to the 1^0- primer locations. Amplification runs for 35 cycles using syber green as the real-time detection agent. B.) A strand-specific nested RT-PCR assay was performed on synthetic HCV RNA, representing the 5'UTR in conditions that define assay specificity and sensitivity limits. A 7 log distribution of correct or incorrect primer usage for each assay was amplified using the procedure described in part A. Starting synthetic RNA material was used from 1pg to 0.001fg in the presence 50ug/ul of total cellular RNA. Results represent typical results of the strand-specific assay comparing correct and incorrect primer based amplification. Each point on the graph represents the cycle number where the corresponding amount of starting material was initially detected.

Ex-vivo differentiation profile analysis using flow cytometric analysis

PBMCs from healthy donors or chronic, treatment-naïve, HCV patients were subjected to flow cytometric analysis for B cell specific activation marker expression. Standard experimental design utilized multicolor flow cytometric antibodies to several, researcher defined, activation or maturation markers predetermined to possibly exist on the surface of B cells. PBMCs were 'thawed' from a cryogenic state by slowly applying warmed (37^0C) RPMI + 5U/ml DNase I (Invitrogen, San Francisco, CA), washing 2-3 times in cold PBS for 10 minutes at 1400 RPM (vendor), and re-suspended in FACS buffer (10X PBS, 1% BSA, 0.1% Sodium azide) at a 2 x10^6/ml concentration. Approximately 2 x 10^5 cells (100ul) were partitioned into 5 ml polypropylene FACS tubes (BD Falcon) and then stained with fluorescently tagged antibodies for surface CD19, CD23, CD25, CD27, CD38, CD69, CD71, CD86, CD81, and HLA-DR for 30 minutes. Cells were subsequently washed and fixed in 1% Para formaldehyde (Becton Dickinson). Flow cytometric data was acquired on a BD LSRII flow cytometer using BD FACS Diva software and was analyzed using the FlowJo software program (TreeStar, OR). Analysis was performed on no less than 50,000 total collected events, restricted by gating on the characteristic pattern of non-granulated 'live' lymphocytes on a forward vs. side scatter plot. B cells were identified using CD19 gating and all down stream analysis was applied to this isolated population. Statistical inferences were calculated on both the mean

fluorescence intensity (MFI) and percent expression of all markers. Nonspecific antibody binding was compensated for using fluorescent labeled mouse isotype controls.

In-vitro cell activation assays

PBMCs from healthy donors or chronic, treatment-naïve, HCV patients were subjected to mitogen induced activation and flow cytometric analysis for B cell specific activation marker expression. PBMCs were thawed and exposed to either pokeweed (5ug/ml), phytohemaglutin (5ug/ml), multivalent IgM (± CD40L), or formalin-fixed *Staphylococcus aureus* (Calbiochem, Gibbstown, NJ) (1:1000 dilution of stock in RPMI, 5% heat-inactivated human AB serum, penicillin, streptomycin) for up to 48 hours in 96 well, polystyrene plates. Analysis of resultant B cell phenotype utilized the same flow cytometric readout described in *Ex-vivo differential profile analysis.*

3H-thymidine based mixed lymphocyte reaction assays (MLR)

Purified B cells from healthy donor or chronic, treatment-naïve, HCV patients were evaluated as APC in an allogeneic mixed lymphocyte reaction. Third party, total T cells or CD4+25- T cells were cultured together with B cells at a 10:1 ratio in 96-well, polystyrene microplates (2×10^5 T cells: 2×10^4 B cells).

All MLRs (13 total) used 1 of 2 third party T cell sources to reduce the variability in individual MHC allogeneic properties. Cultures were pulsed with 1uCi of 3H-thymidine on day 5 and harvested on day 6 for counting using a wallac scintillation counter (TomTec, CT).

3H-thymidine based B cell proliferation assays

B cell proliferation was assessed using 3H-thymidine incorporation. CD19 (+) B cells were purified to $\geq$ 90% purity and subjected to B cell receptor (BCR) crosslinking using Pansorbin (formalin-fixed *Staphylococcus aureus)* (Calbiochem, Gibbstown, NJ). Pansorbin is unique in inducing proliferation based on the properties of the bacterial surface protein, protein A. Protein A binds the Fc receptor portion of Immunoglobulin molecules IgG, IgM, and to a lesser extent IgE. Protein A binding of the BCR surface bound Ig molecules induces a broad, antigen independent, crosslinking event culminating in the proliferation of responding B cells. B cell proliferation was assessed in both healthy donors and chronic, treatment-naïve HCV patients. Approximately 2 x 10^5 B cells were incubated with a diluted stock pansorbin (1:1000 dilution in RPMI + 10% FBS/Penicillin/Streptomycin) in 200ul total volume. Cells were incubated for 72 hours at 37^0C/5%CO_2 in 96 well polystyrene microplates. 12 to 16 hours prior to the endpoint, cultures were mixed with 25ul of 3H-thymidine.

Cells were subsequently harvested (TomTec, CT) and radioactive counts were determined using a wallac scintillation counter (PerkinElmer).

Flow cytometric apoptosis assays

B cells from healthy donors or chronic, treatment-naïve, HCV patients were evaluated for apoptosis using flow cytometric analyis of cell surface phosphatidylserine levels. Either PBMCs or microbead purified B cells (CD19) were exposed to pansorbin in an experimental design similar to *B cell proliferation assays.*

Crosslinking assays

CD19 (+) B cells were purified to $\geq$ 90% purity and subjected to CD81 or IgM crosslinking in the following protocols. In one approach, 0.02ug/ul of antibody in BBS buffer (0.17M Boric acid, 0.12M NaCl, 1% bovine serum albumin) and was applied to 96 well 'polysorb' ELISA plates (NUNC, Rochester, NY) for 1 hour at room temperature. Following antibody absorption, 200ul of 2 x 10^6/ml B cells were applied in the wells up to 72 hours, followed by flow cytometric analysis of CD25 (APC, Becton Dickinson), and CD69 (PE, Becton Dickinson) activation marker expression. A second design utilized sulfate

modified polystyrene beads (Sigma-Aldrich) suspended in PBS at a 2 x 10^5 /ul. Beads were subsequently incubated with 20ug/ml of anti-CD81 or anti-IgM antibody for 1 hour at 370C in RPMI + 10% FBS, then resuspended in a final concentration of 1 x10^7 beads/ml. Antibody coated beads were then incubated with 2 x 10^5 CD19-isolated B cells in a 96 well polystyrene microplate (BD Falcon). Cells were incubated for up to 72 hours and analyzed, again, for CD25 and CD69 activation marker expression. IgM was utilized, in this case, as a positive control, while negative controls used a nonspecific mouse IgG antibody subjected to the same experimental parameters.

CFSE based proliferation assays

The magnitude and quantification of allogeneic CD4+25- T cell responses were determined by flow cytometric assays, utilizing a green fluorescent dye, termed carboxyfluorescein succinimidyl ester (CFSE – Invitrogen, CA)(Crawford, Yan et al. 2004). Cells were re-suspended in phosphate-buffered saline (PBS- Sigma Aldrich) at 1 x10^6 cells/mL. Cells are incubated at 37^0C with 0.25μM CFSE for 7 minutes (in the dark). After a series of washes, CFSE labeled cells are re-suspended in cell culture media (RPMI, 5% heat-inactivated human sera, penicillin, streptomycin) at 2 x 10^6 cells/ml, and mixed with allogeneic B cells or Monocytes from healthy donors or chronic, treatment-naïve HCV patients at a 1:10 ratio (2 x10^5 T cells: 2 x 10^4 APCs) in 96-well plates. The mixed

lymphocyte reactions were incubated for 7 days; followed by cell surface staining for cell markers CD4-PE, CD25 APC, and CD19-PE cy7 (BD Biosciences). Subsequent to cell surface staining, the cells are subjected to FOXP3 intracellular staining using anti-FOXP3-AlexaFluor 700 and special permeabilization buffers according to manufactures protocol (eBiosciences). Analysis of resultant cultures was performed on a BD LSR II flow cytometer (BD Biosciences) and relied on CFSE dilution to assess the magnitude of proliferation. As CFSE stained parent cells divide, the level of CFSE concentration is halved in the daughter cells. The readout is a function of the ratio between proliferating cells and that of undivided CFSE 'high' cells.

CFSE based suppression assays

FOXP3 expression in activated CD4+25+ T cells correlates with their capacity to suppress autologus T cell proliferation (Pillai, Ortega et al. 2007). To evaluate the extent of suppressive T cell generation and function, we set up a primary allogeneic MLR using CD19+ B cells or CD14+ Monocytes as APCs and CD4+25- third party T cells as responders. Assay conditions consist of 2×10^4 B cells: 2×10^5 T cells/well in 96 well plates (5-7 well replicates). This initial MLR was incubated at 37^0C/5%CO_2 for 7 days, followed by harvesting and collection of cells into 5 ml polypropylene FACS tubes (BD Falcon). Prior to suppression assay setup, approximately 1 $x10^5$ cells from each MLR condition were set aside

for phenotypic analysis using CD4-PE, CD25-APC, and FOXP3-Alexa 700. The MLR activated T cells were then applied to an anti-CD3 stimulated autologous proliferation assay where they were assessed for suppressive ability. On these assays, the suppressors were washed and re-suspended in PBS at 1 x 106/ml and cell tracker red CMTPX (Invitrogen) was added to the mixture at a final concentration of 0.7uM.. Cells then incubate for 15 minutes at 37^0C, followed by 1mL of human serum for quenching. Quenched cells were washed in PBS, re-suspended in RPMI + 5% human sera + penicillin + streptomycin and incubated for another 30 minutes at 37^0C. The suppressor population was finally washed and re-suspended at 2 x 10^6 cells/ml in RPMI + 5% human sera + penicillin + streptomycin. CMTPX staining allowed for exclusion of suppressors from the analysis. The other 2 cell populations in these cultures consisted of antigen presenting cells and the CD4+25- responder cells. Both populations were from the same healthy donor, yet were unique from any donor used in the primary MLR. The APC population was defined in this experiment as PBMCs depleted of the CD3 expressing cells using CD3+ microbeads and the AutoMACS (Miltenyi Biotech) *deplete* protocol. APCs, once thawed and washed appropriately, were re-suspended in 1mL of a solution of Diluent-C from the PKH staining protocol (Sigma). The cell suspension was mixed with a working concentration of PKH (5ul of stock dye added to 1mL of Diluent-C) and allowed to incubate at room temperature for 5 minutes (in the dark). The mixture was quenched with 1mL of

human sera, washed 3 times with PBS, and re-suspended at 2 x 10^6 cells/mL in RPMI + 5% human sera + penicillin + streptomycin. The PKH stained APCs were then irradiated to avoid any proliferating background from this population. PKH staining allowed for exclusion of APC from the analysis. The responder population was defined in this experiment as CD4+25- cells that were previously isolated using CD4+ selection followed by CD25 depletion, using the AutoMACS (Miltenyi Biotech) *posel d* and *deplete* protocols, respectively. The responders were stained with CFSE using the same protocol setup as described in the *CFSE based proliferation assay* methods. The resultant responder population is re-suspended at 2 x 10^6/mL in RPMI + 5% human sera + penicillin + streptomycin. The suppressor cells were first added, in serial dilution, to a set of 5 FACS tubes starting at 2 x 10^6 cells/mL and ending in 6.25 x 10^4/mL. Both irradiated APCs and responders were added in all FACS tubes at a 2 x 10^6/mL concentration. The total volume in each FACS tube was 1mL and ideally the APC-responder ratio was maintained at 1:1, while the suppressors were serially decreased in manner to titrate the suppressive capacity of MLR generated, transient T regulatory cells. The mixture was activated with the addition of 1uL (of a 1mg/mL stock) of anti-CD3 monoclonal antibody (OKT3), incubated at 37^0C for 5-6 days. At the proliferating endpoint, all tubes were washed with FACS buffer and stained with CD4-PE and CD25-APC for 30 minutes, washed, and fixed with 1%

paraformaldehyde. The events were collected on a BD LSR II flow cytometer (BD Biosciences) and analyzed using Flowjo (Treestar, San Carlos, CA).

JFH-1 preparation and titering

All assays utilizing live or attenuated hepatitis C virus were performed in a laboratory setting which was previously authorized to contain BSL-2 level infectious materials (Laboratory of Julie Pfeiffer, Ph.D.). Any and all assays containing live or attenuated virus were considered infectious or hazardous until material was sterilized, fixed, or treated to eliminate or inactivate all biological activity. Synthetic Japanese fulminate Hepatitis – 1 virus (JFH-1), genotype 2a (graciously provided by the laboratories of Michael Gale, Ph.D. and Julie Pfeiffer, Ph.D.) was propagated in culture using Huh7.5 cells in DMEM (Gale et al.). Approximately $5x10^6$ Huh 7.5/FC cells were seeded in a 10cm dish with 10mL of media (DMEM – Invitrogen, 10% FBS, penicillin, streptomycin) and 12 mLs of stock virus preparation ($1.5 x10^4$ ffu ((focal forming units)) of JFH1 virus). Cells were grown until 80-90% confluence (about 72 hours) then harvested using 0.25% trypsin-EDTA (Invitrogen) and split into 4-10cm dishes (BD Falcon), 3 of which contain 2% FBS media instead of the initial 10%. The 2% batch of cells was harvested approximately every 3 to 4 days and centrifuged at 2000 revolutions per minute (RPM) and the media collected and saved at 4^0C. A new 2% batch is created from the initial 10% propagating culture, replacing the

harvested cells and media from culture is added to the accumulating virus laden stock. Once the collected media reached approximately 400mL, the supernatant was concentrated using ultracentricon filters (Ultracel YM – 50; Millipore) according to manufactures instructions.

Resultant JFH media concentrates were subjected to tittering to determine the focal forming units for downstream applications. Huh7.5 cells were seeded in a 48-well plate (BD Falcon) at 1.5 x 10^4 cells/well and incubated for 24 hours at 37^0. On the following day, a series of 10 fold dilutions of an aliquot of the concentrated virus stock in complete DMEM media (10% FBS, penicillin, streptomycin) was prepared (10^0 to 10^{-3}). The Huh7.5 cells are washed 1x with PBS and then inoculated with 100µl of the virus sample dilutions (in triplicate). The cells were incubated at 37^0 for 3 hours, washed 1x with PBS and incubated again at 37^0 for 2 days in 250µl complete DMEM per well. After the 2 day incubation, media was removed and cells were washed 1x with PBS. Cells were incubated at room temperature with 200ul/well of 4% paraformaldehyde in PBS for 30 minutes. Subsequent to PFA incubations, cells are washed 2x with a PBS-glycine solution (PBS + 10mM glycine + .05% Sodium Azide). Cells were subsequently permeabilized with 0.2% Triton-X (vendor) in PBS for 15 minutes. Cells were then washed 1x in PBS and blocked for 10 minutes in 10% FBS (in complete DMEM). Cells are then incubated at room temperature with a 1:1000 dilution of stock primary antibody; human serum A in 10% FBS (The human

serum has been opsonized from a fulminate chronic hepatitis C patient). After 1 hour and 2 washes with PBS, cells are incubated with a secondary antibody; a 1:1500 dilution of donkey anti-human HRP conjugate. Cells were then incubated at room temperature for 30 minutes. The cells were then washed 2x in PBS and developed using a peroxidase substrate solution from a Vector VIP kit (Vector labs, Burlingame, CA). The resultant focus forming units were counted under a light field microscope. Each well was counted in full for fluorescent patches and the results were tabulated into the equation: $\underline{X}$-ffu x 10^2 x 10 (1000ul total inoculation volume/100ul of added innoculum) – resulting in $\underline{X}$ x 10^3 ffu/mL. X represents the number of focus forming units found in one well of a diluted sample. Since the initial inoculated samples used triplicate wells, X represents the average of ffu from all three wells. The logarithmic denomination in the equation is the same value as used in the dilution series (i.e. – if there were 10 ffu in the 10^{-3} dilution inoculation, the resultant concentration is 100 x 10^3 ffu/mL of virus). In some assays, inactivated virus was required; therefore a stock of concentrated JFH-1 was subjected to 30 minutes of Ultraviolet light exposure. The source of UV light was simply the ultraviolet light found under the standard laminar flow tissue culture hoods (vendor). Virus inactivation was determined by the described tittering method and virus was considered inactivated when there was no focus forming units in any dilution of virus supernatant. Because focus forming units

reflect viral protein production, a lack of units is considered a surrogate marker for the lack of viral replication.

JFH-1 Infection of PBMC assays

Approximately $2x10^6$ PBMCs were incubated with virus at an MOI of 0.1 for 3 hours in complete DMEM (10% heat inactivated fetal bovine serum, 5ug/ml penicillin, 5ug/ml streptomycin) and subsequently washed twice with PBS and then reseeded in 6 well tissue culture plates in complete DMEM up to 7 days at 37^oC/5% CO_2.

Cytokine analysis

Accumulated supernatants from virus infection assays are evaluated for their cytokine profiles. Approximately 50uL of supernatant was prepared in specialized 96 well 10 spot microtiter ELISA plates and analyzed on a Sector Imager 2400 (Meso Scale Discovery, Gaithersburg, Maryland) according to manufactures instructions using a 10-plex TH1/TH2 human cytokine kit (catalogue number-K11010A-4) which detects the following cytokines: INF-γ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, and TNF-α.

Statistical/ Flow Cytometric Analysis

Unpaired (cross-sectional studies) Student t-tests were used to compare the differences between groups and p-values of less than 0.05 were considered significant.

CHAPTER THREE

RESULTS

HCV positive strand RNA is highly associated with B cells from chronic HCV patients

The prospect of non-hepatic HCV replication remains controversial and relies, mainly, on evidence of replication intermediate (negative strand) RNA detection. Previous reports have implicated immune cells as possible reservoirs of viral sequestration during chronic infection (Laskus, Radkowski et al. 2000). The initial findings demonstrating an association of HCV with PBMC suffered from the lack of both assay sensitivity and specificity of viral RNA detection. We adopted an HCV strand-specific RT-PCR protocol from Lanford et al. (summarized in materials and methods) that has been characterized to amplify a minimum of 10 viral RNA copies and has the capacity to detect the correct RNA strand in the presence of 1000 fold excess of the opposite strand (Lanford, Sureau et al. 1994). In a modified version of this approach, we sought to evaluate the presence of HCV RNA in peripheral blood mononuclear cells (PBMC) from chronic HCV patients.

We separated PBMCs into B cell (CD19+), Monocyte (CD14+), and bulk (14/19 depleted) fractions. Standard microbead based isolation techniques were used to isolate or 'enrich' the populations. Figure (4) represents a typical flow cytometric graph of B cell and Monocyte enrichment. These isolated populations

were not considered unique unless the purity was above 85%. The strand specific real-time RT-PCR assay was initially performed on all PBMC populations from 42 treatment naïve chronic HCV patients. In subsequent experiments, only B cells were tested. Thus, in all, B cells from 50 treatment-naïve HCV patients were tested. For the negative control, the strand specific reaction was performed on at least one of 5 PBMC sources from healthy donors during each attempt at HCV RNA detection in HCV patients. Amplification for the negative controls needed to be undetectable before data for HCV RNA was accepted. In some instances, product was detected in the control PBMCs. When this occurred we altered our criteria such that the specific amplification cycle had to be beyond real-time round 30 and the detection of HCV RNA in the HCV patients in the same reaction had to be at least 10 fold higher (at least round 26) in order to 'verify' that the results positively harbored HCV RNA in the analyzed PBMC sample.

Our results showed that HCV positive strand RNA was found in 30% (15 of 50) of the B cells analyzed, and in $\leq$ 5% (3/42) of both the Monocyte and bulk CD19/14-depleted fractions (table 2). Interestingly, in cases of RNA detection in non B cell fractions, RNA was also found in the B cells. Based on the unique and significant association with B cells, it is possible that there was B cell carryover into the other fractions that could explain these findings. Alternatively, there may have been a loose association of virus with nonspecific cells based on the presence of CD81, an HCV co-receptor. The prospect of passive viral association

with cellular receptors was accounted for by trypsin treatment prior to the cellular lysis step, which would eliminate any carryover. Overall, these results suggest that HCV has a high propensity to associate with B cells. We were unable to detect HCV *negative strand* RNA at any appreciable rate in any PBMC fraction (Table 2), and the few instances of detectable negative strand RNA was very close to the sensitivity limits of the assay. Although we were unable to consistently determine viral replication in the analyzed PBMC subsets, detection of HCV specific nucleic acid most likely consisted of RNA found internally and is considered a subjective analysis of HCV virions that productively entered the cell. Overall, theses results suggest that B cells from a subset of HCV patients have associated HCV positive strand RNA. No clear evidence of viral replication was observed in these studies.

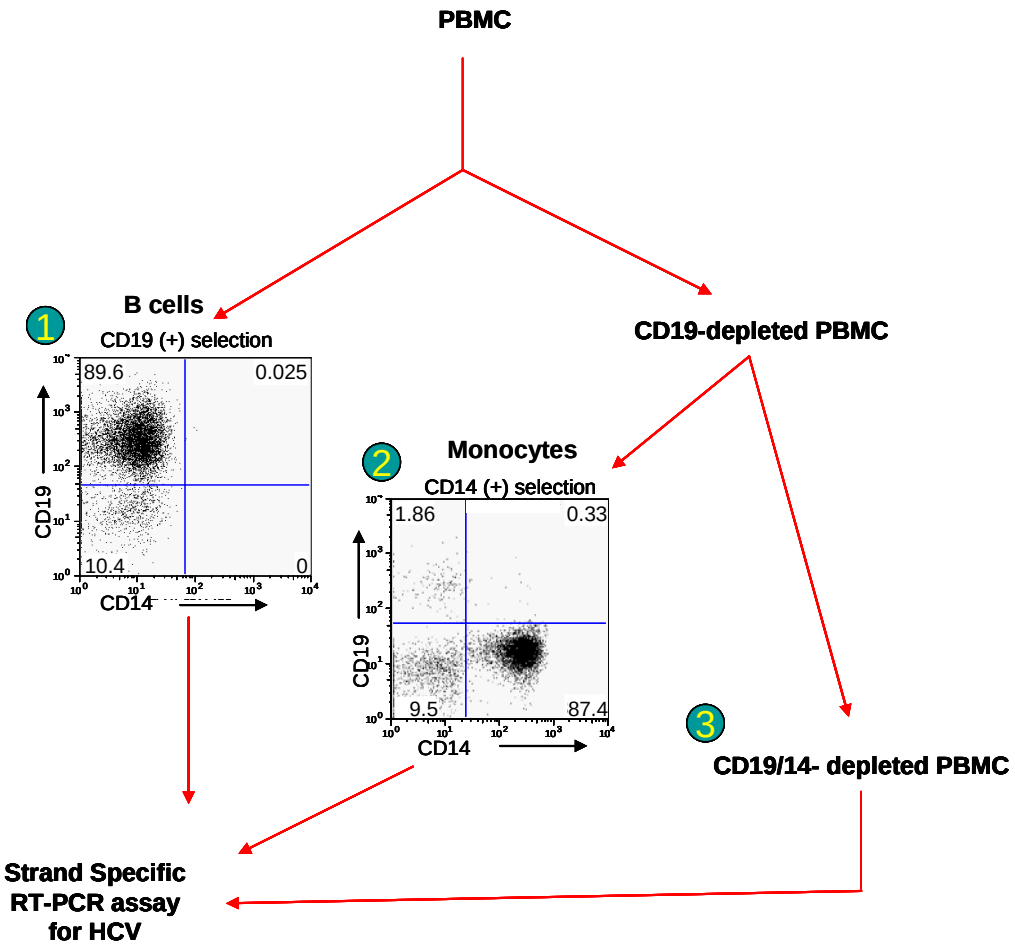


Figure 4. Schematic diagram of PBMC isolation for HCV RNA detection. Flow chart depicts the process where PBMC are separated into fractions prior to cellular lysis and real-time RT-PCR analysis. B cells were isolated using CD19(+) magnetic beads, and monocytes were isolated using CD14(+) magnetic beads. The CD14/19-depleted PBMC population was analyzed as a whole. Prior to cellular lysis, the fractions were incubated with trypsin-EDTA to remove receptors and any associated virus from the cell surface. B cell and Monocyte fractions were typically greater than 85% pure. The 14/19-deplection fraction carried residual B cell and monocyte populations, but they (B or Mono) remained at or below 5% of the population.

Table 2. HCV RNA Detection in cells from treatment naïve, chronic HCV patients.

	+ Strand	- Strand
B cells (CD19)	15/50	2/50
Monocytes (CD14)	3/42	0/42
CD14/19 depleted PBMC	2/42	1/42

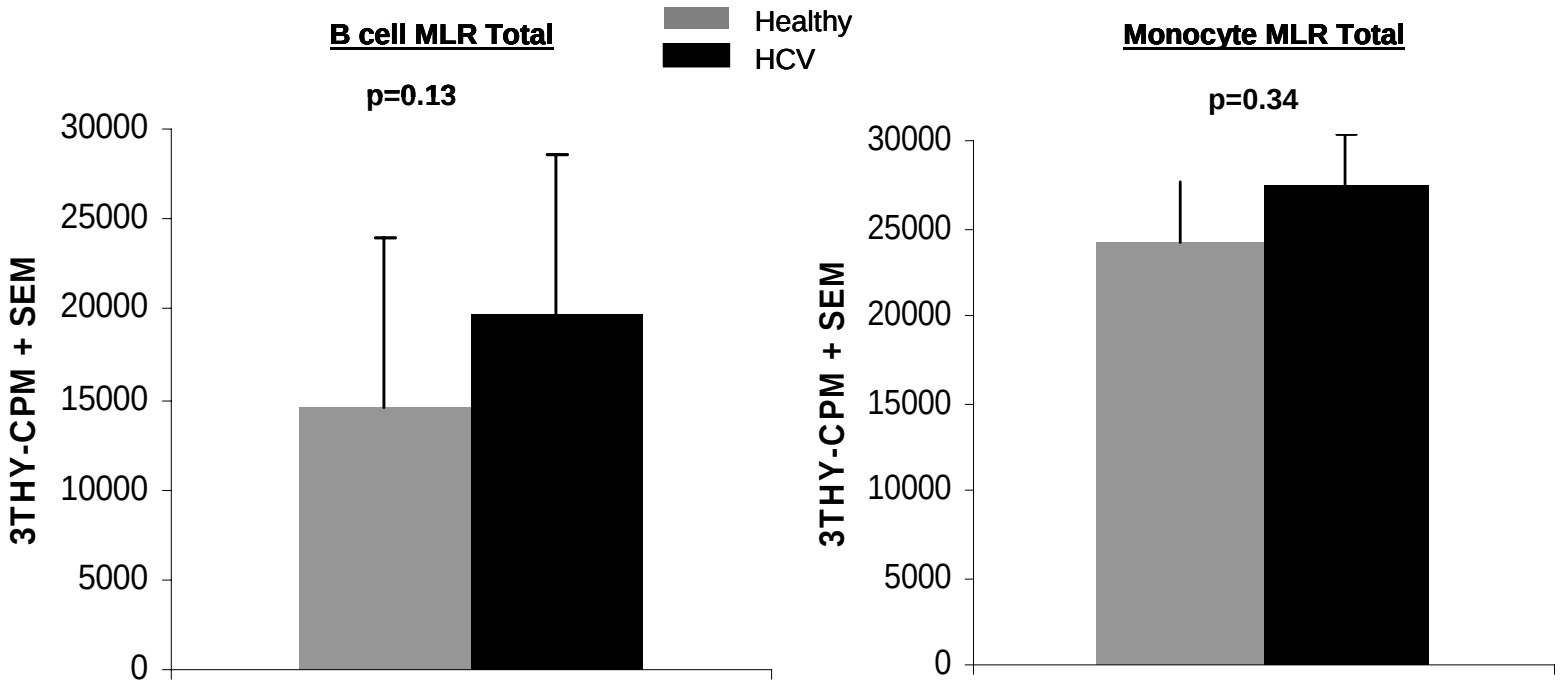
Table depicts the number of chronic HCV patients determined to have HCV RNA in fractioned immune cell populations. B cells were the only fraction in which RNA was consistently found. There was no significant amount of negative strand RNA found in any immune cell population.

B cells from chronic HCV patients stimulate significantly higher T cell proliferation when associated with HCV positive strand RNA

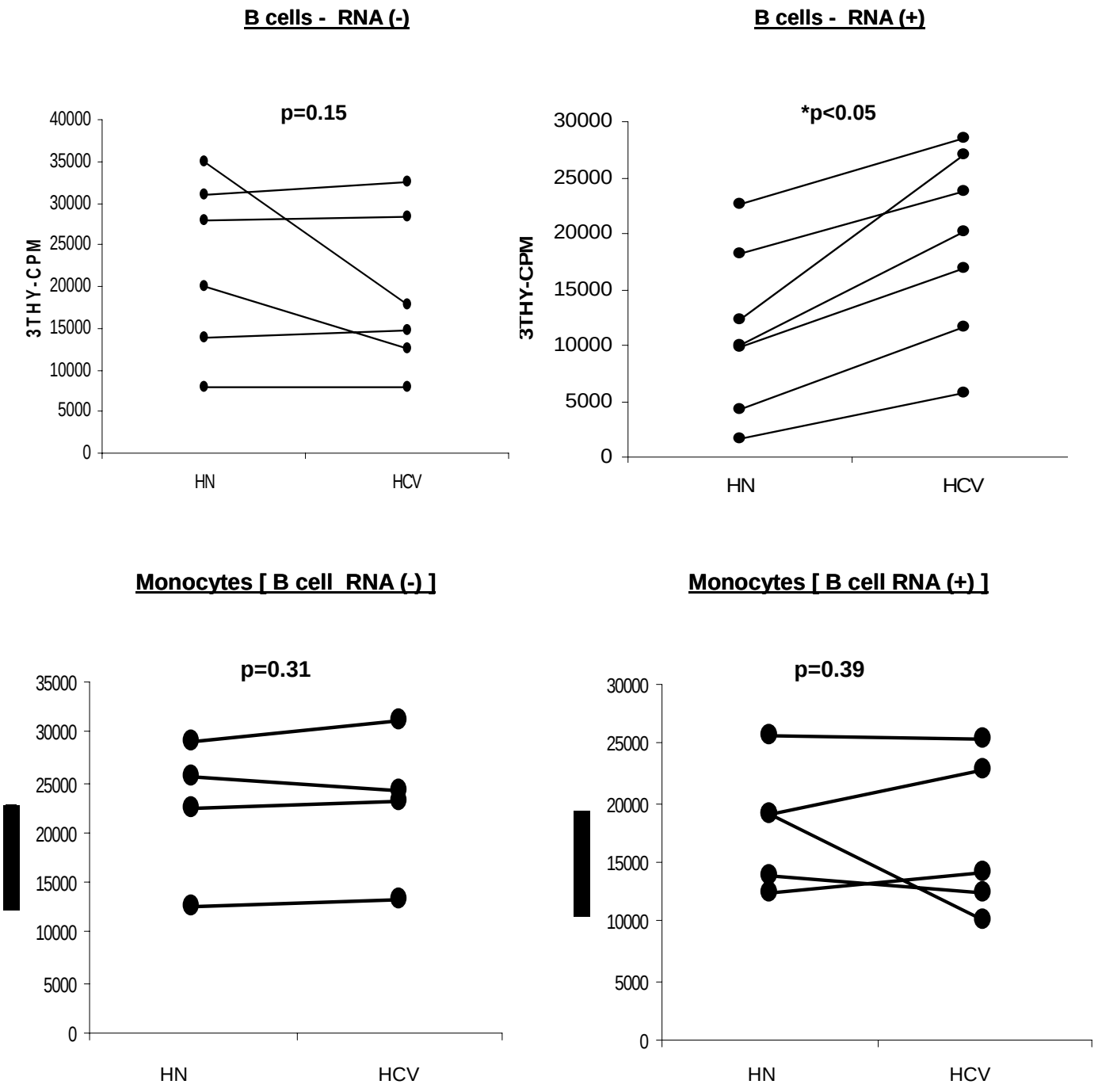
A measure of antigen presenting cell function can be experimentally determined using an allogeneic mixed lymphocyte reaction (MLR). We and others have performed MLR analysis on uniquely defined dendritic cell populations from chronic HCV patients. However, there are conflicting reports on the magnitude of HCV DC dysfunction in an MLR when compared to healthy controls. Delineating chronic HCV dendritic cell function is important in the proposed APC contribution to immune attenuation. However, there is a paucity of information regarding the extent of APC function for B cells and Monocytes during HCV infection. We therefore isolated B cells and monocytes from 13 chronic, treatment-naïve, HCV patients or healthy volunteers and used them as APC in ^{3}H-tymidine based MLR assays. B cells or monocytes from either healthy controls or chronic HCV patients were cultured with third party healthy T cells at a 1 to 10 APC:responder ratio and incubated for 6 days. Each experiment contained one healthy-HCV subject pair as donors. However, one of only two third-party T-cells was used as responders in an effort to reduce experimental variability. The results from these experiments are demonstrated in figure (5). Neither monocytes nor B cells from HCV patients were significantly different in their ability to induce T cell proliferation, when compared to healthy ($p=0.34$ and 0.13, respectively). However, B cells from patients that harbored detectable HCV RNA in B cells showed significantly higher MLR stimulation, compared to healthy subjects (figure XXB)($p \leq 0.05$). Conversely, B cells without detectable

HCV RNA or monocytes from either group showed no significant differences compared to heathy subjects. Figure 5C shows the same data normalized to MLR data from the corresponding healthy B cell control (designated as 100). This shows a significant difference between T cell proliferation from HCV B cells with undetectable viral RNA found and HCV B cells where there was RNA detected ($p<0.001$). In contrast, monocyte-induced T cell proliferation (based on the presence of viral RNA in those B cells from the same PBMC source) was not significantly different in the two groups ($p= 0.15$). Thus, B cells from patients who have detectable HCV RNA in their B cells appear to have a hyperactive APC phenotype. The finding of hyperactive B cells contrast with the known attenuation of T cell responses that characterize chronic HCV infection. However, our lab has recently shown that activation of CD4+25- T cells may result in the formation of CD4+25+Foxp+ suppressor/regulatory T cells (Pillai, Ortega et al. 2007). This may explain the paradoxical observation that HCV+ B cells were hyperactive. We thus hypothesize that hyperactive B cells from HCV patients may induce greater numbers of regulatory T cells.

A.


B cell MLR Total
p=0.13
Healthy
HCV
3THY-CPM + SEM
30000
25000
20000
15000
10000
5000
0
Monocyte MLR Total
p=0.34
3THY-CPM + SEM
30000
25000
20000
15000
10000
5000
0

B.


B cells - RNA (-)
p=0.15
3THY-CPM
40000
35000
30000
25000
20000
15000
10000
5000
0
HN
HCV
B cells - RNA (+)
*p<0.05
3THY-CPM
30000
25000
20000
15000
10000
5000
0
HN
HCV
Monocytes [B cell RNA (-)]
p=0.31
35000
30000
25000
20000
15000
10000
5000
0
HN
HCV
Monocytes [B cell RNA (+)]
p=0.39
30000
25000
20000
15000
10000
5000
0
HN
HCV

C.

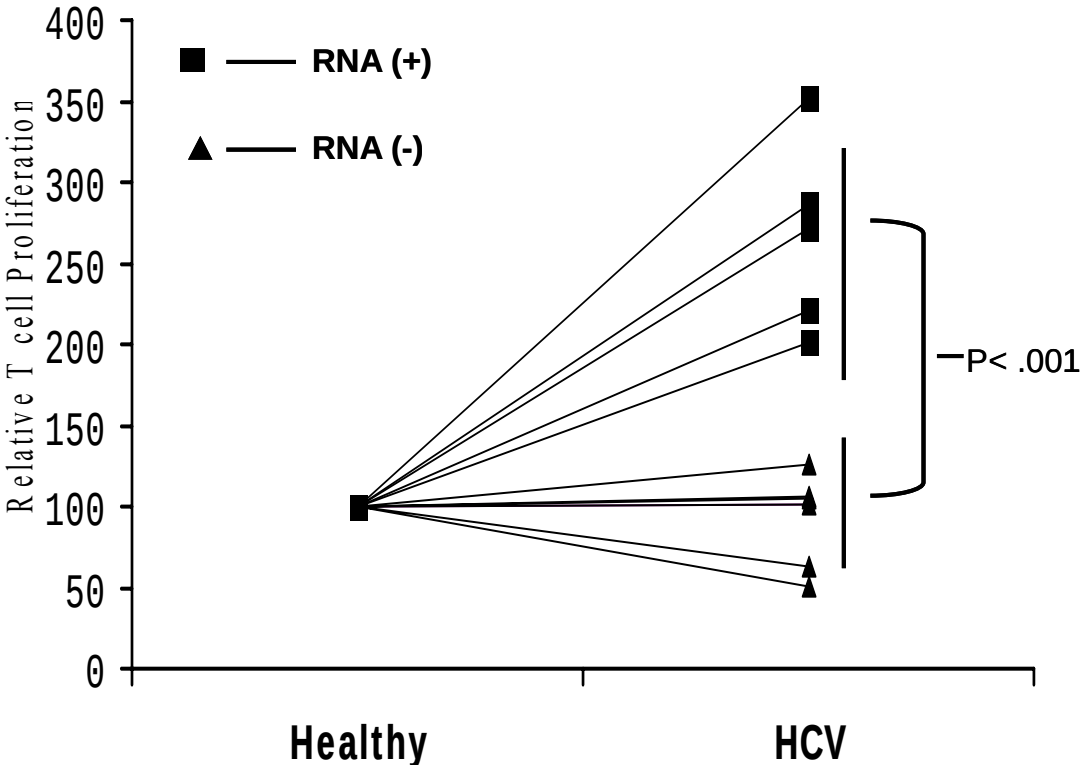

400
350
300
250
200
150
100
50
0
Relative T cell Proliferation
RNA (+)
RNA (-)
P< .001
Healthy
HCV

Figure 5**. B cells from Chronic HCV treatment naïve patients stimulate a higher T cell Mixed Lymphocyte Reaction** A.) Purified B cells and Monoctyes from up to13 healthy donors or 13 HCV-infected patients were used to stimulate purified allogeneic $CD3^{+}$T-cells from one of two healthy third party subjects. Allogeneic cultures were pulsed with ^{3}H-thymidine on day 5 and harvested on day 6. Graphs represent counts of radiolabeled thymidine which was incorporated into proliferating T cells. The setup represents a stimulator:responder ratio of 1:10. B.) Results from the B cell MLR were allocated based on detection of HCV RNA and graphed in a pair-wise manner which illustrates the extent of T cell proliferation based on the presence or absence of HCV RNA. Monocytes from the same cohort of donors were also divided based on the presence or absence of HCV RNA in the corresponding B cells in the same HCV donor and are represented in a pair-wise manner with Monocytes from the healthy control counterpart. C.) Graphical representation of data when T cell responses from healthy subjects are normalized to an arbitrary base of 100 and the pair-wise HCV T cell responses subsequently represented as a measure of the magnitude from a base of 100. The statistical inference is calculated as the significance of relative T cell proliferation between HCV B cells with or without the detection of HCV RNA ($p<0.001$).

HCV B cell activation results in the generation of CD4+25+Foxp3+ regulatory T cells.

Based on the premise of T regulatory cell generation, we decided to evaluate the phenotype of MLR activated T cells in a B-cell induced MLR. We used the same setup as designed for the 3H-thymidine MLR, mixing 2 x 10^4 B cells with 2 x 10^5 T cells in a 96-well format (in triplicate) and allowing 7 day incubation. In these experiments T cells were first labeled with CFSE to allow for flow cytometric analysis to determine T cell proliferation and activation. We found that HCV RNA+ B cells induced a greater percentage of CD25+, Foxp3+ allogeneic T cells which correlated with an increase in CFSE diluted T cells, explaining the higher rate of proliferation seen in ^{3}H-thymidine assays (Figure 6). We further evaluated the suppressive capacity of the in-vitro generated, activated T-cells to confirm that these were indeed suppressor T cells. At the end of the MLR, cultures were harvested, washed, and used as 'suppressors' in an autologous T cell proliferation Setup for the suppression assay consisted of third party T cell depleted- PBMCs as APCs and CD4+25- T cells from the same source (as the APCs and primary MLR T cells) as the responding population. The suppression assay was activated using anti-CD3 and incubated for 7 more days. Results showed that the HCV B cell-activated CD4+25+Foxp+ T cells are indeed suppressor cells (Figure 7). While the B cells from these experiments were HCV RNA (-), the results are adequate to show that the in-vitro generated CD4+35+Foxp3+ T cells are suppressive in nature. Therefore, we conclude that MLR cultures stimulated by HCV RNA (+) B cells generated greater suppressor T cell potential.

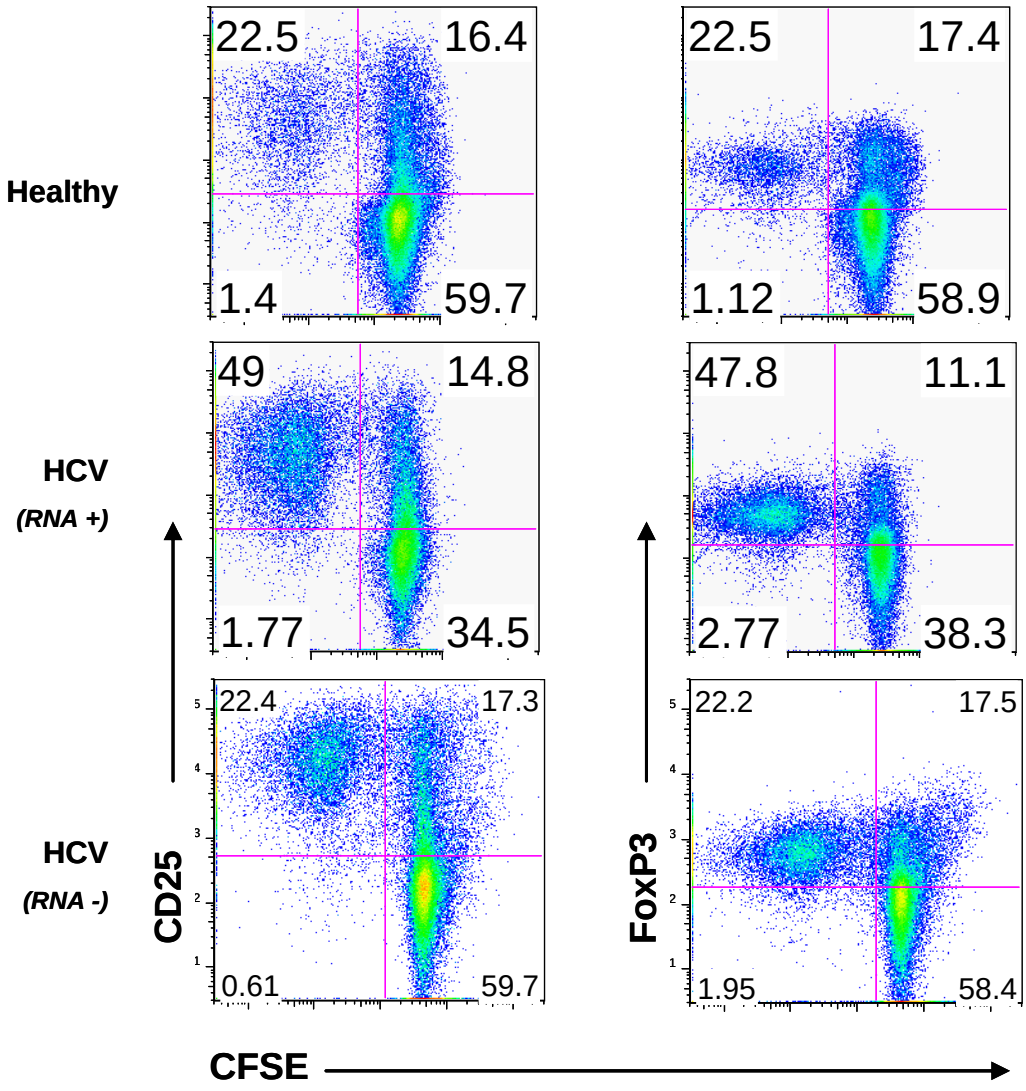

Healthy
HCV
(RNA +)
HCV
(RNA -)
CD25
FoxP3
CFSE
22.5
16.4
1.4
59.7
22.5
17.4
1.12
58.9
49
14.8
1.77
34.5
47.8
11.1
2.77
38.3
22.4
17.3
0.61
59.7
22.2
17.5
1.95
58.4

Figure 6. The magnitude of T cell proliferation from a B cell MLR correlates with the magnitude of CD25 and Foxp3 expression in proliferating cells. The figure is a graphical representation of the proliferating T cell response from a B cell MLR. CD4+25- T cells were stained with CFSE and analyzed for CD25 cell surface expression and intracellular Foxp3 expression after 5 days in culture. Data is representative of typical findings from a healthy donor compared with HCV patient B cells that either harbored or was void of HCV positive strand RNA. The presence of HCV RNA from HCV patient B cells are denoted in the graph.

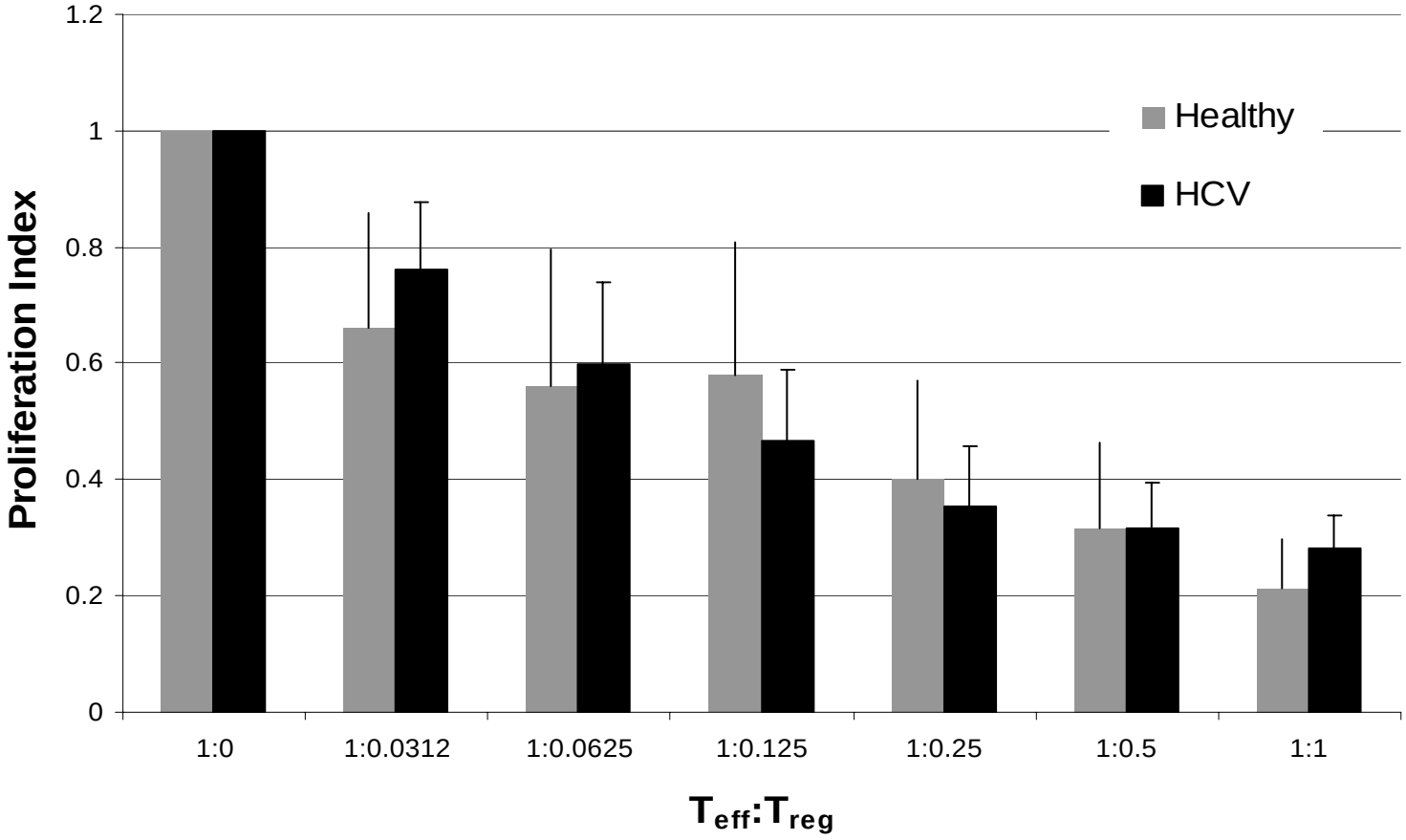

1.2
1
0.8
0.6
0.4
0.2
0
Proliferation Index
Healthy
HCV
1:0
1:0.0312
1:0.0625
1:0.125
1:0.25
1:0.5
1:1
Teff:Treg

Figure 7. Suppression profile of MLR generated T cells from a chronic naïve or healthy control B cell show similar capacities. T cell/B cell mixtures from the B cell MLR were harvested and used as a suppressor cell source for an autologus T cell proliferation assay. Plot represents the proliferation index from CFSE labeled proliferating T cells in an anti-CD3 activated autologus MLR. Proliferating T cells were incubated with a serial dilution of suppressor T cell mixtures, and the proliferation index represents the ratio of T cell proliferation from cultures with varying degrees of suppressors over cultures with no suppressors present. Data represents results from 5 healthy and 5 HCV B cell generated T 'suppressor' cells against 1 $CD3^-$ PBMC:$CD4^+25^-$ responder cell source. $CD3^-$ PBMC (as APC source) and CD4+25- responder ratio's were used at 1:1.

Phenotypic B cell comparisons between HCV patients and healthy subjects reveal a slight activation phenotype in chronic HCV cohorts.

A great deal of chronic HCV sequelae derives from B lymphocyte function and activation. Mixed cryoglobulinemia, B cell lymphomas, and an increase in the production of autoantibodies are all prominent pathological characteristics associated with chronic untreated HCV infection (Agnello, Chung et al. 1992; De Rosa, Gobbo et al. 1997). Based on the nature of B cell functionality and the propensity of chronic infection to correlate with alterations thereof, we tested the phenotypic attributes of ex vivo derived B cells. Flow cytometric analysis was used to determine expression levels of key activation and/or differentiation markers that could potentially reveal activated B cell subsets (Table 3). The results showed that HLA-DR expression (Mean fluorescence intensity) was higher in chronic HCV B cells when compared to healthy controls (Figure 8). Interestingly, the magnitude of MHC intensity correlated with those patients whose B cells harbored HCV positive strand RNA ($p<0.05$) (figure 8). This finding validated the previous experimental results, showing the HCV B cell potential to induce a greater T cell proliferation. MHC class II levels (represented here by HLA) is a solid indication of the antigen presentation potential and its correlation with both higher T cell generation and HCV RNA accumulation suggest , again, direct viral mediation of APC potential. Interestingly, B cells from patients that did not show detectable HCV RNA also

showed some phenotypic differences, compared to B cells from healthy donors. Thus, for CD86 and CD71 there was a significant increase in RNA (-) B cells from HCV patients compared to healthy controls ($p<0.05$). Similarly, CD27 and CD38 expression levels were very close to significant ($p=0.07$) between healthy and RNA (-) B cells from HCV patients. While these phenotypic features did not translate into higher APC function, they clearly indicate an activated phenotype among B cells of all HCV patients as a whole. These findings support a model wherein HCV-associated B cell activation may be an early event and some sequelae of activation may remain even after such association has terminated, or has resulted in B cell selection.

Table 3. Markers used in the analysis of B cell phenotype

HLA-DR	-Part of MHC class II surface antigen; Marker for immune stimulation
CD23	-Low affinity IgE receptor; B cell growth and activation factor; promotes plasma cell differentiation
CD25	-IL-2 receptor alpha chain; lymphocyte activation marker
CD27	-Tumor necrosis factor superfamily member 7; memory B cell marker
CD38	-ADP-ribosyl cyclase 1; plasma cell differentiation marker
CD71	-Transferrin receptor; Marker for actively proliferating cells
CD86	-T cell co-stimulatory molecule

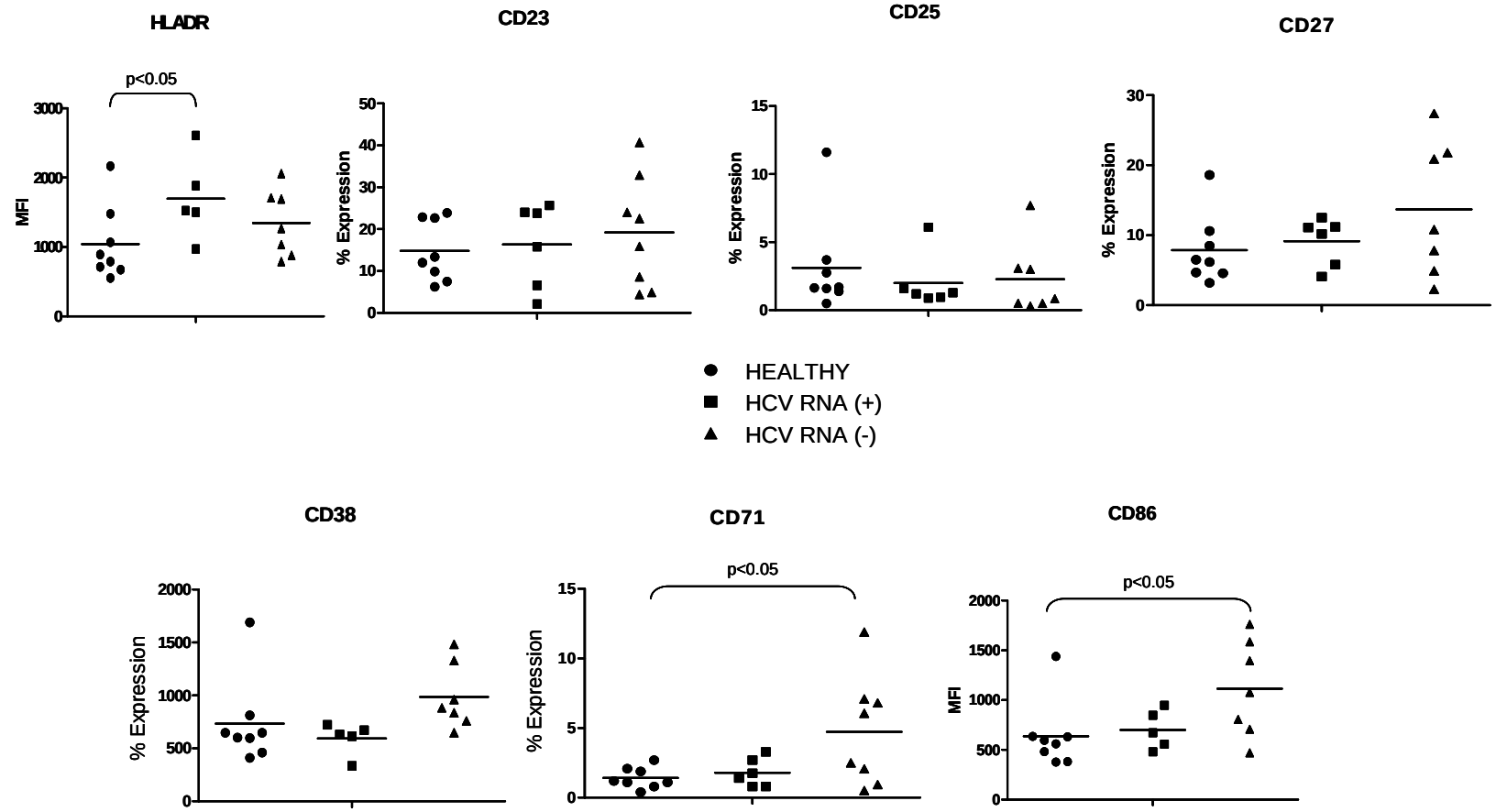

HLADR
p<0.05
MFI
3000
2000
1000
0
CD23
% Expression
50
40
30
20
10
0
CD25
% Expression
15
10
5
0
CD27
% Expression
30
20
10
0
HEALTHY
HCV RNA (+)
HCV RNA (-)
CD38
% Expression
2000
1500
1000
500
0
CD71
p<0.05
% Expression
15
10
5
0
CD86
p<0.05
MFI
2000
1500
1000
500
0

Figure 8. Ex-vivo profile of B cells from healthy donors and HCV patients show slight activation differences.
PBMCs from 14 HCV patients (6 RNA+/8 RNA -) or 8 healthy donors were examined ex-vivo with the following panel of B cell activation markers: **1**-CD19 PE cy7, CD25 APC, CD69 PE; **2**-CD19 PE cy7, CD27-FITC, CD23 PE; **3**-CD19 PE cy7, CD 71 FITC, CD 86 PE, CD 38 APC; 4-CD19 PE cy7. Mouse Isotype Ig controls were used for PE, APC, and FITC channels. MFI levels were determined for HLADR and CD86, while percent expression was used for the remaining markers.

B cells from chronic HCV patients are attenuated in their response to polyclonal stimulation.

Some of the main characteristics of polyclonal stimulation of B cells include increased proliferation, activation marker up-regulation, and nonspecific antibody production (Nashar, Hirst et al. 1997; Hunziker, Recher et al. 2003). There are immune mechanisms in place that regulate the magnitude of nonspecific activation events, including clonal deletion, anergy, and activation-induced apoptosis. The aforementioned characteristics are considered a consequential relationship between chronic viral infection and B cell function. For instance, during HIV infection, B cells are hyporesponsive to T -cell independent in-vitro polyclonal stimulation (Miedema, Petit et al. 1988). Based on these findings, it is reasonable to predict that B cells from chronic HCV infected persons may also show signs of chronic antigenic stimulation.

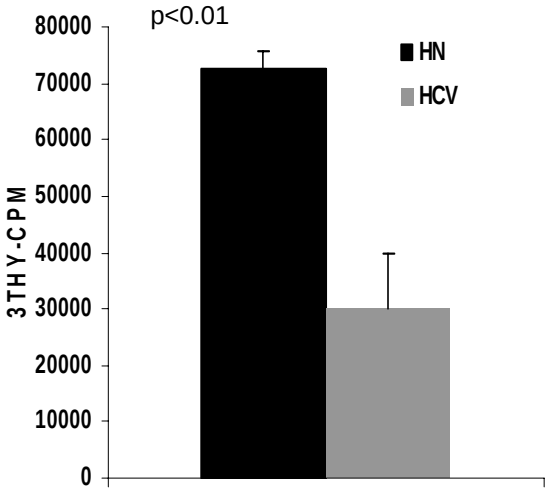
It is well known that a measure of B cell functionality resides in their proliferative response to stimulation. We therefore analyzed the proliferative capacity of chronic HCV B cells using formalin fixed *Staphylococcus aureus* (pansorbin). S aureus produces a ubiquitously expressed surface protein, termed *protein A*. Protein A has many experimental uses based mainly on its high affinity toward the Fc region of immunoglobulins; mainly IgG1, IgG2 and IgM (Goodyear and Silverman 2003). Because the B cell receptor is composed of membrane bound IgM, exposure to protein A induces crosslinking, which leads to antigen independent activation and proliferation. We measured the extent of polyclonal B cell proliferation from 5 sets of healthy or chronic HCV patient B

cells using 3H-thymidine uptake. We found that B cells from chronic HCV patients showed a distinct attenuation of proliferation after three days of stimulation, when compared to healthy controls, $p< 0.05$ (figure 9A). It is possible that the rapidity and/or duration of B cell proliferation may be delayed in chronic infection, so we extended the time course of proliferation analysis in one experiment up to 5 days, taking 24 hour time points, and found that all B cell proliferation events waned after day 4. We evaluated whether this reduction in proliferation was via an increase in the apoptosis/ cell death of chronic HCV B cells, by staining for Annexin V. Thus, we flow cytometrically evaluated the extent of annexin V accumulation after 2 days of B cell exposure to formalin fixed S aureus. From 4 separate examples of apoptotic staining, we observed a trend toward a higher percentage of Annexin V staining on B cells from chronic HCV patients, indicating a higher rate of apoptosis (Figure 10). However, this trend did not reach statistical significance.

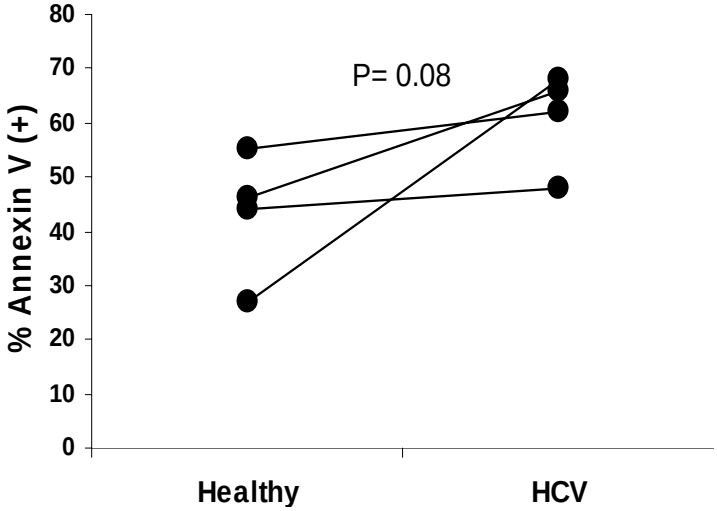
Polyclonal B cell activation is a result of innate immune response mechanisms triggered by the presence of a microbial pathogen-associated molecular pattern (Montes, Zuniga et al. 2002). Besides proliferation, an important aspect of this response involves an increase in activation marker expression and cellular differentiation (Janossy and Greaves 1975). Although protein A (pansorbin) induced activation of B cells is sufficient to induce proliferation, its use in the assessment of B cell activation was problematic. There frequently was considerable background staining artifacts that were probably a result of the affinity of protein A toward Fc regions of the fluorochrome labeled Ig molecules. Therefore, alternative B cell stimuli were

used, such as pokeweed mitogen (PWM), a lectin from *Phytolacca Americana.* PWM is well known for its ability to stimulate B cell differentiation and antibody production. To evaluate the activation and differentiation potential of ex-vivo, peripheral derived B cells we incubated PBMCs from either healthy donors or chronic HCV patients with 5ug/ml of PWM up to 48 hrs and analyzed B cell activation marker expression (listed in Table 3) using flow cytometric analysis. We found that, at 24hrs, chronic HCV B cells (compared in total) trend toward an attenuation of activation marker up-regulation (Figure 10). What is more interesting is the analysis of activation when separating HCV B cells on the basis of HCV RNA detection. In those B cell cohorts where no RNA is detected, there is a significant difference of attenuation in several markers when compared to healthy controls. CD69, 25, and 71 are all significantly reduced in the percentage expression difference between healthy controls and HCV patients where no RNA is detected ($p<0.05$). There were no significant differences between cohorts of HCV RNA positive patients and any other (healthy, RNA -) group. In contrast, it is interesting to point out that CD27 is the only activation marker whose expression was up-regulated more in B cells from chronic HCV patients. Moreover, the significance of CD27 expression between healthy controls and HCV patients is maintained only in the cohort of chronic HCV patients where there was no HCV RNA detected. The analysis of B cells using PWM is a useful tool for determining the capacity of naïve B cells to differentiate into antibody secreting cells. Antibody production was not addressed in these studies as our study was mainly focused on APC function.

A.


p<0.01
HN
HCV
3TH Y-CPM
80000
70000
60000
50000
40000
30000
20000
10000
0

B.


P= 0.08
% Annexin V (+)
80
70
60
50
40
30
20
10
0
Healthy
HCV

C.

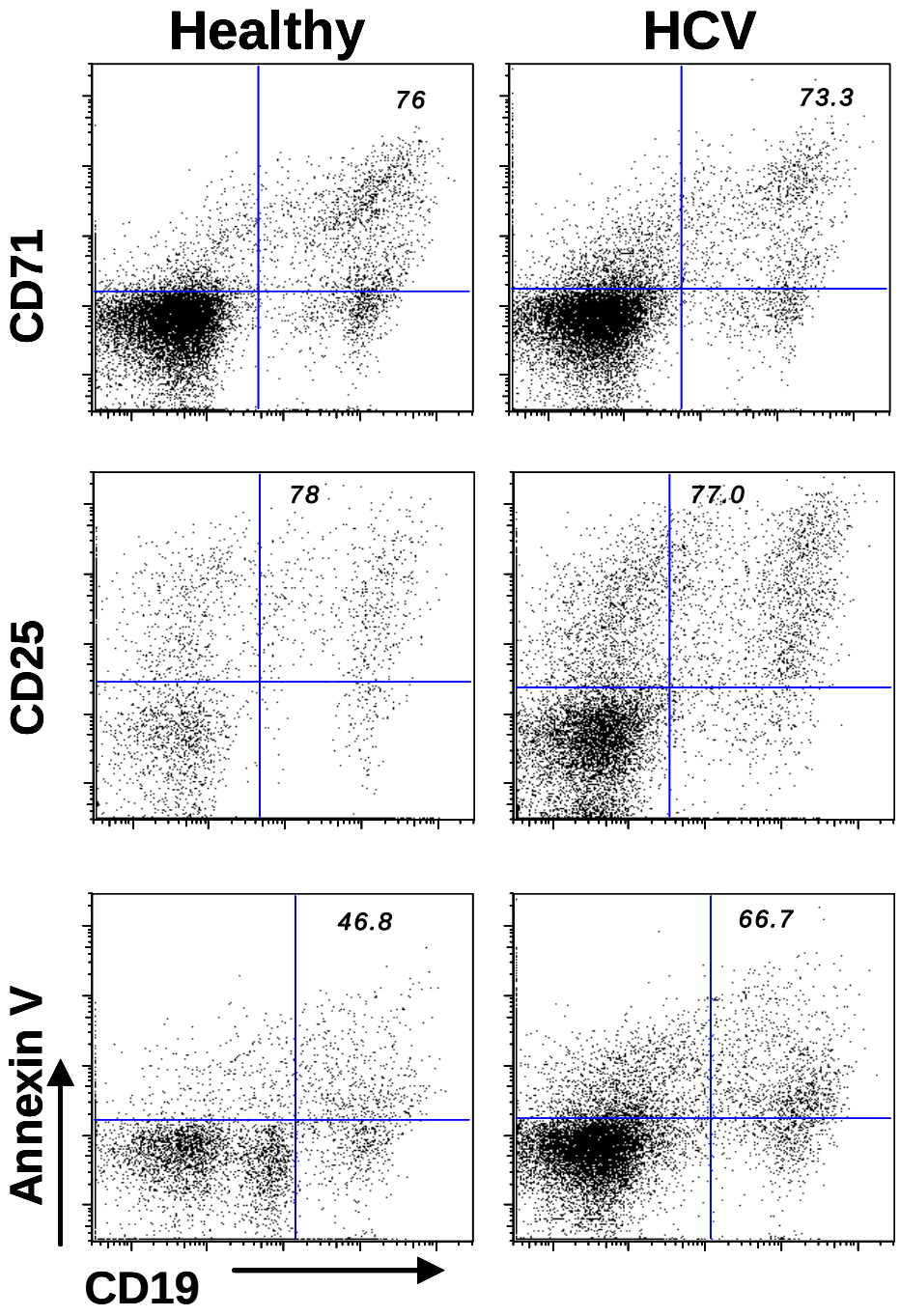
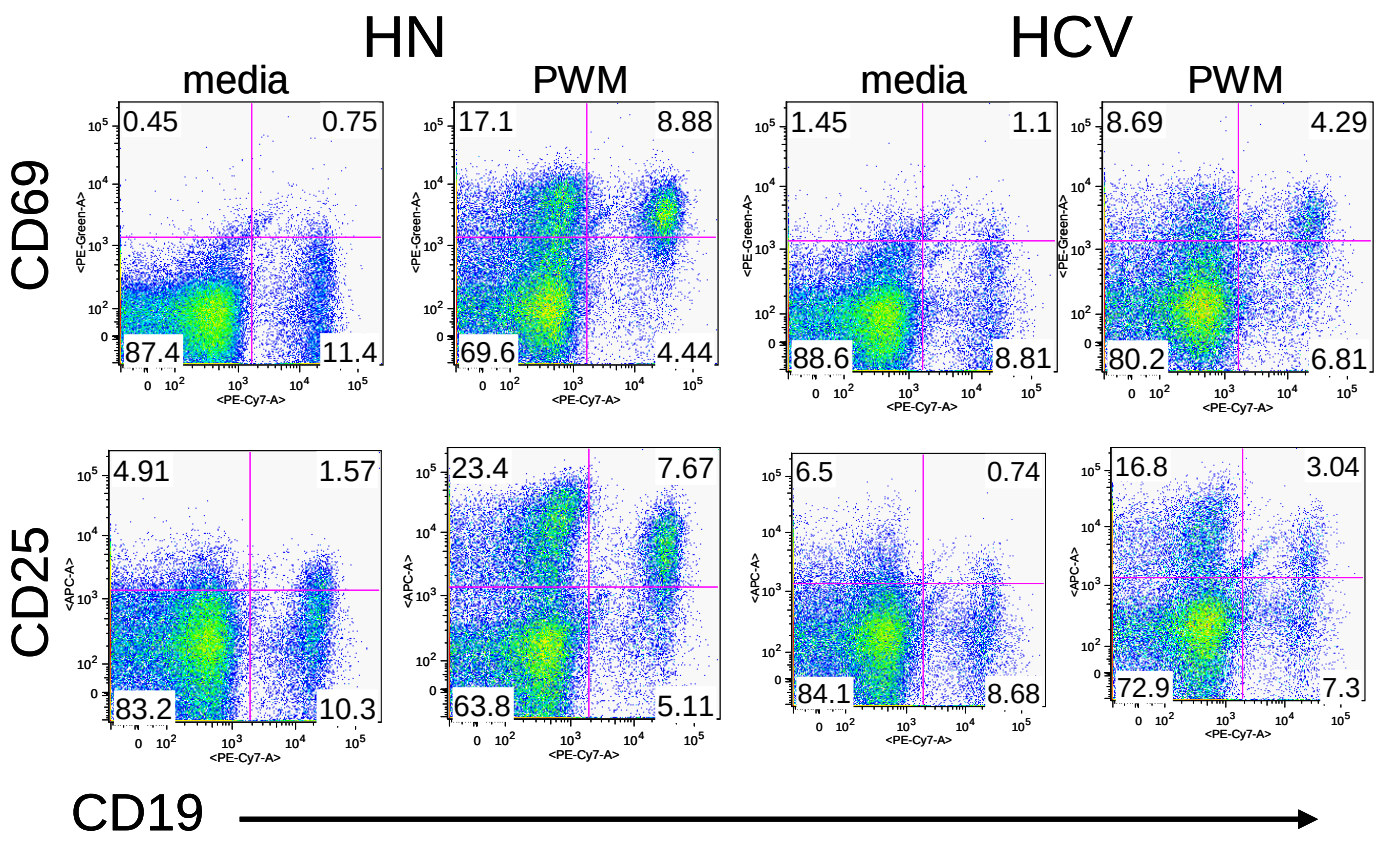


FIGURE 9. **B cells from chronic HCV patients show attenuation in proliferation to Staphylococcus protein A**. A.) B cells from chronic HCV patients or healthy donors were isolated using CD19+ magnetic bead separation. $2x10^5$ B cells (in triplicate) were incubated for 3 days with a 1 to 1000 dilution of pansorbin (Protein A) with a 16hr pulse of 3H-Thymidine. B.) PBMCs from chronic HCV patients or healthy donors were incubated for 2 days with a 1 to 1000 dilution of pansorbin (protein A). Cells were then stained with Annexin V-FITC and CD19 PercP. Analysis of percent expression of annexin V staining is shown from 4 separate experiments. C.) Representative dot plot of pansorbin activated PBMCs after 2 days of culture. B cells are separated on the X- axis by CD19 PE-cy7 and Annexin-FITC, CD71-PE, and CD25-APC are represented on the Y axis.

A.


HN
HCV
media
PWM
media
PWM
CD69
CD25
CD19
0.45
0.75
87.4
11.4
17.1
8.88
69.6
4.44
1.45
1.1
88.6
8.81
8.69
4.29
80.2
6.81
4.91
1.57
83.2
10.3
23.4
7.67
63.8
5.11
6.5
0.74
84.1
8.68
16.8
3.04
72.9
7.3
<PE-Green-A>
<APC-A>
<PE-Cy7-A>

B.

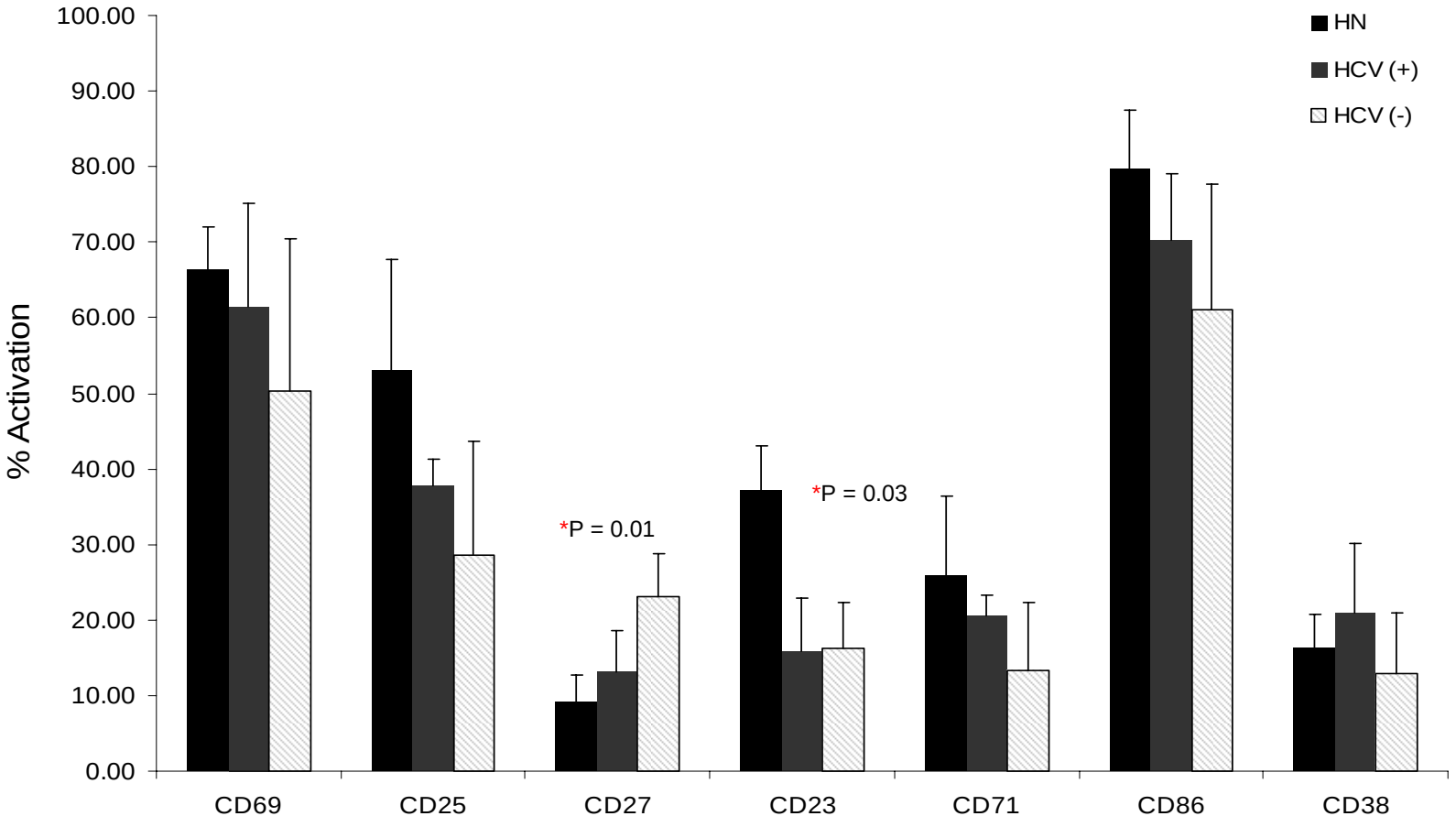

100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
% Activation
HN
HCV (+)
HCV (-)
*P = 0.01
*P = 0.03
CD69
CD25
CD27
CD23
CD71
CD86
CD38

Figure 10. HCV B cells have a slight attenuation in response to pokeweed mitogen (pwm) stimulation. A.) Representative FACS plot of PWM activated PBMCs. The plots illustrate B cells (CD19+) from a healthy donor or HCV patient that express CD25 and CD69 24 hours after stimulation. B.)PBMCs from 7 chronic HCV patients or 4 healthy donors were incubated for 24 hours with 5ug/ml of pokeweed mitogen. Cells were subsequently stained with the following 3 panels of activation markers: 1-CD19 PE cy7, CD25 APC, CD69 PE; 2-CD19 PE cy7, CD27-FITC, CD23 PE; 3-CD19 PE cy7, CD 71 FITC, CD 86 PE, CD 38 APC; 4-CD19 PE cy7. Mouse Isotype Ig controls were used for PE, APC, and FITC channels. Statistical significance was achieved for CD27 between healthy and HCV patients whos B cells do not harbor HCV RNA ($p=0.01$) and for CD23 between healthy and both cohorts of HCV patients ($p=0.03$).

In-vitro exposure to JFH-1 virus activates B cells and results in enhanced APC function

As mentioned previously, there is considerable evidence supporting the direct interaction and possible HCV infection of immune cells. Transplanted liver re-infection, viral subspecies tissue diversity, and multi-cell sources of viral co-receptor expression are all major factors that support the possibility of extra-hepatic viral propagation. There are numerous reports that either confirm or contradict the hypothesis that HCV infects immune cell populations. Most are variations on experimental designs that assess the presence of HCV RNA or viral protein production. Until recently, the lack of an in vitro propagating system capable of supporting full length HCV replication made it difficult to conduct direct infection experiments. A pilot study on a subgenomic HCV genotype 2a replicon from a Japanese fulminant hepatitis patient (JFH-1) demonstrated, for the first time, in vitro evidence of virus propagation using a full length genomic sequence and no tissue culture adaptations (Kato, Date et al. 2003). In an effort to test the effect of HCV infection on immune cells, we evaluated the ability JFH-1 to affect the phenotype/ function of PMBCs. JFH 2a is the first Hepatitis C viral clone shown to propagate in cell culture without the addition of tissue culture adapted mutations in either the virus or host cell (Zhong, Gastaminza et al. 2005). We exposed freshly isolated PBMCs from healthy donors to JFH for 3 hours. These cultures were then washed to clear unbound virus that may not have been

absorbed or taken up by cells. The cells were then incubated up to 7 days post infection, separated into B cell, monocyte, and bulk fractions, and then analyzed by real time RT-PCR for viral RNA (positive and negative strand). From multiple such attempts ($\geq$5) we were unable to detect either positive or negative strand RNA in these cells (data not shown). CD8 T cell removal prior to exposure also failed to show evidence of viral propagation.

However, flow cytometric analysis revealed that virus exposure (even at an MOI of 0.005) induced B cells to highly express activation markers CD25, CD27, CD69, CD71, CD86 and CD38 (Figure 11). The activation occurred in response to both viable and UV-inactivated virus, and was blocked when cells were pre-exposed to anti-CD81 antibody (Figure 12). This suggested that B cell activation did not require viral replication, but was dependent on HCV-CD81 interactions. Interestingly, when B cells were incubated alone without JFH , or when PBMCs were incubated with JFH in the absence of monocytes (as illustrated by CD14 depletion), there was no significant activation , suggesting that accessory cells were also required for B cell activation. In fact, activation of B cells minimally requires the presence of monocytes and T cells (Figure 14A). We further found that levels of interferon, TNFa, IL-10, and IL-13 correlated with the magnitude of B cell activation (figure 14B). In conditions where B cells, monocytes, and T cells were present together, there was also a significantly higher production of these four cytokines and a significantly higher expression of CD71.

The level of cytokine production dropped when monocytes were removed from culture, and was similarly weak when B cells and monocytes were incubated together without T cells. The level of cytokine production was restored only when T cells and monocytes were added to the culture conditions. The apparent reliance upon cytokine factors suggest an intricate coordination of multiple cell types in the distinct B cell activation event that occurs in the course of exposure to virus.

Finally, in an attempt to detect HCV protein in virus-exposed B cells, we incubated PBMC with 0.01 MOI of JFH-1 for 7 Days. The experimental parameters are similar to all previous infection assays, as virus was incubated with cell for 3 hours and washed away. Cells were then incubated in culture for the remaining 7 days, at which time PBMC were washed and stained intracellulary using a polyclonal serum from an HCV genotype 2a-infected patient (graciously provided by Dr. Michael Gale). In a preliminary experiment, HCV protein was detected in exposed and activated B cells (Figure 14).

A.)

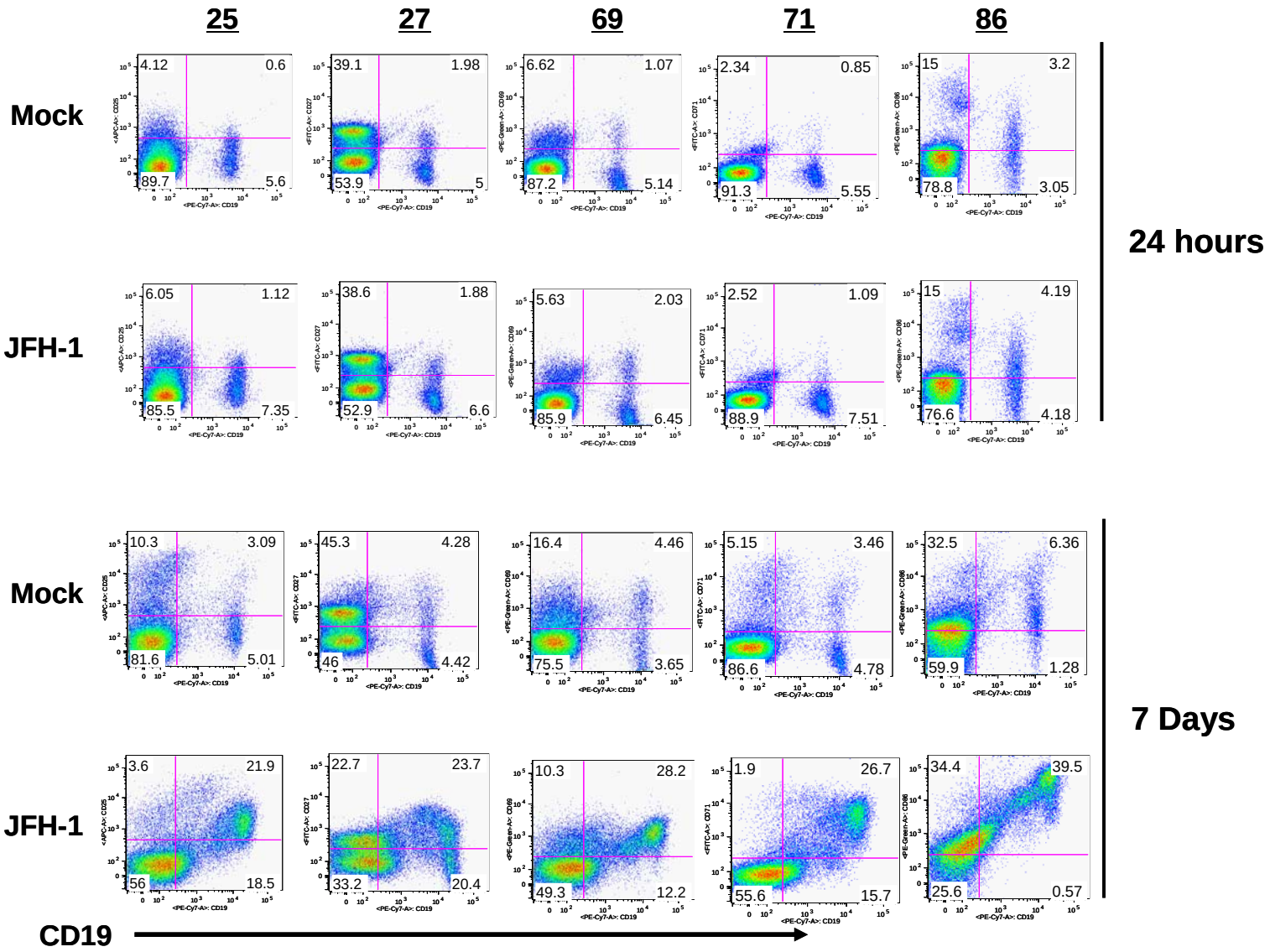

25
27
69
71
86
Mock
JFH-1
Mock
JFH-1
24 hours
7 Days
CD19
4.12
0.6
89.7
5.6
39.1
1.98
53.9
5
6.62
1.07
87.2
5.14
2.34
0.85
91.3
5.55
15
3.2
78.8
3.05
6.05
1.12
85.5
7.35
38.6
1.88
52.9
6.6
5.63
2.03
85.9
6.45
2.52
1.09
88.9
7.51
15
4.19
76.6
4.18
10.3
3.09
81.6
5.01
45.3
4.28
46
4.42
16.4
4.46
75.5
3.65
5.15
3.46
86.6
4.78
32.5
6.36
59.9
1.28
3.6
21.9
56
18.5
22.7
23.7
33.2
20.4
10.3
28.2
49.3
12.2
1.9
26.7
55.6
15.7
34.4
39.5
25.6
0.57

Figure 11. B cells are highly activated when exposed to JFH virus in vitro.
Representative dot plots of JFH exposed PBMCs at 1 day and 7 days post exposure. In the assay, PBMCs were exposed to virus for 3 hours, washed 2x and reseeded for up to 7 days. FACS plots illustrating the activation of B cells are shown. On the x-axis is CD19 and each column shows the assessment of different activation markers (represented at the top of each column). The first two rows are the 1 day plots for mock vs. JHF infection and the bottom two rows are mock vs. JFH at 7 days post infection. Mock infection is defined here as cells in culture with no exogenous stimulation.

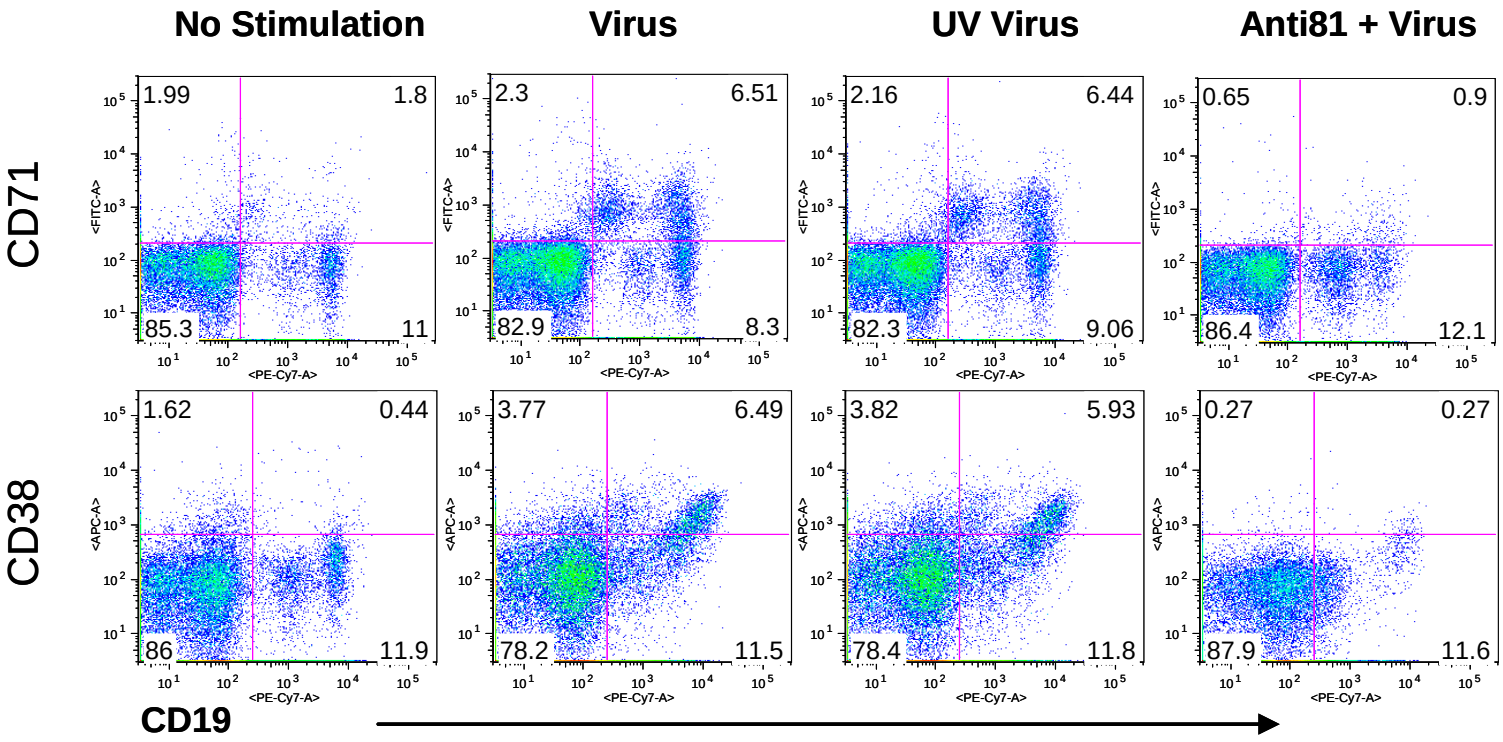
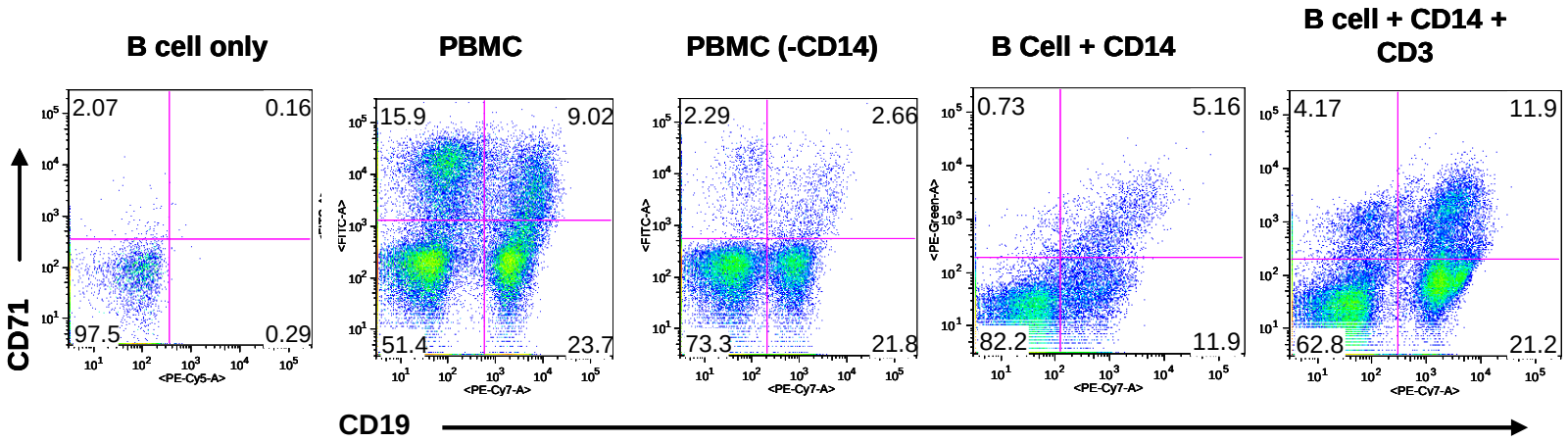

No Stimulation
Virus
UV Virus
Anti81 + Virus
CD71
CD38
CD19
1.99
1.8
85.3
11
2.3
6.51
82.9
8.3
2.16
6.44
82.3
9.06
0.65
0.9
86.4
12.1
1.62
0.44
86
11.9
3.77
6.49
78.2
11.5
3.82
5.93
78.4
11.8
0.27
0.27
87.9
11.6
<FITC-A>
<APC-A>
<PE-Cy7-A>

Figure 12. JFH induced activation of B cells requires CD81 but not viable virions. PBMCs from a healthy donor were incubated with viable or UV inactivated virus at a 0.01 virus-to-cell ratio for 3 hours at 37^0C. In one setup, PBMCs were incubated with anti-CD81 antibody for 1 hour prior to the addition of viable virus. Cells were subsequently washed 2x in PBS and incubated at 37^0C for 7 days. PBMCs were then harvested and analyzed for CD71 and CD38 activation marker expression on B cells, which are represented by CD19 expression.

A.)


B cell only
PBMC
PBMC (-CD14)
B Cell + CD14
B cell + CD14 + CD3
2.07
0.16
97.5
0.29
15.9
9.02
51.4
23.7
2.29
2.66
73.3
21.8
0.73
5.16
82.2
11.9
4.17
11.9
62.8
21.2
CD71
CD19
<PE-Cy5-A>
<FITC-A>
<PE-Cy7-A>
<PE-Green-A>

B.

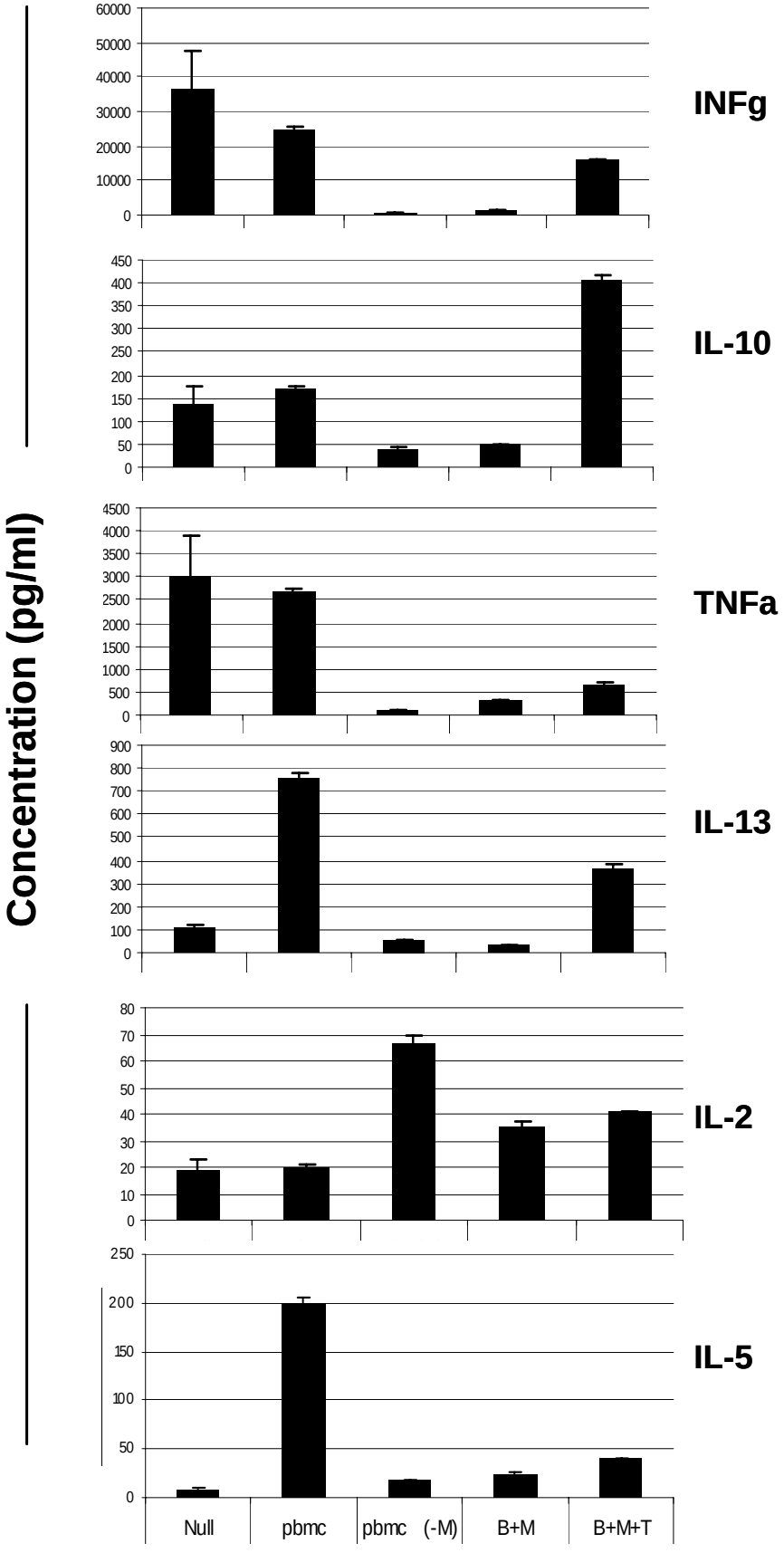

Concentration (pg/ml)
INFg
IL-10
TNFa
IL-13
IL-2
IL-5
Null
pbmc
pbmc (-M)
B+M
B+M+T

Figure 13. JFH induced activation of B cells requires accessory cell factors. A.) B cells were incubated alone, with CD14 microbead isolated monocytes, or with CD14 isolated monocytes and CD3 isolated T cells all with JFH-2a viable virus at a 0.01 virus-to-cell ratio. Also, PBMCs alone or PBMCs depleted of CD14+ monocytes were incubated with JFH-2a viable virus at a 0.01 virus-to-cell ratio. All cell combinations were incubated for 7 days and B cells were subsequently analyzed for CD71 expression. B.) Supernatant from cell combination cultures defined in part A was analyzed for cytokine expression profiles. The following cytokines were analyzed: INFg, IL-10, TNFa, IL-13, IL-2, and IL-5.

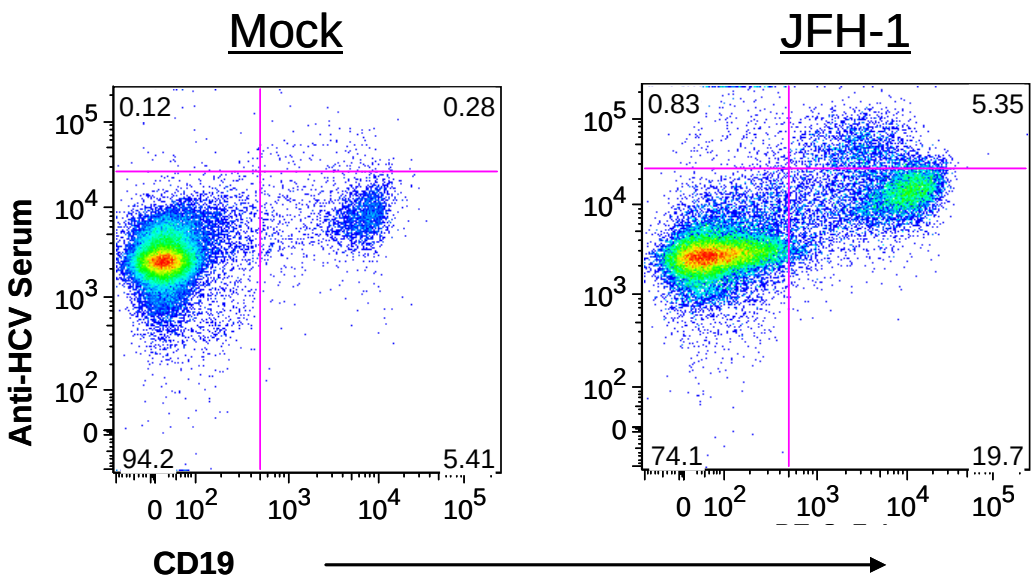

Mock
JFH-1
Anti-HCV Serum
CD19
0.12
0.28
94.2
5.41
0.83
5.35
74.1
19.7
0
10^2
10^3
10^4
10^5

Figure 14. A subset of JFH-1 exposed B cells show signs of viral protein production. Healthy PBMC were exposed to either JFH-1 viral particles (MOI 0.01) or tissue culture media for 3 hours. PBMC were subsequently washed and incubated at $37^0C/5\%CO_2$ for 7 days. At the termination of incubation, cells were washed several times in PBS and stained, intracellulary, for HCV proteins. Graph depicts B cells isolated on the x-axis using anti-CD19 PE Cy7 and HCV protein on the y-axis using an anti-human sera/ secondary anti-IgG Alexa700 complex.

Virus-exposed B cells generate Foxp3 expressing suppressor T cells in MLR cultures

In an effort to corroborate our findings from HCV-infected patients and strengthen our proposed model of T regulatory cell generation, we determined the effect JFH-activated B cells have on the production of Foxp3 expressing, suppressor T cells. We exposed PBMC to viable virus, UV-inactivated virus, or tissue culture media for 7 days. A portion of PBMC were also pre-incubated with anti-CD81 antibody prior to exposure to viable virus. B cells were isolated from the 7 day experiment and used as APC in a mixed lymphocyte reaction against CD4+25+Foxp3+ T cells that were labeled with CFSE. We found that, similar to HCV RNA (+) B cells from patients, B cells from JFH-exposed cultures induced a greater percentage of activated, Foxp3-expressing T cells, when compared to baseline conditions (Figure 15). Interestingly, the magnitude of proliferation of T cells from UV virus-exposed B cells was comparable to untreated cells, yet B cells from these conditions were equally activated in our previous analysis. This suggests that live virus was required to replicate the full effects found in ex-vivo derived HCV B cells that harbored HCV positive strand RNA.

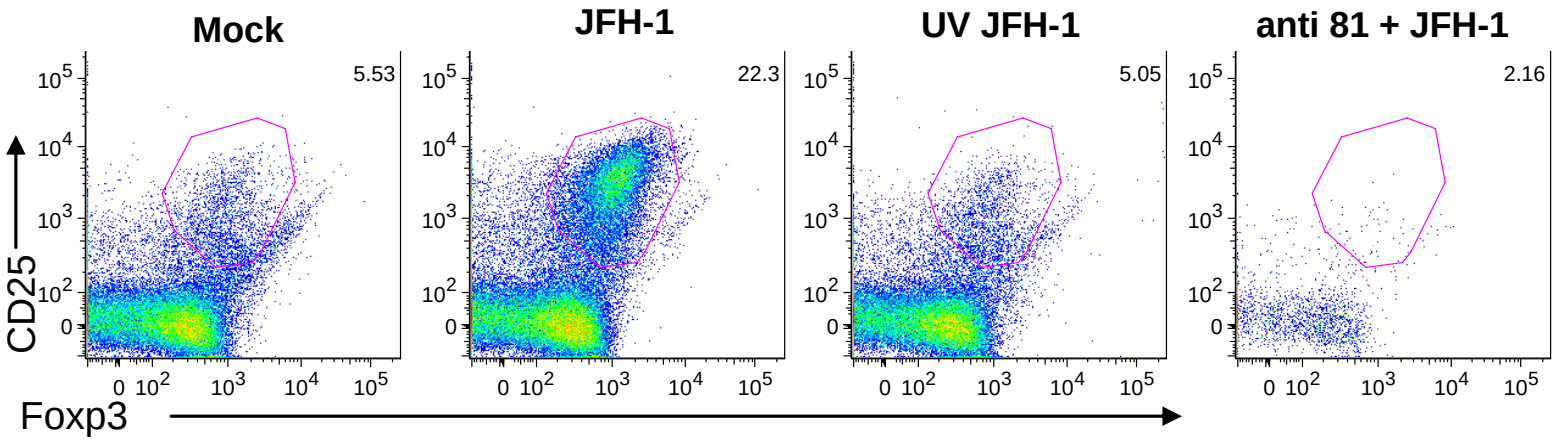


Figure 15. JFH induced B cell activation of allogeneic CD4+25+Foxp3+ T cells relies on virus viability. PBMCs from a healthy donor were incubated with viable or UV inactivated virus at a 0.01 virus-to-cell ratio for 3 hours at 37^0C. In one setup, PBMCs were incubated with anti-CD81 antibody for 1 hour prior to the addition of viable virus. Cells were subsequently washed 2x in PBS and incubated at 37^0C up to 7 days. B cells were subsequently isolated using CD19 microbeads and used as APCs in an allogeneic reaction against CFSE labeled $CD4^+25^-$ T cells. After 7 days of incubation at 37^0C, cultures were harvested and stained for extracellular CD25 and intracellular Foxp3 expression.

CHAPTER FOUR
Conclusions and Recommendations

The immune system as an extra-hepatic site of HCV replication

The possibility of extra-hepatic HCV replication is a topic widely disputed. One of the most studied locations is cells of the immune system. Extra-hepatic HCV tropism has been implicated in bilary, salivary, neuronal and lymphatic tissue. Mainly due to its ease of cultivation, cells of the peripheral immune system have been studied the most in this context. There were many examples of immunotropism for specific HCV RNA sequences, but these early studies lacked the ability to discern between passive viral absorption and true infection (Zignego, Macchia et al. 1992; Muller, Pfaff et al. 1993; Willems, Moshage et al. 1993). However, the possibility of direct infection garnered support with the finding of HCV RNA species in PBMC populations in the absence of serum HCV RNA (Schmidt, Wu et al. 1997). Also, evidence supporting infection of immune cells comes from the finding that in-vitro mitogenic activation of HCV seropositive patient lymphocytes increased the incidence of HCV RNA detection in these immune cells. This suggests that HCV may replicate more efficiently in pre-activated immune cells. Our results provide evidence to the contrary in that ex-vivo analysis shows lack of HCV RNA in phenotypically activated B cells, while patients with detectable HCV RNA showed B cell profiles similar to the naïve B cell state seen in healthy subjects (Figure 8). It is possible that HCV infection of B cells precludes activation, thereby preventing the full differentiating potential of naïve B cells. Other lines

of evidence lend further support to the lymphotropic strategy of HCV. Experiments evaluating HCV quasispecies sequences show that serum-associated sequences are a product of extra-hepatic origin, suggesting lympho-specific contribution (Pal, Sullivan et al. 2006). Major, indirect, evidence of lymphotropism is supported by the viral receptor usage for cell infection. Both CD81 and LDL have been established as co-receptors necessary for viral uptake into viable cells (Pileri, Uematsu et al. 1998; Agnello, Abel et al. 1999). In fact, our data shows that CD81 is necessary to establish the activation phenotype seen in B cells as a result of exposure to JFH-1 virus. Therefore, there seems to be mounting evidence of direct immunotropic viral associations.

The validity of viral lymphotropism is argued against by the lack of evidence showing productive replication. Many early studies into immune cell reservoirs lacked evidence of HCV negative intermediate strand detection in any cell type outside of hepatocytes (Lanford, Chavez et al. 1995; Boisvert, He et al. 2001). Even in those few studies where negative strand RNA was found, many still argue that excessive genomic strand HCV RNA could act as a template for 'false' strand detection (McGuinness, Bishop et al. 1994). Strand-specificity of assay procedures was a major caveat of strand-specific RNA detection. Landford et al. adopted a strand-specific assay which could amplify positive or negative strand RNA with high fidelity based on the unique polymerase and reverse-transcriptase function of the thermostable rTth enzyme (Lanford, Sureau et al. 1994). The advent of this thermostable PCR increased the range HCV RNA discrimination. Therefore, we used a modified version of this strand-specific method and identified HCV positive strand RNA in B cell populations of

chronically infected patients (table 2). However, we could not consistently detect HCV negative intermediate strand RNA. The detection limits of our assay were capable of uniquely amplifying at least 100 copies of negative strand RNA (methods and materials), suggesting that if HCV is replicating, it occurs at levels below our detection criteria. In support of this, there is evidence suggesting that negative strand detection is generally only found in a minority of cells predicted to be infected by HCV(Agnello, Abel et al. 1998). Combine this argument with reports that large amounts of progeny genomic RNA is amplified from a single intermediate RNA species, and HCV RNA is found at low levels in PBMC populations, it is easy to explain that negative strand detection in PBMC relies on very sensitive methods that may be beyond current experimental capabilities.

We show that HCV has a unique affinity for B cells over other peripheral blood populations. This was rewarding because there has been previous evidence supporting B cell tropism using different methods (Muller, Pfaff et al. 1993; Zignego, Giannelli et al. 2000). What is interesting about this distinct niche is the rarity of the B cell in PBMCs (<10%). A surface level analysis that compares B cells, T cells, and DCs as a potential viral reservoir does not immediately suggest any significant advantages. In fact, when taking into account that CD81, a known viral co-receptor, is expressed on most leukocytes, it should be highly unlikely that virus only be found associated with one cell type. So even as CD81 has been shown to be important for viral attachment and entry, there are apparently other cellular factors that mediate successful viral uptake. Although the finding of virus association with B cells is not novel, it is still relatively unknown what determines this affinity. The possibility of passive viral sequestration relies on the idea that

the immune cells act as carriers of virus and do not necessarily act as reservoirs. We accounted for this by trypsinizing all cells prior to PCR analysis to remove cell-surface associated virus. We cannot conclude on the replicative competence of B cells in chronic HCV infection, but we do provide evidence of a propensity of HCV to associate with B cells over all other PBMC cell types.

B cell dysfunction as a consequence of direct viral association

The mounting evidence of immunotropism begs the question of direct viral mediation of immune cell function. The preferential infection (association) of HCV with B cells suggests a pathogenic implication of their interaction. In fact, there is a large body of evidence showing significant correlations of chronic HCV infection and B lymphoproliferative disorders (LPD). The contribution of B cells in the resolution of acute HCV infection is debatable. There is no significant correlation of antibody levels to HCV antigens and the resolution of infection (Bowen and Walker 2005). Never the less, a multitude of extra-hepatic disease is related to abnormal B cell function. The characteristics of B cell associated sequelae are suggestive of polyclonal activation. For example, mixed cryoglobulenemia (MC) typically presents with immune complexes of monoclonal IgM and polyclonal IgG containing HCV RNA (Mayo 2003). The mechanism of proposed polyclonal B cell activation is still unclear, but may involve CD81 crosslinking. CD81, a ubiquitously expressed receptor that is

known to bind HCV glycoprotein E2, is associated with CD19 and CD21 on B cells (Levy, Todd et al. 1998). Engagement or crosslinking of this complex results in several B cell activation events, including enhanced TNF alpha production, cellular proliferation and Ig gene hyper-mutation (Altomonte, Montagner et al. 1996; Machida, Cheng et al. 2005). We speculated that B cell hyper-activation of T cell proliferation associates with HCV RNA detection may be facilitated by similar events. We evaluated this hypothesis by incubating anti-CD81 antibodies with naive B cells and could not find a distinguishable B cell activation phenotype when assessed by CD25 and CD69 activation marker expression. It is possible that complete crosslinking was not achieved, as previous authors show that CD81 crosslinking on B cells requires at least two antibody species with affinity to distinct CD81 epitopes (Rosa, Saletti et al. 2005). Another proposed mechanism of B cell hyper-activation involves chronic antigenic stimulation (Suarez, Lortholary et al. 2006). A result of chronic antigen stimulation of naïve B cells results in an attenuated B cell response phenotype (Montes, Acosta-Rodriguez et al. 2007). This immune mechanism is necessary to control the inadvertent activation of B cells that are specific to host antigens, as demonstrated in lupus and multiple sclerosis. A major phenotype of chronically activated or exhausted B cells is a reduced capacity to proliferate in response to exogenous stimulation. We found a low level of chronic HCV B cell proliferation in response to B cell receptor crosslinking, suggesting a vast population of

exhausted B cells. We also could not rescue these B cells from eventual apoptosis with the addition of IL-4 and IL-2, which suggests that the fate of chronically activated B cells in HCV could not be immediately rescued. What is still undetermined is the possible reversion of function based on successful antiviral therapy or spontaneous resolution of virus. Regardless of the extent of B cell dysfunction during infection, it would be useful to determine if normal B cell function can be restored after successful therapeutic intervention. In this scenario, it would also be interesting to determine if viral RNA is maintained in B cells, serving as possible reservoirs of re-infection.

Although antibody production does not correlate with HCV resolution, the improper activation of B cells may modulate T cell responses to infection. In support this, it has been shown that B cell deficient mice exhibit reduced functional T cell responses to both viral and bacterial burdens (Yang and Brunham 1998). Also, there is a significant increase in the regulatory T cell population of B cell non-Hodgkin's lymphomas that appear to correlate with levels of intra-tumoral activated CD70+ B cells (Yang, Novak et al. 2006). From these findings, it is reasonable to speculate that HCV mediated B cell dysfunction may contribute to the resultant T cell abnormalities. It is also possible that chronic B cell activation is a direct result of contact with and low level protein production of internalized HCV virions. Future studies into the nature of B cell-

HCV interactions may lead to a better understanding of direct viral mediation of immune responses as a result of unique immune cell tropism.

Until recently, there has not been an in vitro model of HCV replication capable of propagating full length HCV. Production of infectious HCV was achieved with the discovery and cell culture propagation of JFH-1 (Lindenbach, Evans et al. 2005; Wakita, Pietschmann et al. 2005; Zhong, Gastaminza et al. 2005). Replicon systems utilizing full length sequences from JFH-1did not require tissue culture adaptations. This suggested that the viral clone where the sequence derived had inherent adaptive mutations allowing for efficient infectivity both in vivo and in vitro (Wakita, Pietschmann et al. 2005). Conclusive evidence for HCV immunotropism lacked in-vitro support due to the relative inefficient replication and mutational augmentation of prior HCV tissue culture studies. The discovery of JFH-1 prompted our interest in its capacity to infect PBMC in vitro. From 6 separate attempts, we were unable to detect positive or negative strand HCV RNA in JFH exposed PBMCs. In all of our infectivity assays, we used a viral MOI of 0.01 or lower. Based on our cell numbers, this resulted in approximately 2×10^4 virions at the start of each infection assay. Our real-time strand specific PCR assay had the capacity to detect down to 250 copies of positive strand HCV RNA. In order to detect HCV RNA, approximately 1%, or 200 copies of starting virus had to infect the PBMC. If the results from our initial screening was any indication of viral tropism, it

would stand that B cells would be the main source of in-vitro infected PBMCs. B cells constitute, on average, about 10% of the PBMC population, providing only about 2 x 10^4 cells hypothetically capable of harboring virus. Therefore, JFH-1 had a 10% success rate at infecting PMBC in-vitro. This explanation is only speculative, but does provide the framework for experimental conditions that will maximize the potential to examine JFH-1 immunotropism. What we discovered was a surprisingly robust B cell activation that occurred as early as 4 days post exposure to JFH-1. Interestingly, this B cell phenotype did not require viable, replication competent virus. Yet, availability of CD81 suggests that B cells required direct contact with virus. As this is a novel in-vitro finding, it does correlate with current experimental evidence of B cell phenotype as a result of CD81 crosslinking. A recent study evaluating CD81 crosslinking on naïve B cells by recombinant E2 protein showed robust B cell activation complete with CD25 and CD69 expression and B cell proliferation (Rosa, Saletti et al. 2005). This suggests an intimate association with HCV induced B cell activation and availability of CD81. Also, the down-stream consequence of CD81 crosslinking on B cells was polyclonal proliferation and hypermutation of the Ig gene (Machida, Cheng et al. 2005; Rosa, Saletti et al. 2005). We did not evaluate B cell proliferation as a result of exposure to JFH-1 virus, but we did find CD25 and CD69 activation marker expression. We also found that the activation of B cells required secreted factors (Figure 15), that correlated with the presence of both

monocytes and T cells. Previous experimental evidence shows that polyclonal B cell activation requires the presence of Monocytes and monocyte secreted factors (Petersen, Kieffer et al. 1982). Future studies into the consequential association between B cells and HCV will benefit from the results obtained from our in-vitro JFH-1 studies.

T Regulatory Cells in the modulation of Chronic HCV Infection

A major pathological result of chronic infection is the continued damage of host tissue. The ability to curtail the magnitude of an immune response is a mechanism designed to prevent overt host damage or autoimmune diseases as a result of chronic immune stimulation. The immune system has regulatory mechanisms in place to prevent the onset of these pathological endpoints. The definition of these 'regulatory' immune cells is a topic of increased interest. The initial focus of T regulatory cells was on their role in preventing tissue damage from autoreactive T cells (Suri-Payer, Amar et al. 1998). Evidence is mounting regarding their role in the modulation of chronic viral infections. For instance, Suvas et al found that depletion of 25+ Treg cells increases the magnitude of antigen specific CD8+ T cell responses during a mouse model of HSV-1 infection (Suvas, Kumaraguru et al. 2003). The phenotype of antigen specific T cells in chronic HCV infection has garnered interest in the role of Treg cells as a possible mechanism of viral persistence. In fact, there are several reports finding an

increase in the overall CD4+25+ phenotypical regulatory T cell population in chronic HCV infection (Sugimoto, Ikeda et al. 2003; Cabrera, Tu et al. 2004; Boettler, Spangenberg et al. 2005; Rushbrook, Ward et al. 2005). What is still undetermined is the source of Treg cells in the context of infection, as CD4+25+ T cells are either naturally occurring or generated in response to infection. Interestingly, these Treg cells do not discriminate on their mechanisms of suppression as isolated regulatory cells from HCV infected patients suppressed EBV and CMV T cell responses in vitro (Rushbrook, Ward et al. 2005). Regardless of the source and function of T regulatory cells, it would be relevant to determine if chronic infection leads to the overproduction of suppressive T cells, thereby preventing the necessary activation threshold for clearing virus. Indeed, it has been shown that peripherally derived CD4+25- T cells can be activated to perform transient regulatory T cell functions (Pillai, Ortega et al. 2007). Interestingly, Treg production occurred regardless of the nature of activation (anti-CD3, PHA, or allogeneic). Since there is very little data regarding the source of the increased Treg population in chronic infection, the observation of transient Treg production lead us to speculate on their potential generating sources. We showed that ex-vivo derived B cells that harbored HCV positive strand RNA generated a higher production of CD4+CD25+Foxp3+ T cells. Although we did not show conclusive evidence on the suppressive capacity of these B cell generated Foxp3+ T cells, one can reason based on previous evidence that they

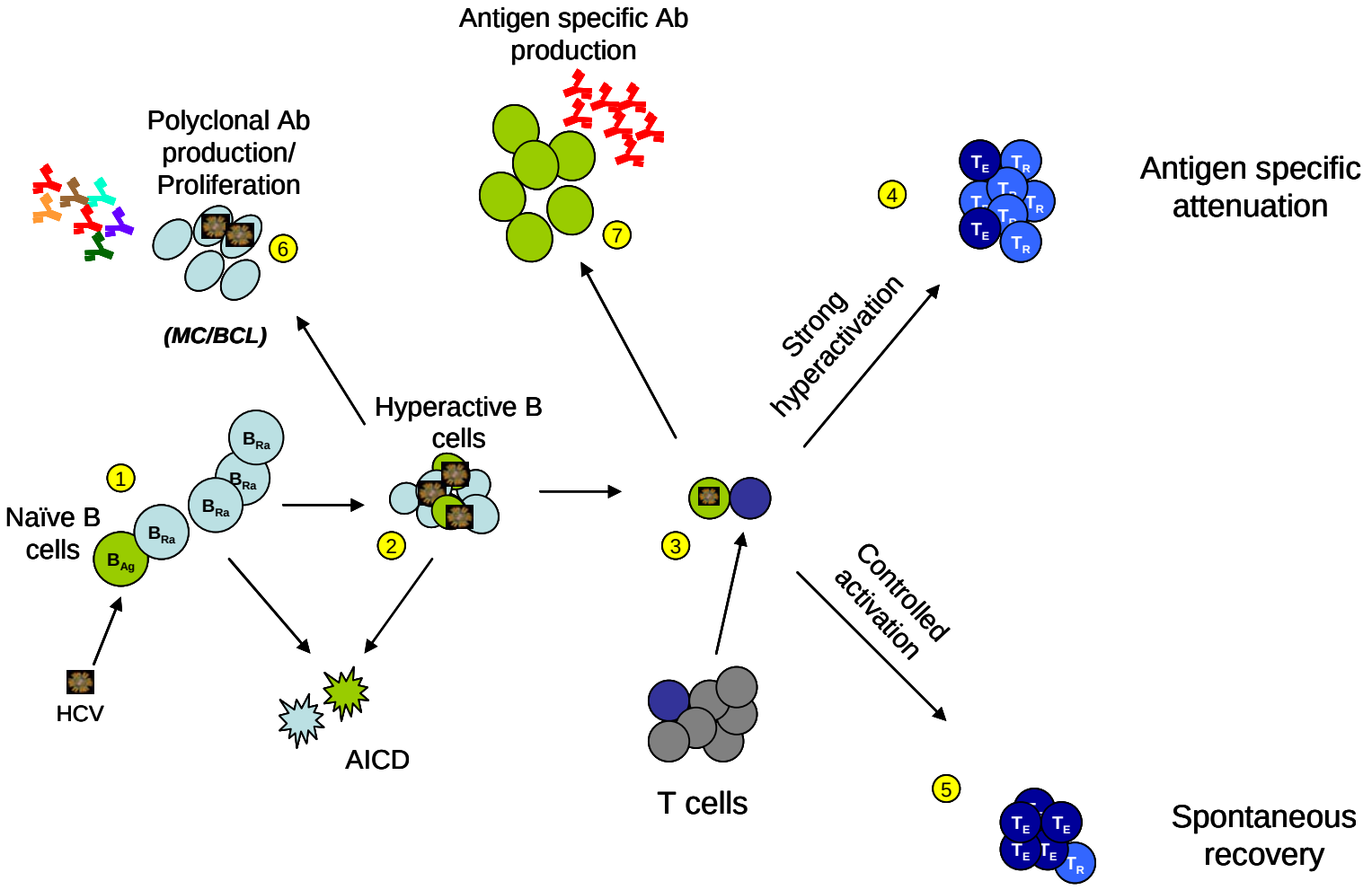
maintain suppressive capacitance. Also, we showed that JFH-1 activated B cells have a greater capacity to induce MLR generated CD4+25+Foxp3+ T cells, corroborating with our ex-vivo derived B cell data. Interestingly, this suppressor T cell generation required live virus, as B cells exposed to UV-inactivated virus showed similar CD4+25+Foxp3+ T cell generating capabilities as unaltered B cells. Our results suggests that B cell induction of suppressor T cells in this assay requires a distinct mechanism exclusive of B cell virus induced B cell activation.

Based on our findings, we surmise that B cells may serve not only as extra-hepatic HCV reservoirs, but may consequently become 'hyperactivated' contributors to antigen specific transient regulatory T cell generation (summarized in model A). The results of this project provide evidence to warrant a deeper analysis of the immunological and pathological consequences of commonly found immune cell tropisms resulting in paradoxical innate hyper-activation and adaptive overt suppression. These results provide fodder for future clinical contentions on therapies designed to tip the balance in favor of viral clearance, thereby restoring natural mechanisms of antiviral immunity.

Figure 16. Model of hyperactive B cell induction of T regulatory cells during HCV infection

[Question for potential future studies are posed in parentheses]

1. During infection, naïve B cells encounter HCV virus. CD81 interactions are critical in viral uptake. HCV-specific B cells (B_{Ag}) may have enhanced possibility of viral uptake through B-cell receptor (compared to random B cells (B_{Ra}). B cells associated with virus attain a hyperactive phenotype. *[Question: What are the molecular interactions leading to B cell activation following viral association?]*
2. The 'hyperactive' B cells may have various fates. Productive internalization of virus combined with the proper autologous cell factors create conditions of unrestricted lymphoproliferation and/or differentiation. B cells may undergo activation-induced cell death (AICD) in the absence of autologous cell factors (or availability of other signals). *[Question: Can these stages be tracked after on vitro JFH infection? Can they be modulated by extrinsic factors (Cellular contact/cytokines)?]*
3. B cells that produce viral protein may be capable of presenting HCV antigen to HCV-specific T cells (depicted in blue). Presumably, these T cells have been already primed during the infection or, potentially, are naïve T cells. Again, HCV-specific B cells may have a higher rate of interaction with HCV-specific T cells, allowing for predominantly HCV-specific immune perturbation.
4. Hyperactive B cells induce unduly sustained T cell activation, possibly skewing the T cells into a sustained T regulatory (T_R) state. This leads to suppression of T cell responses (predominantly suppressing HCV-specific T cells), contributing to chronicity of infection. *[Question: Patients with HCV RNA(-) B cells also show increased numbers of T_R ex vivo. Is B cell interaction an early event? Are T_R sustained despite lack of continued B cell-HCV association?]*
5. In a different setting, appropriate T cell activation would promote effector T cell (T_E) function, leading to viral clearance. *[Question: Do patients who clear virus not have productive B cell-HCV interaction?]*
6. Random antigen B cells (B_{Ra}) that have been 'hyper-activated' by HCV contact give rise to spontaneous antibody production. Internalized HCV protein products counteract antigen induced cell death pathways, which results continuous lymphoproliferation and eventual oncogenesis. *[Question: Is there evidence of consistent HCV-B cell association in patients with mixed cryoglobulinemia (MC)/B cell lymphoma (BCL)?]*
7. HCV antigen-specific B cells (B_{Ag}) are still capable of antibody production, although the presence of these antibodies does not correlate with the outcome of infection. Antibodies may enhance greater uptake of virus by other B cells/APC.


Antigen specific Ab production
Polyclonal Ab production/ Proliferation
(MC/BCL)
Hyperactive B cells
Naïve B cells
HCV
AICD
T cells
Strong hyperactivation
Controlled activation
Antigen specific attenuation
Spontaneous recovery
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