

Generalized impairment of CD8⁺ T-cells in HCV mono- and HIV-HCV co-infection

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Abstract

Chronic hepatitis C virus (HCV) infection has global effects on the immune system. CD8⁺ T-cells, responsible for viral clearance and control, are dysfunctional for as yet unknown reasons. It is hypothesized that IL-7 signaling pathway deficiencies contribute to this impairment. Blood-derived CD8⁺ T-cells in chronic HCV mono- and HIV-HCV co-infection had lower IL-7-induced activation of STAT5 and production of Bcl-2, and lower proliferation in co-infection, compared to controls. Lower Bcl-2 production was also associated with increased fibrosis. These changes were independent of the IL-7 receptor α expression and suppressor of cytokine signaling 1 or 3 expression. Intrahepatic CD8⁺ T-cells in HCV-infection did not activate STAT5 above basal levels with cytokine stimulation and had lower Bcl-2 expression than blood-derived cells. In conclusion, bulk CD8⁺ T-cells were impaired in response to IL-7 and the IL-7 signaling pathway may be one mechanism by which CD8⁺ T-cells are impaired in chronic HCV infection.

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List of Abbreviations

AIDS – acquired immune deficiency syndrome
ALT – alanine transaminase
APC – antigen presenting cell
CD127 – IL-7 receptor α
CD132 – IL-2 receptor γ chain (γ_c)
CFSE – carboxyfluoresceinsuccinimidyl ester
CMV - cytomegalovirus
CTL – cytotoxic T lymphocyte (T-cell)
DAA – direct acting antiviral
EBV – Epstein-Barr virus
HAART – highly active anti-retroviral therapy
HBV – hepatitis B virus
HCC – hepatocellular carcinoma
HCV – hepatitis C virus
HIV – human immunodeficiency virus
IFN – interferon
IH – intrahepatic
IL – interleukin
LCMV - lymphocytic choriomeningitis virus
LSEC – liver sinusoidal endothelial cell
MFI – mean fluorescence intensity
NK – natural killer
PBMC – peripheral blood mononuclear cell
PHA – phytohaemagglutinin
SOCS – suppressor of cytokine signaling
SVR – sustained virological response
TCR – T-cell receptor
 T_{CM} – central memory T-cell
 T_E – effector T-cell
 T_{EM} – effector memory T-cell
 T_{EMRA} – terminally differentiated T-cell
 T_N – naïve T-cell

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Chapter 1: Introduction

1.1. Hepatitis C virus infection

The World Health Organization estimates that approximately 2-3% of the world's population is hepatitis C virus (HCV) positive (Public Health Agency of Canada, 2010). HCV is a blood borne virus, with a frequently asymptomatic acute infection. Depending on the genotype of HCV, the immune system is unable to clear the virus in the acute phase and 50-80% of infected individuals develop a persistent, chronic infection (Kaplan et al., 2007). Persistent infection leads to chronic hepatitis, liver fibrosis and can result in cirrhosis and an increased risk of developing hepatocellular carcinoma (HCC) (Rehermann, 2007). It can take 20-30 years after infection for cirrhosis to develop in up to 50% of patients, but once that occurs, up to 6% of individuals per year will develop liver failure and up to 5% will develop HCC (Maasoumy and Wedemeyer, 2012; Wong and Lee, 2006). Chronic HCV infection is a leading cause of infection-mediated liver disease, and in Canada, responsible for the most years of lost life of any viral infection (Sherman et al., 2013). Historically, treatment for HCV included pegylated IFN- α and ribavirin, with poor success rates. Recent developments in direct acting antivirals (DAA) have led to very effective inhibitors specific for HCV nonstructural proteins with very high (over 95%) sustained virological response (SVR) rates (Bansal et al., 2015). Unfortunately, it is estimated that many individuals with HCV infection are unaware of their status or live in countries or socio-economic situations where treatment with DAA is unavailable. Therefore, the continued study of the effects of HCV on the immune system, and vice versa, is still important.

1.2. The virology of hepatitis C virus

HCV is a small, positive sense, single stranded, enveloped RNA virus that belongs to the flaviviridae family. There are 6 major genotypes of HCV, with types 1, 2, and 3 most common in North America (Wong and Lee, 2006). HCV contains structural (core, envelope 1 and envelope 2) and nonstructural (P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. Entry of HCV into hepatocytes and the HCV life cycle are tightly linked to host lipid metabolism. HCV is initially captured by glycosaminoglycans and/or lipoprotein receptors, and HCV E2 interacts with high-density lipoprotein receptor scavenger receptor class B type I, CD81, and the tight junction proteins claudin-1 and occludin. The HCV virion enters the cell through clathrin-dependent endocytosis and is uncoated in the cytoplasm of the cell. Its positive sense RNA genome is directly translated into a long polyprotein that is cleaved by viral and host proteases into multiple HCV proteins (Douam et al., 2015). The virus assembles a replication complex in the cytoplasm, consisting of nonstructural viral proteins (NS5B - RNA-dependent polymerase, NS3 – NTPase/helicase, NS4B – initiates formation of complex, NS5A – regulatory protein) and host proteins to replicate its genome (Li and Lo, 2015). Assembly of the virion occurs on the membrane of the endoplasmic reticulum and is tightly linked to the lipoprotein pathway of the host cell; mature virions form lipoviroparticles and are secreted from the cell along with the secretion of lipids (Jones and McLauchlan, 2010).

HCV is transmitted through the transfer of bodily fluids, contact of contaminated blood with mucus membranes, and breaks in the skin. It is a non-cytopathic virus, whose tissue damage is caused by alterations of the immune system and indirect bystander damage caused by inflammatory mediators released by surrounding cells. As stated, the primary site

of HCV infection is liver hepatocytes. In addition to infection of hepatocytes, HCV is known to infect B cells through the same CD81 binding, leading to cryoglobulemia, a condition highly associated with active HCV infection. HCV RNA has also been found in peripheral blood mononuclear cells (PBMC), though viral replication is almost undetectable in the presence of CD8⁺ T-cells (Li et al., 2005).

There are multiple aspects of the HCV life cycle that are unknown and that complicate its further study; it has a high mutation rate as an RNA virus, it does not grow well *in vitro*, and it only infects humans and chimpanzees (the latter resolves infection spontaneously) (Valiante et al., 2000). Other animal models are limited to immunodeficient mice transfected with human HCV entry genes, transplanted with human chimeric livers, and recent yet limited utility of humanized mice (Akkina, 2013; Dorner et al., 2011; Ploss and Rice, 2009). Advances in cell culture techniques are allowing for better study of HCV virus life cycle (Sarhan et al., 2012).

1.3. Immune response to HCV

1.3.1. Innate immune response

Upon HCV infection, clusters or foci of infected hepatocytes form. The internal ribosome entry site of HCV is recognized by cellular protein kinase R (PKR) and HCV RNA is recognized by RIG-1, leading to the synthesis of interferon (IFN)- β . Initiation of the IFN signaling leads to transcription and translation of interferon stimulated genes (ISGs) in infected and neighboring cells and host-antiviral mechanisms. These include activation of IFN-regulatory factor 3 (IRF-3), increases in antigen presentation and chemokine production by innate cells, effector T-cell responses such as production of inflammatory cytokines like TNF- α , and induction of B cells to produce antibody (Ivashkiv and Donlin, 2014). However,

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HCV can evade some of these mechanisms by its serine protease (NS3-4A), which cleaves components of the pathway. Surrounding cells, like liver resident macrophage Kupffer cells, also express IFN- β and interleukin (IL)-1 β (Park and Rehermann, 2014). Chemokines produced by Kupffer cells and liver sinusoidal endothelial cells (LSEC) recruit liver resident natural killer (NK) cells to the site of infection. NK cells are activated by cytokines produced during the infection, including type 1 IFNs, reductions in MHC expression and expression of activating receptors, and kill infected cells by degranulation and TNF-related apoptosis-induced ligand (TRAIL) (Rehermann, 2013).

1.3.2. Adaptive immune response

Approximately 8-12 weeks following primary infection, an adaptive immune response to HCV is detectable. B cells undergo differentiation and secrete anti-HCV neutralizing antibodies. Neutralizing antibodies for HCV envelope proteins can prevent entry of the virion as these proteins are required for binding and entry to the cell (Wang et al., 2011). Unfortunately, this response can be delayed (Umemura et al., 2006). As B cells express CD81, they are susceptible to HCV infection, which can cause a mutagenic effect on the B cells that can lead to cryoglobulemia and other B cell dysregulation disorders (Ito et al., 2011). Also, the high mutation rate of HCV can render antibodies useless.

CD8⁺ T-cell depletion studies have shown that CD8⁺ T-cells are necessary for controlling viral replication and viral clearance. Specifically, *in vitro* experiments with human PBMC and *in vivo* studies in non-human primates have shown that once CD8⁺ T-cells are removed, HCV RNA levels increase and HCV infection is prolonged, respectively (Li et al., 2005; Shoukry et al., 2003). Also important to the clearance of HCV infection is a CD4⁺ T cell response specific for numerous HCV epitopes, and their support of CD8⁺ T

cells (Park and Rehermann, 2014). The reason for CD8⁺ T-cell failure to clear HCV infection and lack of broadly specific CD4⁺ T-cells is still unknown but may be due to cell intrinsic or virus-mediated mechanisms.

1.4. HIV-HCV co-infection

Human immunodeficiency virus (HIV) and HCV share routes of transmission in humans, and approximately 25% of individuals infected with HIV also are infected with HCV (CDC, 2013). HIV infects CD4⁺ T-cells and macrophages, leading to chronic infection, CD4⁺ T-cell death, and without treatment, acquired immune deficiency syndrome (AIDS). HCV is the most common co-morbidity of HIV infection, and with the increased success of highly active antiretroviral treatment (HAART), the life expectancy of those living with HIV has increased and therefore the number of individuals who progress to liver disease has also increased (Moodie et al., 2009). The risk of developing liver failure is 6 times higher in HIV-HCV co-infection compared to HCV mono-infection due to the liver damaging effects of some HAART drugs such as protease inhibitors (Kared et al., 2014; Sulkowski and Thomas, 2003) and liver failure is one of the main causes of death aside from AIDS in co-infected individuals (Thorpe et al., 2011).

Infection with HIV reduces immune responses to secondary infections. Individuals who are HIV⁺ are less likely to spontaneously resolve HCV infection (5-10% compared to 20-50% in HCV mono-infection) and are more likely to have liver disease and develop cirrhosis (Pol et al., 1998; Sulkowski and Thomas, 2003). While HCV is commonly thought to accelerate HIV progression, less is known about the effect of HIV on HCV infection. HIV infection is a risk factor for recurrence of HCV viremia (i.e. viral relapse) after previous acute infection and spontaneous control, either due to the loss of replication control below

RNA detection levels or higher rates of re-infection (Kim et al., 2006). Another factor to take into account when discussing co-infection is the order in which infection occurred, and time between acquisitions of infection; chronic HIV infection before acquiring HCV, and vice versa. Unfortunately, the time at which an HIV or HCV infection is contracted is not always known by the patient or their caregivers. Both viruses have similar routes of transmission and infected individuals can be asymptomatic for many years. Many HIV/HCV-infected individuals may partake in high risk behavior such as frequent intravenous drug usage and/or have multiple unprotected sexual contacts, hence have no identifiable timepoint of virus transmission.

1.5. The function of CD8⁺ T-cells

CD8⁺ T-cells are a subset of lymphocytes with cytotoxic functions that express T-cell receptors (TCR) which recognize peptides presented by MHC class I molecules present on all nucleated cells. Their cytolytic (CTL) activity is important in anti-viral, anti-tumour, and certain anti-bacterial responses. During an infection, CD8⁺ T-cells are activated by binding of their TCR to peptide-MHC I complexes on antigen presenting cells (APC), and are co-stimulated with B7.1, B7.2 (aka CD80/86) receptors and the cytokine micro-environment. Activating cytokines derived from such APC initially include, but are not limited to, type 1 IFNs (α and β), IL-2, IL-12, and IL-6. Upon activation, CD8⁺ naïve T (T_N) cells undergo clonal expansion and differentiation into short-lived effector cells (T_E). T_E cells will migrate to the site of infection to specifically target affected cells. CD8⁺ T-cells release perforin which forms pores in target cells to allow entry of other cytotoxic particles such as granzymes to disrupt the mitochondria and fragment DNA, and granulysin which has antimicrobial properties. Expression of Fas ligand can also trigger apoptosis of target

cells. In addition, CD8⁺ T-cells produce IFN- γ (blocks viral replication and enhances host anti-viral function), LT- α (kills tumour cells), TNF- α (activates microvascular endothelium) to help clear infection, and IL-2 to augment their other functions. T_E cells then undergo massive contraction whereby 95% of cells die by apoptosis. The remaining T-cells become memory precursors and then central (T_{CM}) and effector memory (T_{EM}) cells, which can quickly expand into T_E cells upon re-stimulation with the same antigen (Cox et al., 2013). Another subset of CD8⁺ T-cells known as terminally differentiated effector memory (T_{EMRA}) cells proliferate less than other memory cells, produce high concentrations of IFN- γ and have increased susceptibility to apoptosis. CD8⁺ T-cells express different surface proteins and exhibit different gene expression patterns and functions at each stage of development. Cell markers to distinguish between stages of human CD8⁺ T-cell differentiation have classically included CD45RA (long isoform of tyrosine phosphatase) and CCR7 (chemokine receptor that allows for homing to the lymph nodes), but also include many others (Sallusto et al., 1999).

1.6. CD8⁺ T-cells and their response to IL-7

Generation and maintenance of CD8⁺ T-cells are centrally controlled by the common γ chain cytokines interleukin-2 (IL-2), IL-7, and IL-15. The receptor complexes for these cytokines all contain the IL-2 receptor γ chain (γ_c , CD132), along with one or more other components specific for each cytokine. Of particular interest is the pleotropic nature of IL-7; IL-7 signaling is critical in early T-cell development, is important in the generation of memory cells, and promotes T-cell survival (Hofmeister et al., 1999; Schluns and Lefrancois, 2003). IL-7 is produced by many tissue types, including bone marrow stromal cells, intestinal epithelium, follicular dendritic cells, thymic cortical epithelial cells, and

hepatocytes (Sawa et al., 2009). The receptor complex for IL-7 is a heterodimer of CD132 and IL-7 receptor α (CD127). Signaling through IL-7 and CD127 triggers production of anti-apoptotic molecules such as Bcl-2, increased CD8⁺ T-cell proliferation, and augments CTL function (Crawley et al., 2014; Sin et al., 2000; Smyth et al., 1991). Along with the constitutively expressed γ_c , CD127 is highly expressed on T_N and T_{CM} cells, and its expression is variable during an immune response, perhaps as a means to regulate responses to a limited amount of IL-7 (Hofmeister et al., 1999; Mazzucchelli and Durum, 2007). Regulation of CD127 expression is explained in part by multiple mechanisms such as proteosomal degradation, decreased CD127 mRNA transcription, and release of soluble CD127 (Faller et al., 2010; Ghazawi et al., 2013; Vranjkovic et al., 2007).

After binding of IL-7 to CD127 in complex with CD132, trans-phosphorylation of receptor associated JAK kinases JAK1 and JAK3 occurs. These in turn phosphorylate tyrosine residues on the receptors. Receptor phosphotyrosines act as docking sites for the SH2 domains of STAT5, which are phosphorylated by the JAK kinases. The phosphorylated STAT5 (pSTAT5) proteins dimerize and translocate into the nucleus where they act as transcription factors to initiate gene expression for T-cell differentiation, homeostasis (proliferation and glucose metabolism), survival (anti-apoptotic Bcl-2), as well as regulation of signaling (suppressors of cytokines (SOCS)) and enhancement of CTL proteins such as perforin (Crawley et al., 2014; Smyth et al., 1991). As IL-2, IL-15 and IL-7 all use CD132 in their receptor complexes, there is overlap of downstream signaling as well as competition for CD132 (Palmer et al., 2008) that may lead to indirect or unintentional changes in the IL-7 signaling pathway.

1.7. Generalized liver functions and pathologies

The liver is composed of two lobes with connecting ligaments, and has two blood supplies, the portal vein carrying blood from the intestines and spleen and an artery from the abdominal aorta, that filters 30% of the body's total blood volume every minute (Valiante et al., 2000). It acts to filter the blood coming from the gut to metabolize and assist in the storage of nutrients, to produce hormones, metabolize medication, and remove toxins such as alcohol and excess medication (Ramadori et al., 2008). Liver function is vital and significant liver injury or permanent damage is irreversible and fatal if widespread. The bulk of the liver is composed of hepatocytes, with some LSEC, Kupffer cells, and hepatic stellate cells. Cycles of inflammation seen in chronic hepatic infection lead to hepatocyte apoptosis and necrosis, and eventually tissue scarring or fibrosis which is thought to be caused by hepatic stellate cells. Cirrhosis is severe fibrosis associated with an increased risk of developing HCC and end stage liver disease (Wong and Lee, 2006). Fibrosis, cirrhosis, and HCC can occur in hepatic infections as well as with alcohol abuse and intake of hepatotoxic chemicals (overuse or long-term use of medications such as acetaminophen and HAART, and exposure to industrial toxins such as arsenic, etc) (Moodie et al., 2009; Simeonova et al., 2001). Combinations of HCV infection, drug use or medication, and/or alcohol can lead to exacerbated damage to the liver (Pol et al., 1998).

1.8. Intrahepatic Lymphocytes

In health, there are few lymphocytes present in the liver, those that do infiltrate into the liver are typically of a memory or antigen experienced phenotype (Heydtmann et al., 2006). In chronic HCV infection, or other liver tropic infections, lymphocytes migrate from

lymph nodes to the liver via the blood and infiltrate into its lobes. These intrahepatic (IH) lymphocytes have a higher proportion of innate immune (NK, natural T (NT), and T-cells with NK-cell receptors (NKT)), CD4⁺ (Th1 and Th0), and CD8⁺ T-cells compared to lymphocytes present in the periphery (Valiante et al., 2000).

Under non-inflammatory conditions, LSEC act to create CD8⁺ T-cell tolerance to antigen coming from the gut (e.g. food, medication), by presenting the antigen in the context of inhibitory ligand PD-L1 to PD-1 on the CD8⁺ T-cell. In inflammatory conditions, the liver is capable of acting as a tertiary lymphoid organ and LSEC can activate naïve CD8⁺ T-cells directly in the liver. Kupffer cells can also migrate towards the LSEC and interact with CD8⁺ T-cells circulating through the liver. Unique to the liver, hepatocytes are also capable of expressing the requisite MHCs and co-stimulatory receptors required to activate naïve CD8⁺ T-cells. Once activated, the CD8⁺ T-cells will migrate past the LSEC and into the liver lobes and localize to areas of inflammation or infection (Bertolino et al., 2002; Jenne and Kubes, 2013). The CD8⁺ T-cells found in the liver, in health or hepatic infection, are almost exclusively T_{EM} cells, and have high expressions of inhibitory, exhaustion markers PD-1 and 2B4 (Kroy et al., 2014).

1.9. Immune dysfunction during acute HCV infection

The frequent failure of CD8⁺ T-cells to clear HCV infection during the acute phase of infection may be due in part to inefficient HCV-specific CD8⁺ T-cell responses (Meyer-Olson et al., 2004; Thimme et al., 2001), insufficient CD4⁺ T-cell help, and viral mechanisms of immune evasion such as rapid mutation rates and changes in antigenic epitopes (Bull et al., 2015; Kaplan et al., 2007; Meyer-Olson et al., 2004; Thimme et al., 2001). Evaluation of HCV immune response in acute infection is challenging as this stage is

commonly asymptomatic and individuals are typically not diagnosed with HCV infection until chronic infection sets in. Nevertheless, close monitoring of high risk groups has enabled the study of some immunological parameters in acute infection.

HCV-specific CD8⁺ T-cell impairment is evident in acute infection. When stimulated with peptide, HCV-specific CD8⁺ T-cells do not produce as much TNF- α and IFN- γ as CD8⁺ T-cells specific for Epstein-Barr virus (EBV) and cytomegalovirus (CMV) when stimulated with their cognate peptides, and this difference remains through chronic HCV infection (Gruener et al., 2001). Differences in CD8⁺ T-cell response in acute infection between individuals who spontaneously clear or develop persistent infection may predict the development of chronic infection. Production of IFN- γ in response to HCV epitopes in acute infection is much stronger, and decreases significantly over time in individuals who progress to chronic infection (Bull et al., 2015). Early development of polyfunctional HCV-specific CD8⁺ T-cells (IFN- γ , IL-2 production and expression of the degranulation marker CD107a) and higher levels of CD127 and Bcl-2 expression correlate with spontaneous resolution of infection (Badr et al., 2008). HCV-specific CD8⁺ T-cells in acute infection that later becomes chronic also have higher PD-1 expression compared to those that will spontaneously clear the virus (McMahan et al., 2010).

1.10. Impaired CD8⁺ T-cells in chronic HCV infection

1.10.1. HCV-specific CD8⁺ T-cell impairment

During chronic infection, HCV-specific CD8⁺ T-cells make up 0.05-2% of circulating CD8⁺ T-cells (Abel et al., 2006; Lechner et al., 2000; Wedemeyer et al., 2002). These CD8⁺ T-cells have been the most thoroughly investigated type of CD8⁺ T-cells in HCV infection. In general, HCV-specific CD8⁺ T-cells produce less perforin, have reduced

CTL function, and reduced ability to produce IL-2, TNF- α and IFN- γ (Rehermann, 2007). When stimulated with their cognate antigen, fewer HCV-specific CD8⁺ T-cells produce IFN- γ , the cells proliferate less than flu-specific cells (Wedemeyer et al., 2002), and they produce less perforin than CMV-specific cells (Lucas et al., 2004) in the same individual. Therefore, the function of HCV-specific CD8⁺ T-cells is decreased compared to other antigen-specific CD8⁺ T-cells within a given individual.

1.10.2. Intrahepatic CD8⁺ T-cell impairment

The number of liver infiltrating CD8⁺ T-cells in HCV infection can differ depending on the severity of liver inflammation, but the proportion of HCV-specific CD8⁺ T-cells in the liver ($\approx 2\%$) is consistently higher than in the blood. In general, IH-lymphocytes are more apoptotic, and areas of the liver with a greater number of total but fewer HCV-specific CD8⁺ T-cell infiltrate have more apoptosis than areas with more HCV-specific CD8⁺ T-cells (Abel et al., 2006; Nuti et al., 1998). IH-CD8⁺ T-cells have lower CD127 expression, and higher Tim-3 and PD-1 expression than their blood-derived counterparts. Exhaustion marker expression is associated with alanine transaminase (ALT) levels (measure of liver injury that can fluctuate over time) and lower CD127 expression is associated with increased viral load (Bensch et al., 2007; Golden-Mason et al., 2009; Larrubia et al., 2013; Owusu Sekyere et al., 2015). The majority of HCV-specific IH-CD8⁺ T-cells are PD-1 and Tim-3 positive and express multiple exhaustion markers per cell, and many of these cells express the immunoregulatory cytokine IL-10 compared to non-HCV specific cells (Abel et al., 2006; Kroy et al., 2014; McMahan et al., 2010). Lower expression of mRNA transcripts encoding IL-7 in hepatocytes in chronic HCV infection may also lead to less activation and reduced survival of T-cells in the liver (Larrea et al., 2014).

In infectious hepatitis (HCV or HBV infection), intrahepatic lymphocytes show increased activation (e.g. decreased CD28 expression), and a higher proportion of these cells secrete IFN- γ compared to peripheral lymphocytes (Valiante et al., 2000). While more cells may be IFN- γ^+ , in chronic HCV infection, IH-CD8 $^+$ T-cells stimulated with HCV-peptide produce less IFN- γ compared to flu-peptide stimulated cells (Spangenberg et al., 2005) and produce less IFN- γ following anti-CD3/antiCD28 (TCR) stimulation compared to blood-derived CD8 $^+$ T-cells (Sumida et al., 2013). Bulk IH-CD8 $^+$ T-cells also have more double strand DNA breaks than blood-derived CD8 $^+$ T-cells which has been associated with reduced STAT1 and STAT5 phosphorylation and less IL-2 production (Hoare et al., 2013). Poor function and high levels of exhaustion markers contribute to the decreased ability of these CD8 $^+$ T-cells to control HCV infection occurring in the liver.

1.10.3. Bulk CD8 $^+$ T-cell impairment

While less studied, bulk CD8 $^+$ T-cells are also impaired during chronic HCV infection for multiple reasons, such as increased double strand DNA breaks, bystander chronic infection and direct effects of HCV proteins, along with many as yet unknown causes. As HCV infection progresses, the proportion of CD8 $^+$ T-cells whose DNA have double strand breaks increases leading to apoptosis and decreased function; these CD8 $^+$ T-cells have reduced STAT1 and STAT5 phosphorylation in response to IL-6 and IL-2 respectively, and a reduced production of IL-2 overall (Hoare et al., 2013). CD8 $^+$ T-cells also have increased apoptosis in response to α CD3/28 (TCR) stimulation (Zhao et al., 2013). The inflammatory environment during chronic infection has effects on all the T-cells present, not just those specific for HCV. This could be contributing to the observed increase in exhaustion marker expression, such as Tim-3 on bulk CD8 $^+$ T-cells in HCV infection

compared to healthy individuals (McMahan et al., 2010). In addition, regulators of transcription for T-box 21 (T-bet) involved in the differentiation of effector to memory CD8⁺ T-cells are altered in non-HCV-specific CD8⁺ T-cells during chronic HCV infection compared to health, similar to observations in a murine model of chronic lymphocytic choriomeningitis virus (LCMV) infection (Stelekati et al., 2014).

1.11. CD8⁺ T-cells in HIV-HCV co-infection

HIV infection causes major changes to the immune system, which can indirectly affect CD8⁺ T-cell function. For example, CD4⁺ T-cell numbers are significantly reduced in untreated HIV infection, diminishing their ability to support CD8⁺ T-cell function (Levy et al., 2009). During HIV-HCV co-infection, blood-derived HIV and HCV-specific CD8⁺ T-cells are more impaired than in each of their respective mono-infection states. In co-infection, HIV-specific CD8⁺ T-cells produce less perforin and express lower levels of CD57, a marker of T-cell senescence and pre-disposition to activation-induced cell death (AICD), compared to HIV mono-infection (Harcourt et al., 2005). Spontaneous control of HCV in HIV-HCV co-infection is associated with higher lymphoproliferative responses to HCV peptides (Kim et al., 2006). Also, very few blood-derived HCV-specific CD8⁺ T-cells are detected in HIV-HCV co-infection compared to HCV mono-infection, potentially because they preferentially localize to the liver (Kared et al., 2014). These differences in HCV-specific CD8⁺ T-cells in co-infection are not due to differences in the fine specificity of the cells, as no significant difference has been observed in specific T-cell clones between HCV mono- and HIV-HCV co-infection (Barrett et al., 2011). The majority of studies which evaluated CD8⁺ T cell responses in HIV-HCV co-infection have identified more pronounced impairment of antigen-specific responses compared to mono-infection states.

In the liver, infiltrating CD8⁺ T-cells differ subtly between HCV mono- and HIV-HCV co-infection. More CD8⁺ T-cells have been observed in liver biopsies of HIV-HCV co-infected individuals than in HCV mono-infection. In addition, co-infected individuals have fewer CD4⁺ T-cells and more apoptotic lymphocytes than HCV mono-infected individuals (Canchis et al., 2004; Vali et al., 2008), potentially leading to reduced CD4⁺ T-cell help for CD8⁺ T-cells in HIV-HCV co-infection. There are more regulatory and exhausted (PD-1⁺) T-cells in the liver in HIV-HCV co-infection than in HCV mono-infection (Barrett et al., 2014). This suggests that CD8⁺ T-cells in blood and liver may be further impaired in HIV-HCV co-infection than in HCV mono-infection.

1.12. Potential contributors to immune impairment in HCV infection

1.12.1 HCV proteins

The HCV core protein has been found in the plasma of individuals with chronic HCV infection, forming nucleocapsids that may or may not contain viral RNA and hence bear potential for infectivity (Maillard et al., 2001). Non-enveloped nucleocapsids present in the bloodstream correlate with viral load (Aoyagi et al., 1999). Core has been reported to bind to the complement receptor gC1qR on T-cells. Binding of core inhibits TCR-mediated activation of Src Kinase Lck and ZAP-70, and phosphorylation of Akt (Yao et al., 2004), unregulates PD-1 and PD-L1 expression (Yao et al., 2007), and functions inhibited by core (e.g. IL-2 production, proliferation, ERK/MEK phosphorylation, PD-1 expression) are rescued with anti-gC1qR antibody (Yao et al., 2001, Yao et al., 2007). In CD8⁺ T-cells, IFN- α binding leads to activation of the JAK/STAT signaling cascade, but the HCV core protein can prevent the phosphorylation of STAT1 (Lin et al., 2006) and inhibit proliferation, IL-2, and IFN- γ production of blood-derived HCV-specific CD8⁺ T-cells (Kittlesen et al., 2000;

Rehermann, 2007). Core-mediated impairment of T-cells may be through PD-1 and PDL-1 as treatment with anti-PD1 and PDL-1 antibodies rescues the core-mediated impairment (increased apoptosis, decreased T-cell proliferation) (Yao et al., 2007). Also, HCV protein effects on T-cell function are not limited to the core protein as HCV protein pool (core, NS3 and NS4) induces IL-10 production by PBMC in a direct fashion (antigen presentation was inhibited) (Barrett et al., 2008).

1.12.2. Mutating epitopes

HCV is an RNA virus with a poor fidelity RNA polymerase and no proof reading capability, leading to high rates of error in the replication of its genome; sequence analysis of viral isolates from a single individual may differ by as much as 35%. Along with immunological pressure by HCV-specific CD8⁺ T-cells, this leads to escape mutations with greater fitness that antigen-specific memory CD8⁺ T-cells cannot recognize (Valiante et al., 2000).

1.12.3. Immune suppression mediated by the liver

As mentioned, LSEC can mediate the response of CD8⁺ T-cells to antigen. In non-inflammatory conditions, LSEC tolerize CD8⁺ T-cells to antigen as an unnecessary immune response could cause serious damage to the organ (Jenne and Kubes, 2013). Under inflammatory conditions, this shifts to activation of CD8⁺ T-cells to antigen presented in the liver. However, the quality of the activation CD8⁺ T-cells receive in the liver is less than when activated in conventional lymph nodes. After primary hepatocyte activation, CD8⁺ T-cells express lower levels of IL-2 and Bcl-xl, leading to premature apoptosis, and *in vivo*, hepatocyte activated CD8⁺ T-cells produce less TNF- α and IFN- γ (Bertolino et al., 1999). This is theorized to be due to the lack of proper co-stimulation present in the liver, compared

to the costimulatory molecules and cytokines which are professionally expressed in the lymph node. In a mouse model it was shown that CD8⁺ T-cells activated in the liver by bone marrow derived cells that were used to reconstitute an irradiated mouse's liver had lower IL-2 expression, intermediate IFN- γ and high Bim expression (pro-apoptotic) compared to CD8⁺ T-cells activated in lymph nodes (Holz et al., 2012).

1.12.4. Exhaustion markers

While exhaustion markers may not be a cause of dysfunction, they may reflect a mechanism of continued CD8⁺ T-cell impairment. For example, PD-1 expression is typically increased on early effector CD8⁺ T-cells in acute infection but then decreases, except in chronic infection it remains high. Both PD-1 and Tim-3 are upregulated in chronic HCV infection, particular on HCV-specific CD8⁺ T-cells (Barber et al., 2006; Barrett et al., 2014; Golden-Mason et al., 2009; McMahan et al., 2010). PD-1 and Tim-3 blocking antibodies are capable of reversing some of the proliferation and IFN- γ impairment and IL-10 production seen by their increased expression on CD8⁺ T-cells, in a dose dependent manner (Golden-Mason et al., 2007; McMahan et al., 2010; Urbani et al., 2006). A lower expression of PD-1 on HCV-specific CD8⁺ T-cells in acute infection has been associated with spontaneous HCV clearance (Urbani et al., 2006). Exhaustion is typically thought to be due to persistent antigen stimulation, but in the case of chronic HCV infection, KLRG1 (killer cell lectin like receptor G1, found on antigen experienced cells) expression on HCV-specific CD8⁺ T-cells is lower than on antigen-specific CD8⁺ T-cells in other chronic viral infections, EBV and HIV infection (Bensch et al., 2007), indicating there may be another cause of increased exhaustion markers in HCV infection, or exhaustion markers are a characteristic and not a mechanism of impairment.

1.12.5. Impaired CD8⁺ T-cell responses to IL-7

In chronic viral infections, such as EBV, CMV, and HIV, the expression of CD127 is decreased on CD8⁺ T-cells (Boutboul et al., 2005; MacPherson et al., 2001), changing their ability to respond to IL-7. Expression of CD127 has been tested as a predictor of acute HCV infection outcome. In HCV infection of chimpanzees, animals that later spontaneously cleared the infection had more HCV-specific CD8⁺ T-cells that expressed CD127 than those that later progressed to chronic infection (Shin et al., 2013). In humans, individuals with acute HCV infection that progressed to chronic had the lowest expression of CD127 overall in blood during the acute phase compared to an intermediate level of CD127 expression among those who resolved their acute infection (Golden-Mason et al., 2006).

In chronic HCV infection, there is currently no consensus as to the expression of CD127. Some studies have reported lower CD127 expression on bulk CD8⁺ T-cells in chronic infection compared to healthy or acutely-infected individuals. Others have reported an increased number of CD8⁺ T-cells expressing CD127 in chronic HCV infection (Golden-Mason et al., 2006), while others found no change in CD127 expression on bulk CD8⁺ T-cells (Larrubia et al., 2013), and no correlation between CD127 expression and viral load (Bengsch et al., 2007). While the expression of CD127 is typically highest on naïve and central memory cells, the activity of cells that are CD127⁺ or CD127⁻ is not straightforward. In HCV-infected chimpanzees, more HCV-specific blood-derived CD8⁺CD127⁺ T-cells produce IFN- γ and TNF- α compared to CD127⁻ T-cells in response to stimulation with peptide (Shin et al., 2013) even though CD127⁻ cells would typically be the effector T-cells (Schluns and Lefrancois, 2003).

In addition to receptor expression, the response to IL-7 is controlled by suppressors of cytokine signaling (SOCS). SOCS are negative feedback regulators of signaling in response to cytokine; SOCS1 is involved in T-cell homeostasis, and along with SOCS3, is thought to regulate STAT5 signaling in T-cells (Palmer and Restifo, 2009). In HCV infection, PBMC have higher endogenous levels of SOCS3 mRNA and HCV core upregulates SOCS1 mRNA in T-cells and SOCS3 mRNA in PBMC (Collins et al., 2014; Yao et al., 2008). If SOCS proteins are upregulated in HCV infection, they could inhibit cytokine/receptor initiated signaling to reduce downstream effects, specifically STAT transcription factor driven transcription of genes and impair CD8⁺ T-cell function.

1.13. Rationale and hypothesis

The impairment of bulk CD8⁺ T-cells during chronic HCV infection has been described but the mechanisms of this impairment are still unknown. The impairment is of great interest as it has implications for the overall function of the immune system, such as reduced responses to HBV (Moorman et al., 2011) and influenza (Greenbaum et al., 2004) vaccines. In HIV infection, changes in CD127 expression and cell inherent deficiencies in signalling in response to IL-7 were found to be one cause of generalized CD8⁺ T cell impairment (MacPherson et al., 2001; O'Connor et al., 2010; Vranjkovic et al., 2012; Vranjkovic et al., 2007; Vranjkovic et al., 2011). As HCV is another chronic viral infection with potential global effects on the immune system, it was of interest to determine if CD127 expression and IL-7 signaling were also mechanisms of CD8⁺ T-cell impairment in HCV infection, particularly as HIV-HCV co-infection is common. The expression of CD127 has been evaluated in HCV infection (Bensch et al., 2007; Golden-Mason et al., 2006; Shin et al., 2013), though CD8⁺ T-cell response to IL-7 has not been studied to the best of our

knowledge. Stimulation with IL-7 is also a means to study bulk CD8⁺ T-cell activity without antigen stimulation, particularly as IL-7 is important for many T cell functions. The hypothesis of this thesis is that chronic HCV infection reduces CD8⁺ T-cell activity in response to IL-7 in both HCV mono- and HIV-HCV co-infection.

1.14. Statement of Objectives

In order to evaluate the response of CD8⁺ T cells to IL-7 in chronic HCV infection there were 3 main objectives. Objective 1 was to determine if the activity of blood-derived CD8⁺ T-cells in HCV infection is reduced in response to IL-7 compared to healthy controls. Objective 2 was to determine if the activity of blood-derived CD8⁺ T-cells in HIV-HCV co-infection are also reduced in response to IL-7. Lastly, objective 3 was to determine if the activity of IH-CD8⁺ T-cells in HCV infection were lower in response to IL-7 than blood-derived CD8⁺ T-cells.

Chapter 2: Materials and Methods

2.1. Study Subjects

Individuals included in this study were healthy, HCV⁻ donors, chronically-infected HCV⁺ individuals (i.e. ≥ 6 months HCV RNA-positive, HCV treatment naive), and HIV-HCV co-infected individuals (HAART treated, HCV treatment naive). Exclusion criteria included HBV infection, HCV treatment and HAART non-compliance. Age, gender, and ethnicity are summarized in Table 1, though donors were not recruited for age-gender matching. The study was not designed for correlation analysis with age, HCV genotype or fibrosis score, however, where possible, analysis was completed. Collection of blood and liver biopsy tissue from human subjects was approved by The Ottawa Health Science Network Research Ethics Board and sample collection was done by nurses in the Viral Hepatitis Clinic (blood) and The Ottawa Hospital (TOH) Medical Imaging Department staff (liver). In most cases, fibrosis scores were determined by fibroscan, and individuals were grouped by fibrosis stage (F0-F2 or F3-F4) after sample collection. When a liver biopsy was the diagnostic used, fibrosis was scored using the Metavir system, an algorithm for the evaluation of histological activity and fibrosis range descriptions. In both cases, fibrosis assessment was completed by the Viral Hepatitis Clinic and the TOH Medical Imaging Department.

Table 1. Baseline Characteristics of Study Participants

		HCV ⁻ Controls	HCV ⁺ individuals evaluated in functional experiments	HCV ⁺ individuals included in whole blood phenotype study	HIV-HCV ⁺ Individuals
n		51	29	50	18
Gender (male, female) ^a		M26, F25	M21, F8	M39, F11	M12, F6
Mean age		35.0 ± 10.8	48.9 ± 12.5	49.4 ± 9.9	51.0 ± 9.0
Ethnicity (% Caucasian)		88%	86%	92%	89%
HCV Genotype					
	1		22/29	33*/49	14/18
	2		1/29	6/49	1/18
	3		6/29	8/49	3/18
	4		0/29	3*/49	1/18
Fibrosis Stage ^b					
	0-2		20/28	35/44	11/14
	3-4		8/28	9/44	3/14
Mean HCV RNA (IU/ml)			7.58x10 ⁶ ± 1.1x10 ⁷		1.04x10 ⁷ ± 2.9x10 ⁷
Mean ALT ^b			90 ± 58		81 ± 52

^a M (male), F (female)

^b measured by liver biopsy (Metavir system) or by fibroscan

*One participant with genotype 1 and 4 co-infection

Note: There is no fibrosis or genotype data for some HCV⁺ individuals

2.2. Isolation of peripheral blood mononuclear cells and CD8⁺ T-cells

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation: 30ml of heparinized blood was layered on top of 15ml of lymphoprep (Stemcell Technologies, Vancouver, British Columbia, Canada) and centrifuged for 30 minutes at 470xg with the centrifuge brake in the off-position (Heraeus Instruments Megafuge 1.0). CD8⁺ T-cells were isolated by magnetic bead positive selection or PBMC were frozen for later use at 1×10^7 cells/ml in 10% DMSO FCS (Sigma-Aldrich, Oakville, Ontario, Canada; Gibco, Life Technologies, Burlington, Ontario, Canada).

Isolation of CD8⁺ T-cells from PBMC was completed as described for the human CD8⁺ T-cell positive magnetic selection kit (Stemcell Technologies). Briefly, PBMC were suspended at 1×10^8 cells/ml in MACS buffer (0.5% BSA, 2mM EDTA, PBS). Selection cocktail was added to PBMC (10 μ l/100 μ l PBMC), incubated for 15 minutes, then nanobeads (10 μ l/100 μ l PBMC) added for 10 minutes. In a total volume of 2.5ml of buffer, tubes containing cells were placed in a magnet 3 times and CD8⁺ cells remained in the tube after removal of the liquid. Purity of isolated CD8⁺ T-cells was checked regularly by flow cytometry, with an average of $88.9\% \pm 5.8$.

2.3. Isolation of intrahepatic lymphocytes

IH-lymphocytes were isolated from liver biopsies (approximately 1mm x 1mm x 3cm) collected from HCV-infected individuals as part of their routine care (average size of $0.094\text{g} \pm 0.03$). Samples were processed as described previously (Nascimbeni and Rehmann, 2004). Briefly, tissue was cut into small (1mm) pieces with a sterile scalpel and tweezers. In the well of a 6-well plate, tissue was incubated with 5ml of 500U collagenase

IV and 2% FCS in HBSS (Gibco, Life Technologies), 50U DNase I (Sigma-Aldrich), and 0.6% BSA for 20 minutes at 37°C, shaken every 5 minutes. Tissue was then manually disrupted with a syringe end and filtered through a 70µm filter (Thermo Fisher Scientific, Waltham, MA, USA) to remove undigested tissue. Cells were washed in HBSS and cultured. The percentage of CD8⁺ T-cells in liver biopsy-derived cells was 16.4% ± 13.7, with an absolute number of 96,410 ± 66,861 CD8⁺ T-cells (based on n=5 samples).

Both blood-derived CD8⁺ T-cells and IH-lymphocytes were cultured overnight at 1x10⁶ cells/ml in complete RPMI (supplemented with 20% FCS, 1% L-glutamine, and 0.5% penicillin/streptomycin (Gibco, Life Technologies)) at 37°C, 5% CO₂, before use in experiments. In all experiments evaluating IH-CD8⁺ T-cells, flow cytometry controls were established using blood-derived cells to conserve the limited number of IH-CD8⁺ T-cells available for study.

2.4. Cell surface phenotyping of CD8⁺ T-cells

Subsets of CD8⁺ T-cells were detected in PBMC and IH-lymphocytes by flow cytometry. Cells (1x10⁵ per sample) were incubated in 1% BSA-PBS (100µl) for 30 minutes on ice with antibodies, followed by 2 washes with 1% BSA-PBS. Antibodies used included CD127-PE (clone R34.34, Beckman Coulter, Marseille, France), CCR7-APCCy7 (clone G043H7, Biolegend, San Diego, CA, USA), CCR7-PECy7 (clone 3D12), CD45RA-APC (clone HI100), and CD8-FITC/PeCy5 (clone HIT8a, BD Pharmingen, BD Bioscience, San Jose, CA, USA). These antibodies enabled the distinction of percent of CD8⁺ T cell subsets, defined as follows in this thesis: naïve (T_N, CD45RA⁺CCR7⁺), central memory (T_{CM}, CD45RA⁻CCR7⁺), effector memory (T_{EM}, CD45RA⁻CCR7⁻), and terminally-differentiated effector memory (T_{EMRA}, CD45RA⁺CCR7⁻) (O'Connor et al., 2010; Sommershof et al.,

2009). Initial data acquisition by flow cytometry was completed in the lymphocyte gate (determined by size and granularity of cells) and subsequent gating was determined by FL-1 controls (all antibodies except one of interest) and a total of 3000 events in the CD8 gate were collected.

The expression of CD127 on bulk CD8⁺ T-cells was determined by flow cytometry analysis of whole blood using the antibodies listed above (5µl each antibody/100µl whole blood). Following incubation for 20 minutes with antibodies at room temperature, red blood cells were lysed and remaining cells were fixed using Optilyse C (Beckman Coulter) for 10 minutes at room temperature. Then cells were washed twice in PBS, and evaluated by flow cytometry, using the same FL-1 strategy as above and a total of 10,000 events in the CD8 gate.

2.5. Measurement of pSTAT5

Phosphorylation of the STAT5 signaling protein in response to IL-7 was evaluated after overnight rest at 37°C. Isolated blood-derived CD8⁺ T-cells or IH-lymphocytes (10⁵ cells per sample, 0.5x10⁶cells/ml) were incubated with IL-7 (0.01-10 ng/ml, Sigma Aldrich) for 15 minutes at 37°C. To distinguish CD8⁺ T-cell subsets, cells were stained with CD45RA-APC (3µl/sample) and CCR7-APCCy7 (1.5µl/sample) in 1% BSA PBS on ice for 25 minutes, or went directly to the next step. Cells were washed in PBS and fixed in 100µl of 4% paraformaldehyde for 15 minutes at 37°C. Cells were then washed, incubated with 100% cold methanol (500µl) for 10 minutes on ice, and washed twice. Staining for pSTAT5 was completed in PBS (100µl) with 5µl/sample of anti-pSTAT5 pY694 Alexafluor 488 (clone 47/Stat5(pY694), BD Phosflow, BD Bioscience) for 30 minutes at room temperature, in the dark. After a final wash, pSTAT5 expression was measured as mean florescence

intensity (MFI) by flow cytometry. The same FL-1 gating strategy as above was used and a total of 10,000 events in the lymphocyte gate were collected. To distinguish CD8⁺ T-cells from other IH-lymphocytes, 5µl CD8-PeCy5 was added at the same time as the pSTAT5 antibody. Since the autofluorescence of IH-lymphocytes is higher than that of blood-derived cells, all samples were expressed relative to unstained IH-cells (Sheahan and Rice, 2013).

2.6. Measurement of CD8⁺ T-cell proliferation

Isolated CD8⁺ T-cells (1x10⁷ cells/ml) were labeled with carboxyfluoresceinsuccinimidyl ester (CFSE, 8µM, Cell Trace CFSE cell proliferation Kit, Molecular Probes, Life technologies) in 0.01% BSA-PBS for 10 minutes at 37°C as described previously (Crawley et al., 2010). Cells were washed twice with 15 volumes of cold complete RPMI and then cultured with IL-7 (10ng/ml) and a suboptimal concentration of the T cell mitogen phytohaemagglutinin (PHA, 0.2ug/ml, Sigma Aldrich) for 5 days. Colchicine (100ng/ml, Sigma Aldrich) was used as a negative control for proliferation, as it inhibits microtubule polymerization and arrests mitosis. Proliferation was the percentage of cells with dilution or loss of CFSE signal (halved upon cell division) by flow cytometry, with a total of 3000 events collected in the proliferating cell gate (determined by larger size and granularity of cells than the lymphocyte gate).

2.7. Measurement of Bcl-2 Expression

Ex vivo expression of Bcl-2 in CD8⁺ T-cells (1x10⁵ cells/sample, 0.5x10⁶ cells/ml) was determined after overnight rest of cells at 37°C and after incubation with IL-7 (0.01-10ng/ml) for 48 hours, as previously described (Crawley et al., 2010). At room temperature, cells were fixed in 4% paraformaldehyde (100µl/sample) for 20 minutes, washed in PBS,

then stained in permeabilization buffer (1% saponin in PBS, 100µl, Sigma Aldrich) with anti-Bcl-2 FITC (clone Bcl-2/100, BD Pharmingen) or IgG1-FITC isotype control (clone MOPC-21, BD Pharmingen) antibodies (5µl/sample) for 20 minutes. Bcl-2 expression was measured as mean fluorescence intensity (MFI) by flow cytometry, with a total of 10,000 events collected in the lymphocyte gate. The isotype control was treated with IL-7 (10ng/ml), and analysis of flow cytometry data took this into account. To distinguish CD8⁺ T-cells from other IH-lymphocytes, 5µl CD8-PeCy5 was added before fixation in 1% BSA-PBS (100µl) for 20 minutes at room temperature. The autofluorescence of IH-lymphocytes is higher than that of blood-derived cells, and was taken into account during data analysis (Sheahan and Rice, 2013).

2.8. Measurement of SOCS gene expression

CD8⁺ T-cells were isolated from frozen PBMC (stored in 10%DMSO FCS) as above. PBMC (20×10^6 per donor) were thawed by warming in 37°C bath, adding 1ml warm media (37°C) dropwise to each vial of cells, then adding cells to 13ml of warm media. This was followed by a second wash in warm media (Mallone et al., 2011). CD8⁺ T-cells were isolated from PBMC as above and RNA isolation was completed immediately for baseline expression, or after overnight rest at 37°C, cells were cultured in medium or IL-7 (10ng/ml) for 3 hours, then RNA was isolated.

Total RNA was isolated from 5×10^5 CD8⁺ T-cells using the Rneasy mini-prep kit (Qiagen, Venlo, Limburg, Netherlands). Briefly, 350ul of RLT buffer (supplemented with 2% of 2-mercaptoethanol) was used per sample, mixed, 350µl of 70% ethanol added and mixed by pipetting. The remainder of the protocol following the manufacturer's instructions (Qiagen, 2012) using the Eppendorf Centrifuge 5417R at 8,000xg, with the additional spin

before elution of RNA into 30µl of H₂O. The quantity of RNA was measured using the NanoDrop 1000 (Thermo Fisher Scientific). To determine quality of isolated RNA, 200ng of RNA was mixed with loading buffer and electrophoresed in 1% agarose gel at 60V for 1 hour (PowerPac 1000, Bio-Rad), with the TriDye 2-log DNA Ladder (0.1-10.0kb, New England BioLabs, Ipswich, MA, USA). The gel was stained with vistra green (at 1:25,000 dilution in 50ml TAE, GE Life Sciences, Mississauga, ON, Canada) for 30 minutes and rinsed in TAE. The gel was visualized using the ChemiDoc-It TS2 Imager (UVP, Upland, California, USA).

The same weight of RNA (250ng) was used in all reverse transcription reactions using the iScript Reverse Transcription Supermix for RT kit (Bio-Rad) in a total reaction volume of 20µl according to the kit's instructions using the Eppendorf thermocycler model 5345. Semi-quantitative PCR was completed with the SsoAdvanced SYBR Green Supermix kit (Bio-rad) in a total reaction volume of 20µl with 300nM of primers (Table 2) and 5µl of 1:300 (RPS18), 1:30 (SOCS1), or 1:40 (SOCS3) dilute cDNA. Culture conditions and primer sequences were derived from a personal communication with Dr. P. MacPherson and a University of Ottawa Ph.D. thesis (Al-Ghazawi, 2013).

PCR experiments were completed using the CFX Connect PCR machine (Bio-Rad) with an initial melting for 3 minutes at 95°C, then 40 cycles of the following: 5 second melting at 95°C and 25 second annealing at 60°C. After these steps, samples were heated incrementally between 65 and 95°C to generate a melting curve to determine the specificity of amplification. A two-step reaction (cycles of melting and annealing without elongation) was enabled by short amplicon lengths and the specialized polymerase supplied in the SsoAdvanced SYBR green Supermix kit. Each plate included a calibrator sample of pooled

cDNA to allow analysis between plates. Target gene expression was determined relative to the expression of RPS18 gene (ribosomal protein S18, component of small 40S ribosome subunit), a known housekeeping gene routinely used in RT-PCR. Reaction efficiencies were determined by pooled cDNA dilution curves.

2.9. Analysis and Statistics

Flow cytometry was completed using an FC500 Beckman Coulter flow cytometer and analysis of flow cytometry data was conducted using FCS Express Research Edition 4.0 (De Novo Software, Los Angeles, California, USA). Analysis of QPCR was completed using the gene expression tab of the Bio-Rad CFX Manager software. Graphs and statistics were generated using GraphPad Prism 5.0 software (San Diego, California, USA) and pie charts were constructed with Adobe Illustrator CS6 (Adobe, San Jose, California, USA). Where necessary, statistical analysis included Student's *t*-test, one-way ANOVA with Dunnett post-test, and/or non-linear regression ($p \leq 0.05$), and data are presented as means $\pm$ standard deviation.

Table 2. Primers used for QPCR*

Primer	Sequence (5'→ 3')	Reaction Efficiency
RPS18 forward	CTGCCATTAAGGGTGTGG	100.5%
RPS18 reverse	TCCATCCTTTACATCCTTCTG	
SOCS1 forward	TTTTTCGCCCTTAGCGTGAA	101.2%
SOCS1 reverse	GCCATCCAGGTGAAAGCG	
SOCS3 forward	GAAGATCCCCCTGGTGTGGA	95.0%
SOCS3 reverse	TTCCGACAGAGATGCTGAAGA	

*Primer sequences from (Al-Ghazawi, 2013)

Chapter 3: Results

3.1. Phenotype of blood-derived CD8⁺ T-cells

3.1.1. HCV mono-infection

The distribution of blood-derived CD8⁺ T-cell subsets was evaluated to determine if there were any inherent differences in the types of cells present during chronic HCV mono-infection (Figures 1A, B and C). PBMC were stained with anti-CD8 antibody, and subsets were distinguished on their expression of CD45RA and CCR7. In controls, the subset distribution of CD8⁺ T-cells was as follows (mean $\pm$ SD): T_N (37.1% $\pm$ 2.0) > T_{EMRA} (24.5% $\pm$ 2.8) > T_{EM} (20.12% $\pm$ 2.4) > T_{CM} (18.3% $\pm$ 3.9) (Figures 1D and E), and is consistent with previous reports (O'Connor et al., 2010; Sommershof et al., 2009). In chronic HCV infection, the ranking of subsets was subtly different: T_{EM} (28.9% $\pm$ 3.6) > T_N (26.7% $\pm$ 2.5) > T_{CM} (23.5% $\pm$ 3.2) > T_{EMRA} (20.9% $\pm$ 2.1) (Figures 1D and E). Although there were no significant differences in the proportions of T_{EM}, T_{CM} and T_{EMRA} cells between the groups, the proportion of T_N cells was significantly lower in HCV⁺ individuals (p=0.006 by unpaired Student's *t*-test, Figure 1D).

It should be noted that there were no significant effects of age, HCV genotype and fibrosis score on the subset distribution of CD8⁺ T-cells (Supplemental Table 1). Such analysis was performed where possible although this was limited due to a low distribution of individuals across different ages, genotypes and fibrosis scores. It may be of interest to note that HCV⁺ individuals in the older age group (≥ 58 years of age, i.e. $\approx 75^{\text{th}}$ percentile) tended to have a greater proportion of T_{EMRA} cells than those below 57 years of age (Figure 1F, p = 0.06, unpaired Student's *t*-test).

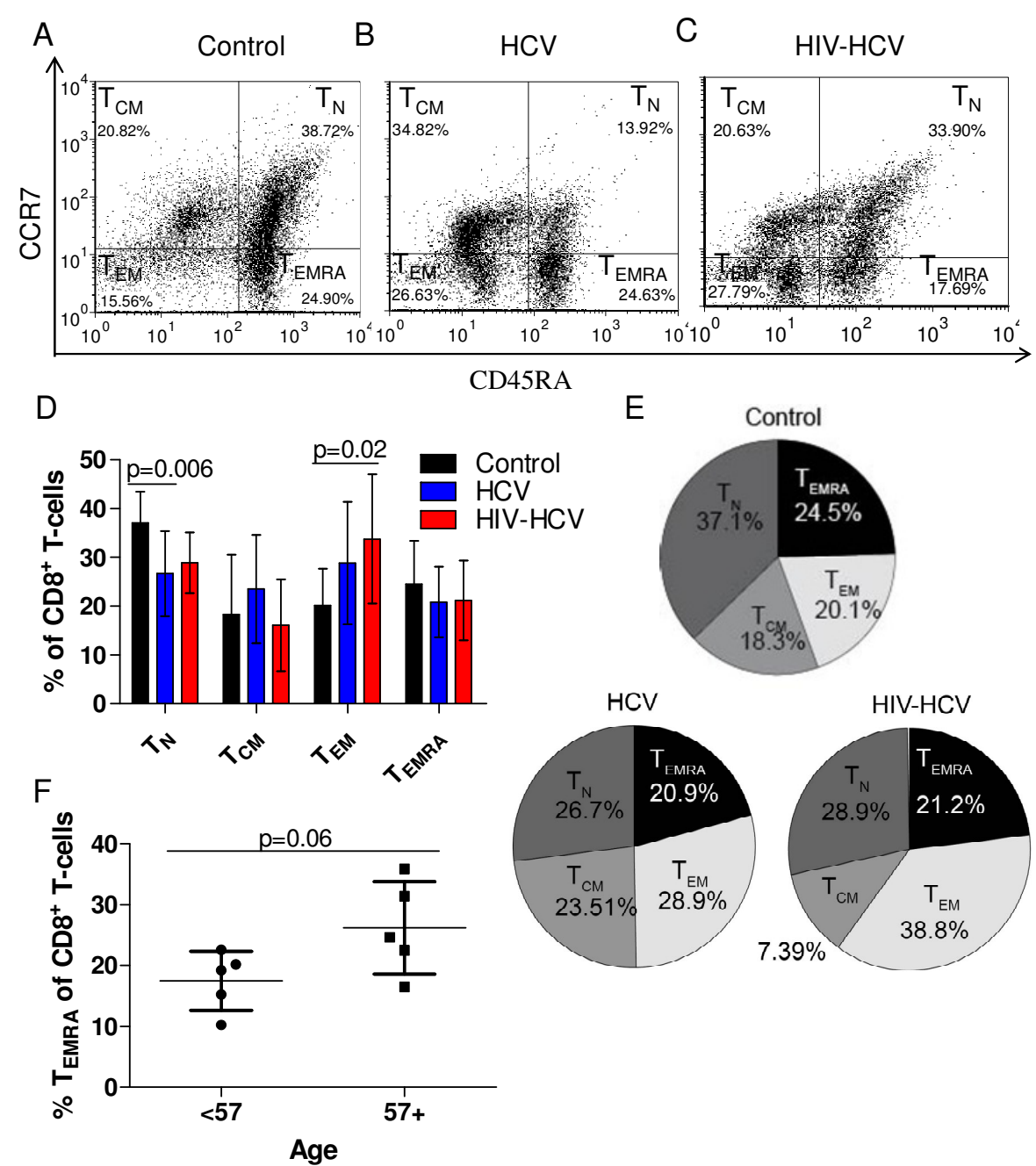


Figure 1. HCV infected individuals have fewer blood-derived CD8⁺ T_N cells while HIV-HCV co-infected individuals have more T_{EM} cells than HCV⁻ controls. Cell surface expression of CD45RA and CCR7 on CD8⁺ T-cells was determined by flow cytometry to distinguish between subsets as follows: naïve (T_N, CD45RA⁺CCR7⁺), central memory (T_{CM}, CD45RA⁻CCR7⁺), effector memory (T_{EM}, CD45RA⁺CCR7⁻), and terminally-differentiated effector memory (T_{EMRA}, CD45RA⁺CCR7⁻). Representative dot plots of CD8⁺ T-cell subset distribution in **A**) controls, **B**) HCV infection, and **C**) HIV-HCV co-infection. **D**) Subset distribution data are graphically represented and **E**) summarized as means in a pie chart (control n=10, HCV n=12, HIV-HCV n=7). **F**) The proportion of T_{EMRA} cells is higher in individuals over 57 years (unpaired Student's *t*-test).

3.1.2. HIV-HCV co-infection

In HIV-HCV co-infection, CD8⁺ T-cell subset distribution was similar to both HCV⁻ controls and HCV mono-infected individuals: T_N (28.9% ± 2.4), T_{CM} (7.39% ± 5.0), and T_{EMRA} (21.2% ± 3.1) cells. An exception to these similarities was the higher proportion of T_{EM} cells compared to HCV mono-infection (33.8% ± 5.1, p=0.02, Student's unpaired *t*-test) (Figures 1D and E). In HIV-HCV co-infection, there was no detected association between CD8⁺ T-cell subset distribution and fibrosis, HCV genotype or age (Supplemental Table 1).

3.2. IL-7-induced signaling in CD8⁺ T-cells

3.2.1. HCV mono-infection

To investigate activation of the JAK/STAT pathway in health and chronic HCV infection, the phosphorylation of STAT5 (pSTAT5) was evaluated in CD8⁺ T-cells stimulated with IL-7. Blood-derived CD8⁺ T-cells isolated from healthy and HCV-infected individuals were incubated with IL-7 (0.01-10ng/ml) or medium alone for 15 minutes, then pSTAT5 was detected by a fix and permeabilization protocol followed by MFI measurement by flow cytometry (Figure 2A). Phosphorylation of STAT5 occurred in a dose dependent manner in CD8⁺ T-cells isolated from HCV⁻ controls and HCV-infected individuals (one-way ANOVA, p=0.0003 and p<0.0001, respectively) (Figure 2B). The level of pSTAT5 in unstimulated cells was similar to unlabeled cells, and there was no difference between the groups. Upon IL-7 stimulation, the minimum concentration of IL-7 required for a significant increase in pSTAT5 was lower for controls (0.1ng/ml) than in HCV infection (1ng/ml) (p≤ 0.05, Dunnett post-test with medium alone as the control). Two doses of IL-7 (0.1 and 1 ng/ml) resulted in the greatest observable difference between the groups. The maximal STAT5 activation was equivalent between the groups following treatment with the highest

concentration of IL-7 tested (10ng/ml). Overall, across these dose responses, less pSTAT5 was detected with IL-7 stimulation in HCV infection compared to controls (non-linear regression, $p=0.005$).

Without cytokine stimulation, STAT5 activation was virtually absent in all subsets and was no different between the two groups. When cells were stimulated with IL-7, the amount of pSTAT5 detected differed across the cell subsets (Figure 2C). In healthy individuals, pSTAT5 expression was highest in T_N cells, followed by T_{CM} and T_{EM} cells, while there were undetectable levels of pSTAT5 in T_{EMRA} cells. In HCV infection, IL-7 stimulation activated STAT5 in T_N and T_{CM} cells but this was significantly lower than that of controls ($p<0.0001$, non-linear regression). Unlike in bulk $CD8^+$ T-cells, $CD8^+$ T-cell subsets stimulated with the highest concentration of IL-7 (10ng/ml) had a lower level of pSTAT5 in HCV infection than HCV⁻ controls.

There were no associations with pSTAT5 production and age detected. There were no individuals with fibrosis scores >2 among those tested in this assay, therefore analysis of any association between the degree of STAT5 activation and liver fibrosis was not possible (Supplemental Table 3 and 4).

3.2.2. HIV-HCV co-infection

Just as pSTAT5 production was lower in HCV infection, the phosphorylation of STAT5 was also lower in HIV-HCV co-infection, compared to HCV⁻ controls. Phosphorylation of STAT5 in bulk $CD8^+$ T-cells cultured with IL-7 (0.1-10 ng/ml) occurred in a dose dependent manner and reached the same level as HCV⁻ controls with the highest concentration of IL-7 (10ng/ml) ($p=0.0002$, one way ANOVA, Figure 2B). By subset, the phosphorylation of STAT5 was significantly lower in T_N , T_{CM} and T_{EM} cells compared to

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those in HCV mono-infection ($p=0.03$, 0.01 , and 0.005 , respectively, non-linear regression, Figure 2C). Unlike in bulk $CD8^+$ T cells, with the highest concentration of IL-7 (10ng/ml), the level of pSTAT5 expression in $CD8^+$ T-cell subsets in HIV-HCV co-infection did not reach the same levels as HCV⁻ controls or HCV mono-infection. The HIV-HCV co-infected individuals tested here did not have fibrosis scores $> F0-1$, therefore correlation analysis of fibrosis effects on STAT5 activation was not possible, and no associations with age were detected (Supplemental Table 3 and 4).

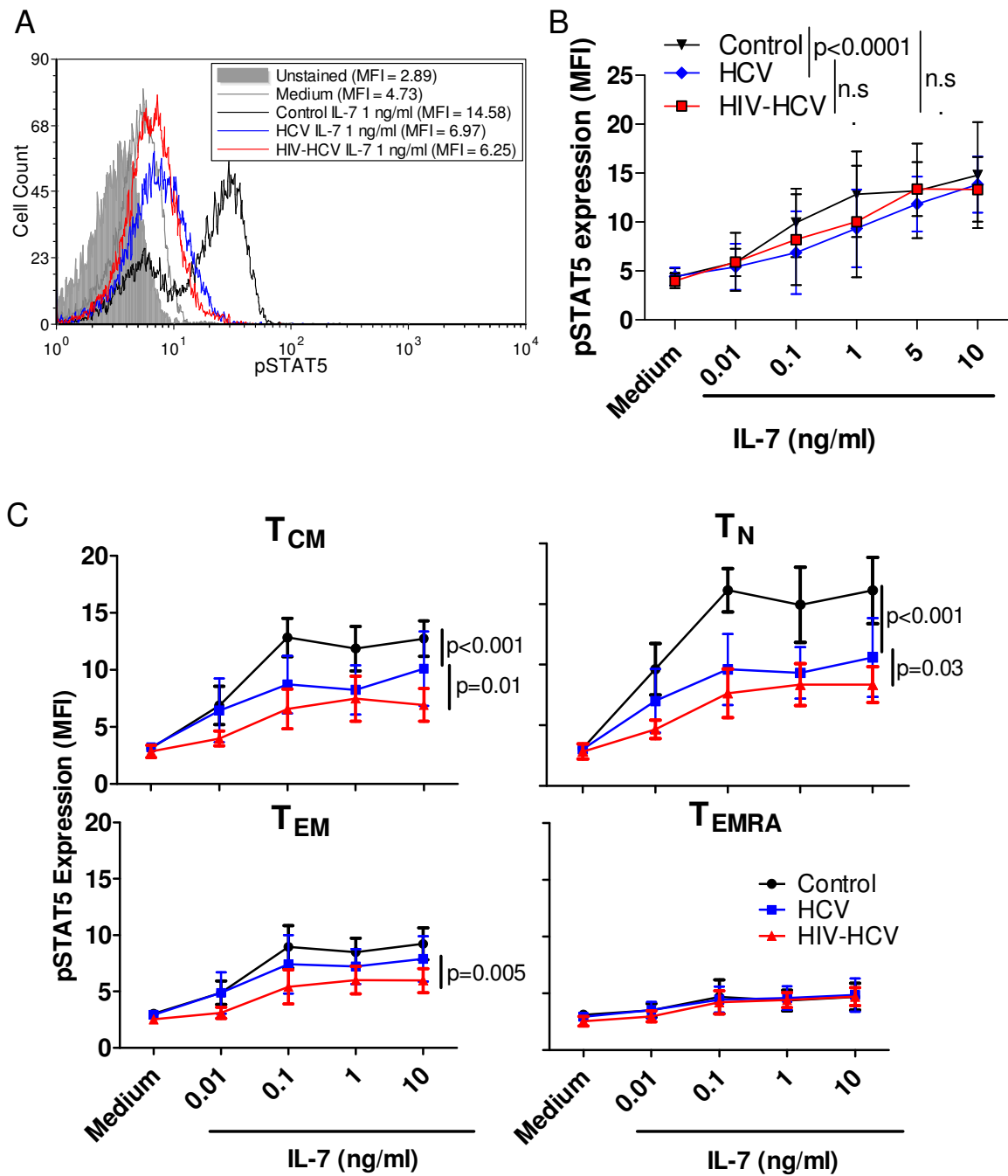


Figure 2. IL-7-induced signaling of blood-derived CD8⁺ T-cells is impaired in HCV mono- and HIV-HCV co-infection. Blood-derived CD8⁺ T-cells were cultured with medium alone or IL-7 (0.01 – 10ng/ml) for 15 minutes and the phosphorylation of STAT5 (pSTAT5) was measured by flow cytometry. **A)** A representative histogram of the mean fluorescence intensity (MFI) of pSTAT5 expression in response to IL-7 (1ng/ml) is shown and **B)** data for pSTAT5 expression by bulk blood-derived CD8⁺ T-cells is summarized (control n=10, HCV n=9, HIV-HCV n=4). **C)** The expression of pSTAT5 in CD8⁺ T-cell subsets (distinguished by CD45RA and CCR7 expression) are summarized as MFI values (control n=7, HCV n=5, HIV-HCV n=5) (non-linear regression, n.s. = not significant).

3.3. Proliferation of blood-derived CD8⁺ T-cells in response to IL-7

3.3.1. HCV mono-infection

Part of the homeostatic function of IL-7 is to stimulate cell proliferation. To quantify the IL-7-induced proliferation of blood-derived CD8⁺ T-cells, cells were labeled with CFSE and proliferation was measured as dilution of the CFSE label (i.e. % CFSE^{low} cells) (Figures 3A, B and C). Equal numbers of cells were stimulated with suboptimal amounts of T-cell mitogen (PHA, 0.2ug/ml), since T-cell activation is required to enable human T-cells to proliferate in response to IL-7 (Kimura et al., 2013). Without stimulation, cells did not proliferate in either group. When cultured with IL-7 alone (10ng/ml) or PHA alone, cells proliferated minimally (paired Student's *t*-test). There was no difference in proliferation in response to PHA alone in controls (19.2% ± 5.6) and HCV⁺ individuals (21.9% ± 3.8). Combining IL-7 + PHA resulted in a significant amount of proliferation in cells from controls. There was no difference in the proliferation in response to IL-7 or IL-7 + PHA between controls and HCV infection (26.9% ± 15.8 and 26.7% ± 10.6, 57.9% ± 7.5 and 58.0% ± 4.5, respectively) (Figure 3D). No association between age and proliferation was detected, and no correlation analysis of fibrosis score with proliferation was possible as only 2 of the HCV⁺ individuals had scores greater than F2 (Supplemental Table 5).

3.3.2. HIV-HCV co-infection

Proliferation of CD8⁺ T cells in response to IL-7 was significantly decreased in HIV-HCV co-infection compared to HCV mono-infection and HCV⁻ controls. With PHA alone there was no difference (22.2% ± 9.4%), yet with IL-7 alone or IL-7 + PHA, the percentage of cells that underwent proliferation was significantly lower than both other groups tested (8.4% ± 6.6, *p*=0.01, 34.8% ± 9.6, *p* = 0.03, respectively, Student's unpaired *t*-test) (Figure

3D). The age distribution and a lack of range in fibrosis scores did not permit analysis of these effects on proliferation of cell from HIV-HCV co-infected individuals (Supplemental Table 5).

3.4. IL-7-induced Bcl-2 production

3.4.1. HCV mono-infection

A major function of IL-7 is to promote the survival of T-cells by inducing the production of anti-apoptotic Bcl-2. Bcl-2 production is dependent on STAT5 activation (Crawley et al., 2014; Tripathi et al., 2010); hence the previous observations of impaired STAT5 activation in HCV infection would suggest a deficiency in Bcl-2 production. To determine if there were differences in *ex vivo* expression of Bcl-2 in blood-derived CD8⁺ T-cells in HCV infection, after isolation, cells were rested overnight at 37°C and Bcl-2 detected by anti-Bcl-2 antibodies and flow cytometry. The basal expression of Bcl-2, both MFI and relative to an isotype control, was not different between HCV⁻ controls and HCV infection (Figures 4A and B).

The ability of isolated CD8⁺ T-cells to produce Bcl-2 with IL-7 stimulation was measured after 48 hours of culture (Figure 4C). The level of Bcl-2 expression was similar in unstimulated CD8⁺ T-cells from controls and HCV-infected individuals. Stimulation with IL-7 (0.01-10ng/ml) increased Bcl-2 production in a dose-dependent manner in controls and HCV infection (one-way ANOVA $p < 0.0001$). However, this response was lower in HCV infection ($p = 0.0006$, non-linear regression) compared to controls. Even with the highest concentration of IL-7 (10ng/ml), CD8⁺ T-cells from HCV infection never produced as much Bcl-2 as cells from healthy controls. Bcl-2 production was not significantly increased in CD8⁺ T-cells of HCV-infected individuals when stimulated with less than 1ng/ml of IL-7,

while cells from controls produced significantly more Bcl-2 with a log lower concentration of IL-7 (0.1ng/ml, $p \leq 0.05$, Dunnett post-test) (Figure 4D). Therefore, production of Bcl-2 by blood-derived CD8⁺ T-cells in response to IL-7 is reduced in HCV infection; cells require more IL-7 to produce similar amounts of Bcl-2 and do not reach the same degree of production as controls.

Of the individuals tested in these Bcl-2 assays, 3 had a higher fibrosis score (F3-F4) and 6 had a low fibrosis score (F0-F2). Relative to the amount of Bcl-2 produced with no stimulation, HCV⁺ individuals with higher fibrosis scores produced significantly less Bcl-2 in response to IL-7 (10ng/ml) compared to those with lower degrees of fibrosis ($p=0.02$, unpaired Student's *t*-test) (Figure 4E). This trend was observed at lower concentrations of IL-7, though was not statistically significant.

3.4.2. HIV-HCV co-infection

In HIV-HCV co-infection, basal levels of Bcl-2 were also similar to HCV⁻ controls, with no difference between HCV mono- and HIV-HCV co-infection (Figures 4A and B). With IL-7 stimulation, Bcl-2 production increased in a dose-dependent manner (one-way ANOVA $p < 0.0001$). The magnitude of this response was significantly lower than in controls but similar between HCV and HIV-HCV co-infection ($p=0.007$, non-linear regression) (Figure 4D). Bcl-2 production was not significantly increased when cells were stimulated with less than 1ng/ml of IL-7, similar to that observed in HCV mono-infection ($p \leq 0.05$, Dunnett post-test).

In HIV-HCV⁺ individuals, all subjects tested for Bcl-2 expression had a fibrosis score of F0-1; hence it was not possible to determine if there was an association between fibrosis and Bcl-2 production in HIV-HCV co-infection (Supplemental Table 6).

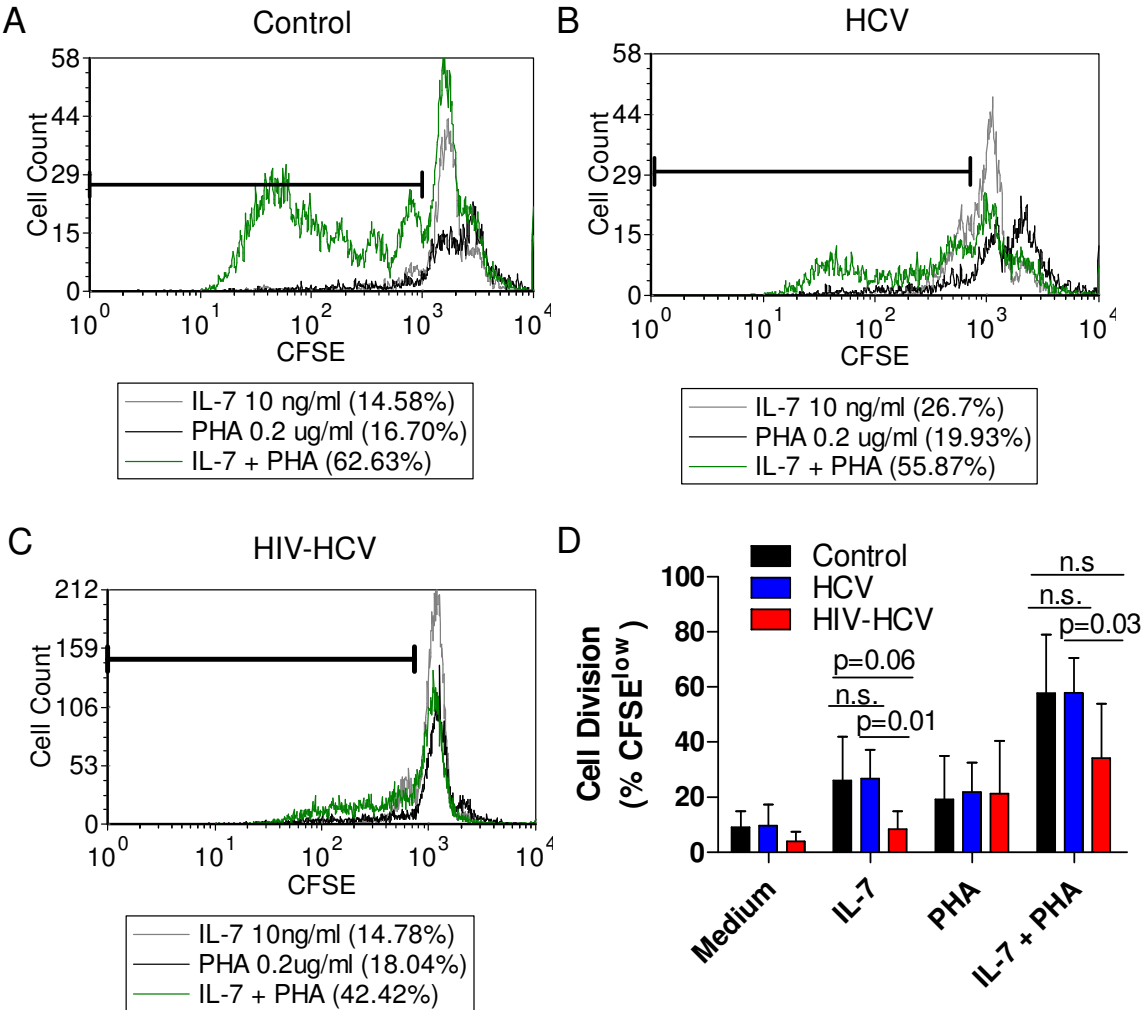
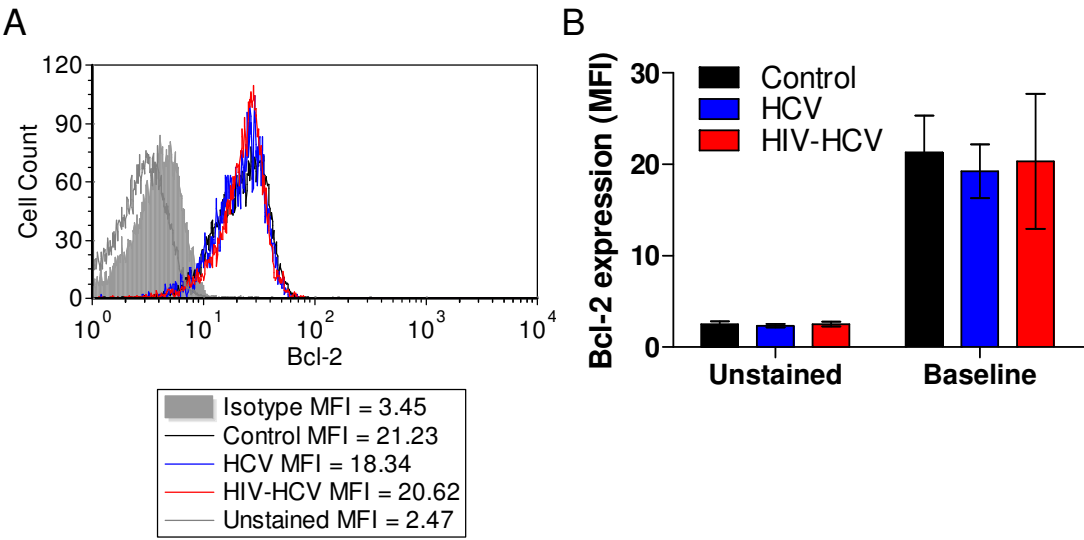


Figure 3. IL-7-induced proliferation is impaired in blood-derived CD8⁺ T-cells from HIV-HCV co-infected individuals but not in cells from HCV mono-infected individuals. Blood-derived CD8⁺ T-cells were labelled with CFSE (carboxyfluoresceinsuccinimidyl ester) and cultured with IL-7 (10ng/ml) and/or suboptimal amounts of T-cell mitogen PHA (phytohaemagglutinin, 0.2ug/ml) for 5 days. Cell proliferation (CFSE dilution) was then measured by flow cytometry. Representative histograms of CFSE dilution for CD8⁺ T-cells isolated from **A)** HCV⁻, **B)** HCV⁺ and **C)** HIV-HCV⁺ individuals are shown with markers indicating the proportion of dividing cells (% CFSE^{low}). **D)** Proliferation of isolated CD8⁺ T-cells from each sampled group is summarized (control n=8, HCV n=8, and HIV-HCV n=4).



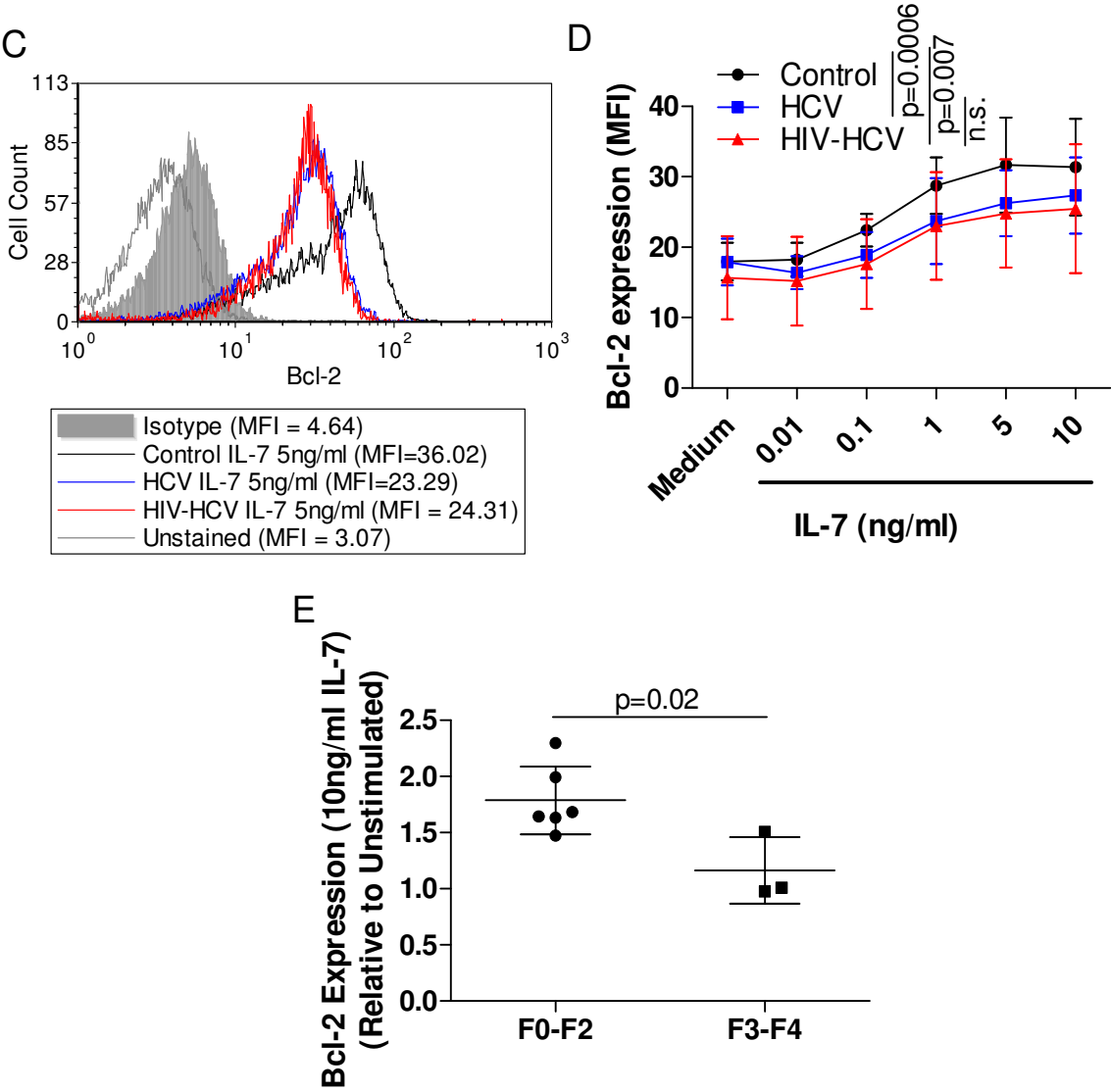


Figure 4. IL-7-induced production of Bcl-2 is reduced in blood-derived CD8⁺ T-cells from HCV mono-infected and HIV-HCV co-infected individuals. Expression of Bcl-2 was measured by flow cytometry in blood-derived CD8⁺ T-cells after overnight rest or after 48 hour culture with medium alone or IL-7 (0.01-10ng/ml). **A)** A representative histogram of CD8⁺ T-cells without cytokine stimulation after overnight rest at 37°C (basal) is shown and **B)** Bcl-2 expression summarized (control n=4, HCV n=5, and HIV-HCV n=5). **C)** A representative histogram of Bcl-2 expression in response to IL-7 (5ng/ml) is shown and **D)** summarized (control n=8, HCV n=9, HIV-HCV n=8, non-linear regression). Values are expressed as MFI. **E)** The IL-7-induced expression of Bcl-2 in blood-derived CD8⁺ T-cells from HCV⁺ individuals with low fibrosis (F0-F2) was compared to that of individuals with high fibrosis (F3-F4) (unpaired Student's *t*-test).

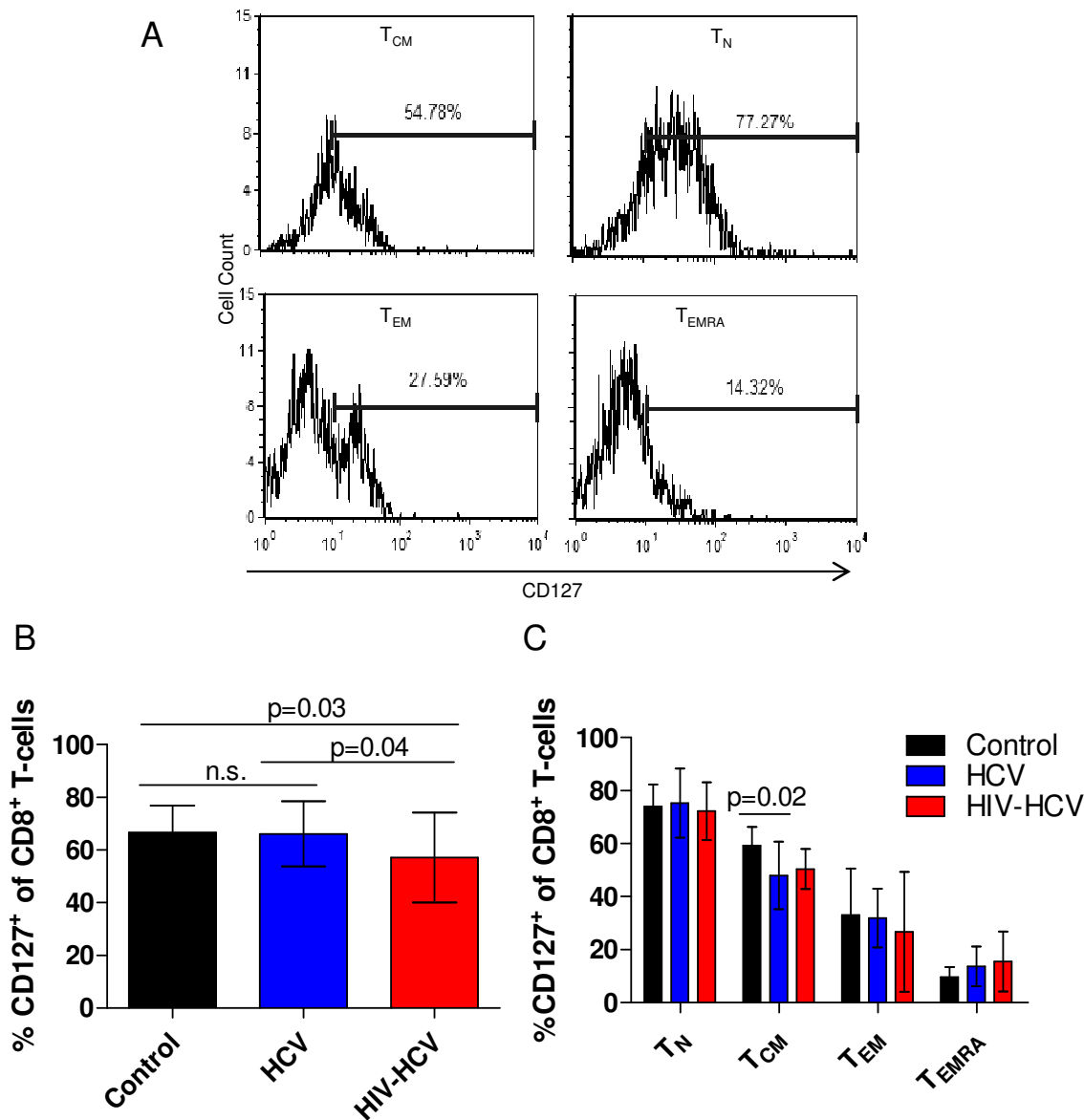
3.5. Possible factors contributing to observed impaired responses to IL-7

3.5.1. Altered receptor (CD127) expression

To determine if the level of CD127 receptor expression could contribute to the observed changes in IL-7 responses, receptor expression was assessed. In bulk blood-derived CD8⁺ T-cells, there was no difference in CD127 expression between HCV⁻ controls and HCV-infected individuals ($66.7\% \pm 1.9$ and $66.1\% \pm 1.8$, $n=30$ and $n=50$, respectively). There was significantly lower CD127 expression on CD8⁺ T-cells in HIV-HCV infection compared to HCV⁻ controls and HCV mono-infection ($57.1\% \pm 4.9$, $p=0.03$ and $p=0.04$, respectively, $n=9$, unpaired Student's *t*-test, Figure 5B).

The proportion of CD8⁺ T-cells expressing CD127 varied by cell subset; in controls, it was highest in T_N ($74.1\% \pm 2.6$), followed by T_{CM} ($59.3\% \pm 2.2$), T_{EM} ($33.1\% \pm 5.5$), and finally T_{EMRA} ($9.6\% \pm 1.2$) cells (Figure 5A). This hierarchical pattern of CD127 expression was similar in HCV infection for 3 subsets: T_N ($75.3\% \pm 3.8$) > T_{EM} ($32.0\% \pm 3.2$) > T_{EMRA} ($13.7\% \pm 2.2$). In HIV-HCV co-infection, the expression of CD127 on CD8⁺ T-cell subsets was similar to HCV mono-infection: T_N ($72.3\% \pm 4.1$), T_{CM} ($50.4\% \pm 2.9$), T_{EM} ($26.7\% \pm 8.6$), and T_{EMRA} ($15.5\% \pm 4.3$). However, fewer T_{CM} CD8⁺ T-cells in HCV infection expressed CD127 compared to controls ($p=0.02$ by unpaired Student's *t*-test) (Figure 5C). The MFI of CD127 expression followed the same trend as percent of CD127⁺ cells (Figures 5D and E).

Levels of CD127 expression on bulk blood-derived CD8⁺ T-cells were not associated with age, fibrosis stage or HCV genotype in HCV infection (Figures 6A, B and C) or HIV-HCV co-infection (Figures 7A, B and C).



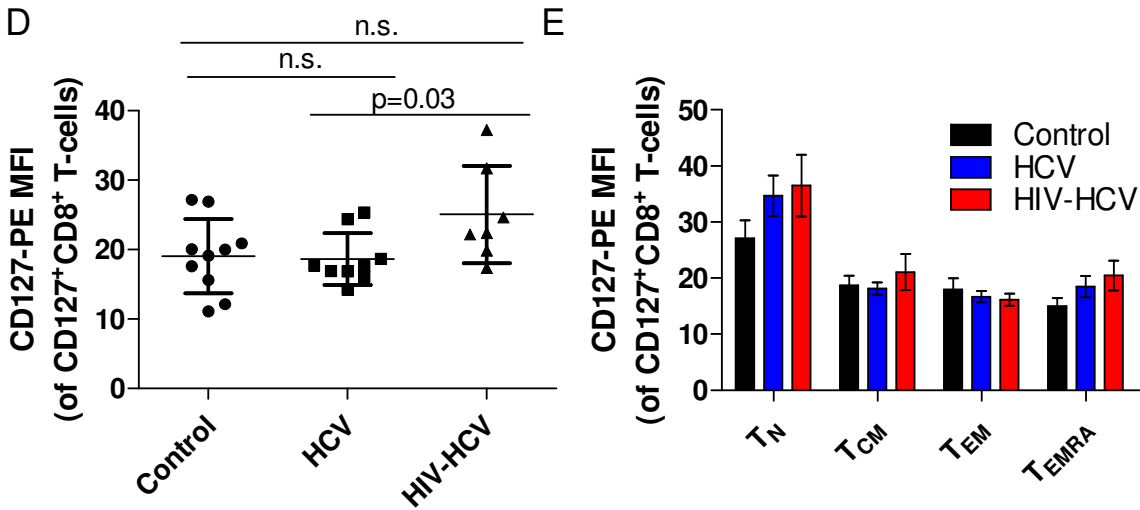


Figure 5. The expression of CD127 is lower on blood-derived T_{CM} cells from HCV mono-infected individuals. A) Cell surface expression of CD45RA, CCR7 and CD127 on CD8⁺ T-cells was determined by flow cytometry. The expression of CD127 between groups is shown in a bar graph on blood-derived B) bulk CD8⁺ T-cells (control n=30, HCV n=50, HIV-HCV n=9) and C) on CD8⁺ T-cell subsets (control n=10, HCV n=12, HIV-HCV n=7). The MFI of CD127 expression followed the same trend as percent of CD127⁺ cells in D) bulk and E) subsets of CD8⁺ T-cells. (Student's unpaired *t*-test).

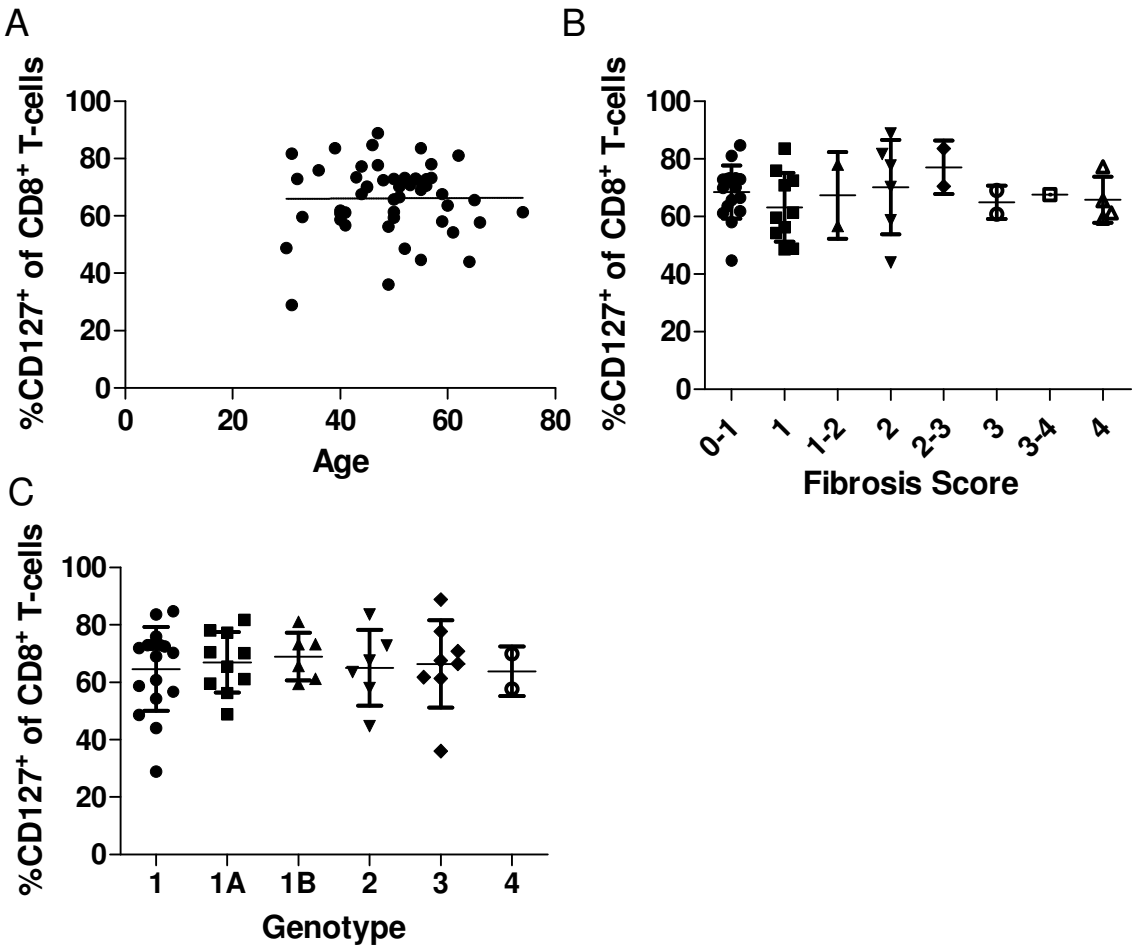


Figure 6. The expression of CD127 on CD8⁺ T-cells is not associated with fibrosis score or HCV genotype in HCV infection. Age, fibrosis score and HCV genotype were provided by the viral hepatitis clinic who recruited the study subjects. Expression of CD127 is not associated with **A)** age, **B)** fibrosis score, **C)** or genotype. Data were analyzed either by linear regression or Student's *t*-test to detect any potential statistically significant differences.

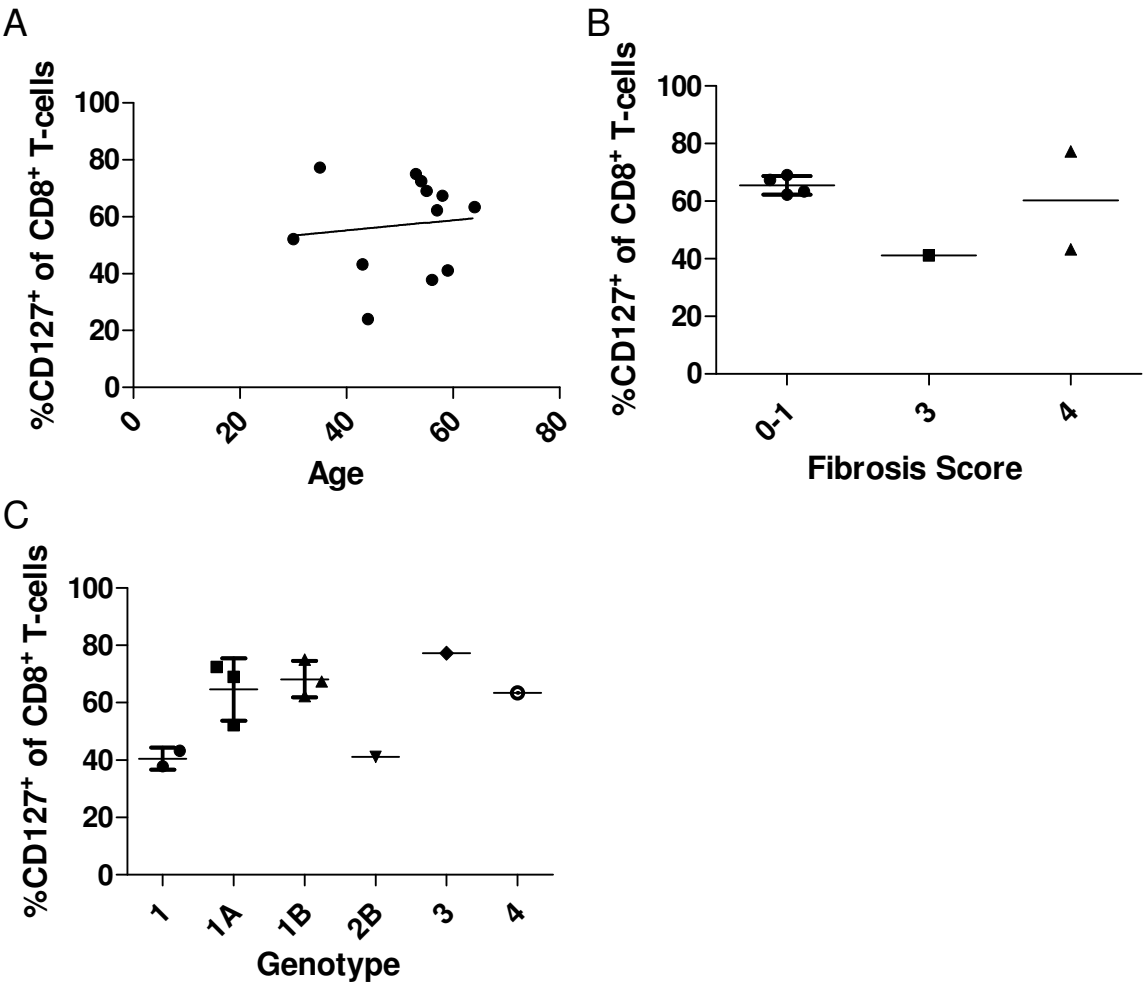


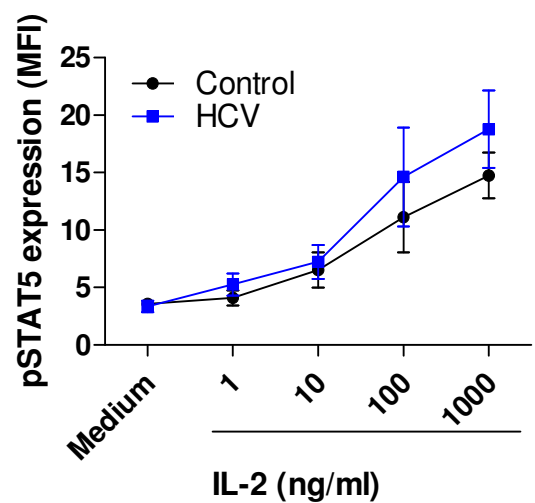
Figure 7. The expression of CD127 on CD8⁺ T-cells is not associated with fibrosis score or HCV genotype in HIV-HCV co-infection. Age, fibrosis score and HCV genotype were provided by the viral hepatitis clinic who recruited the study subjects. Expression of CD127 is not associated with **A)** age, **B)** fibrosis score, **C)** or genotype. Data were analyzed either by linear regression or Student's *t*-test to detect any potential statistically significant differences.

3.5.2. Cytokine specificity of signaling changes

Other γ_c chain cytokines such as IL-2 and IL-15 are also able to activate STAT5. To determine if the lower STAT5 activation observed in HCV infection was specific to IL-7, the expression of pSTAT5 was measured in response to IL-2 and IL-15. As with IL-7 stimulation, there was a dose response to IL-2 and IL-15 (Figures 8A and B). Unlike IL-7-induced pSTAT5, there was no difference between controls and HCV mono-infection in the amounts of pSTAT5 produced by bulk CD8⁺ T-cells.

To determine if JAK/STAT signaling defects in HCV infection were restricted to IL-7, Bcl-2 production in response to IL-2 and IL-15 was measured. As with IL-7 stimulation, Bcl-2 production occurred in a dose dependent manner when cells were stimulated with IL-2 or IL-15 (Figures 9A and B); however, there was no difference between controls and HCV mono-infection in the amounts of Bcl-2 produced by bulk CD8⁺ T-cells. In conclusion, bulk CD8⁺ T-cells were not impaired in their response to other γ_c cytokines in HCV-infected individuals; hence the mechanism of IL-7-mediated impairment of these cells may not target and may not be specific to STAT5 activation, which is common to these cytokines.

A



B

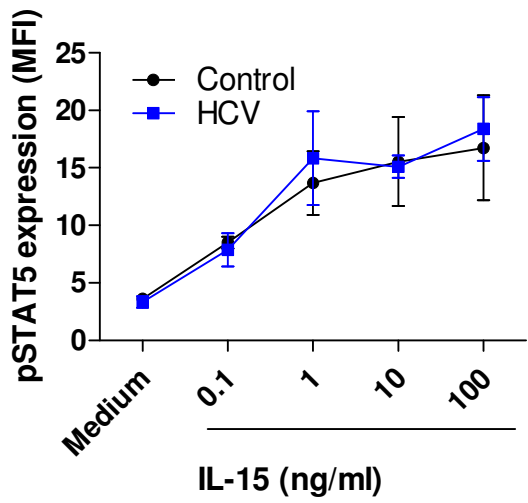


Figure 8. IL-2- and IL-15-induced activation of STAT5 is similar in blood-derived CD8⁺ T-cells from control and HCV mono-infected individuals. Blood-derived CD8⁺ T-cells were cultured with medium alone or **A)** IL-2 (1-1000ng/ml) or **B)** IL-15 (0.1-100ng/ml) for 15 minutes and the phosphorylation of STAT5 (pSTAT5) was measured by flow cytometry (control n=6, HCV n=5).

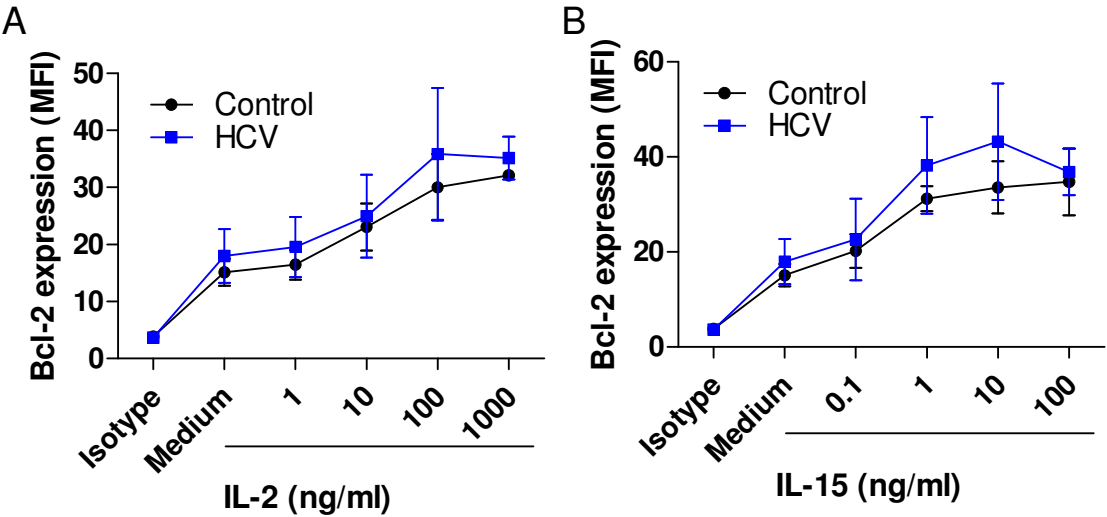


Figure 9. IL-2- and IL-15-induced production of Bcl-2 is similar in blood-derived CD8⁺ T-cells from control and HCV mono-infected individuals. Blood-derived CD8⁺ T-cells were cultured with medium alone or **A)** IL-2 (1-1000ng/ml) or **B)** IL-15 (0.1-100ng/ml) for 48 hours and the expression of Bcl-2 was measured by flow cytometry (control n=7, HCV n=9).

3.5.3. Gene expression of suppressors of cytokine signaling

The changes in IL-7-induced pSTAT5 observed may have been due to changes in the regulation of the signaling pathway by suppressors of cytokine signaling (SOCS). Baseline expression of the genes for SOCS1 and SOCS3 were measured by qPCR, and expressed relative to the expression of the housekeeping gene RPS18. The appropriate melting temperature for the primer sets chosen was determined by thermal gradient, and the efficiencies of the reactions were determined to be between 95 and 101.2% by a standard curve (Figure 10). Isolated RNA was used to measure baseline gene expression by qPCR of SOCS1 and SOCS3 directly after CD8⁺ T-cell isolation (Figures 11A, B and C) and gene expression was not found to differ significantly between controls, HCV mono- and HIV-HCV co-infection (Figures 11D and E).

Gene expression was also measured after IL-7 stimulation for 3 hours, to determine if the dysfunction observed here may be due to IL-7 induced changes in SOCS, as SOCS undergo negative feedback regulation (Figures 12A, B and C). After 3 hours with IL-7 (10ng/ml), only SOCS1 was significantly upregulated in all groups (controls $p=0.007$, HCV $p=0.02$, HIV-HCV $p=0.04$, paired Student's *t*-test, Figure 12D), with no statistical difference in IL-7-induced SOCS1 levels between groups (Figure 12F). The expression of SOCS3 was only significantly increased with IL-7 stimulation in controls ($p=0.007$, Student's paired *t*-test, Figures 12E and G), however the unstimulated level of SOCS3 expression was higher in HCV infection than in HCV controls (Figure 12E).

To ensure the qPCR results were for mRNA expression alone, a control sample of isolated RNA was included in a reverse transcription reaction without any reverse transcriptase. In addition, another control included the direct addition of isolated RNA to a

qPCR reaction. In both cases, it was determined that there was DNA contamination after RNA isolation as the qPCR reaction mixture should not have amplified any PCR products from RNA (Figure 13A). Further, RNA samples were electrophoresed in an agarose gel, and it was confirmed that there was contaminating DNA present, and it was also discovered that the isolated RNA was degraded (Figure 13B).

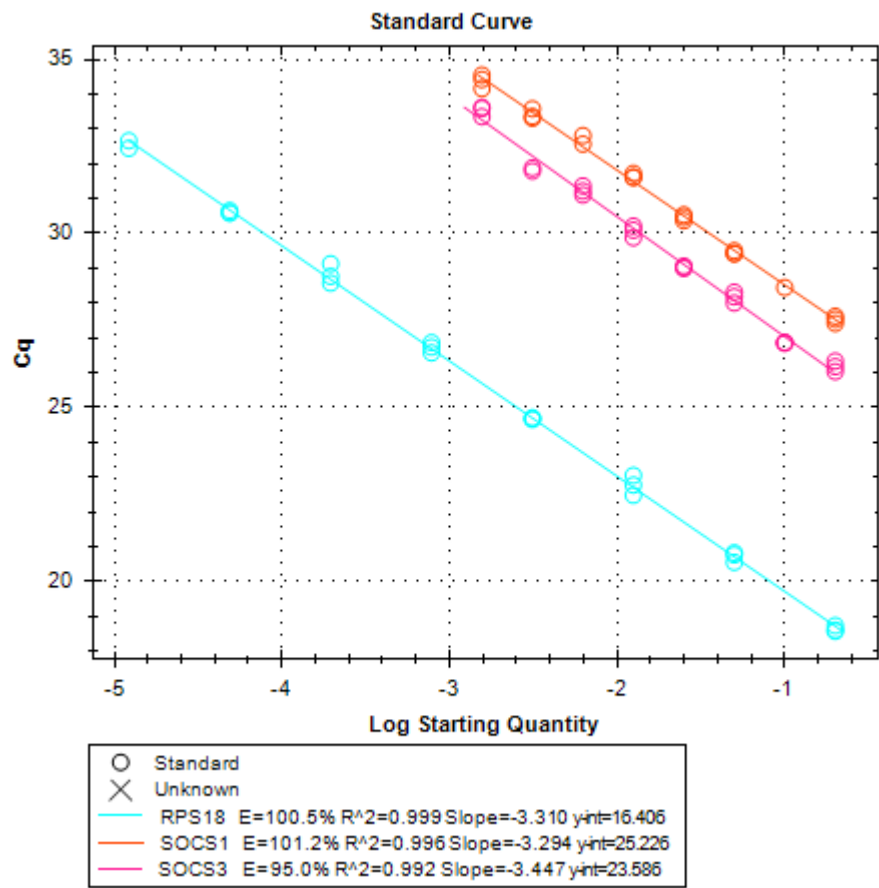


Figure 10. Primer efficiency for qPCR measurement of SOCS gene expression. Semi-quantitative PCR was completed on pooled cDNA serially diluted 4 fold (RPS18) or 2 fold (SOCS1 and SOCS3) from a starting dilution of 1:5. The efficiency of each PCR was calculated from the slope of C_q values vs. the cDNA concentration.

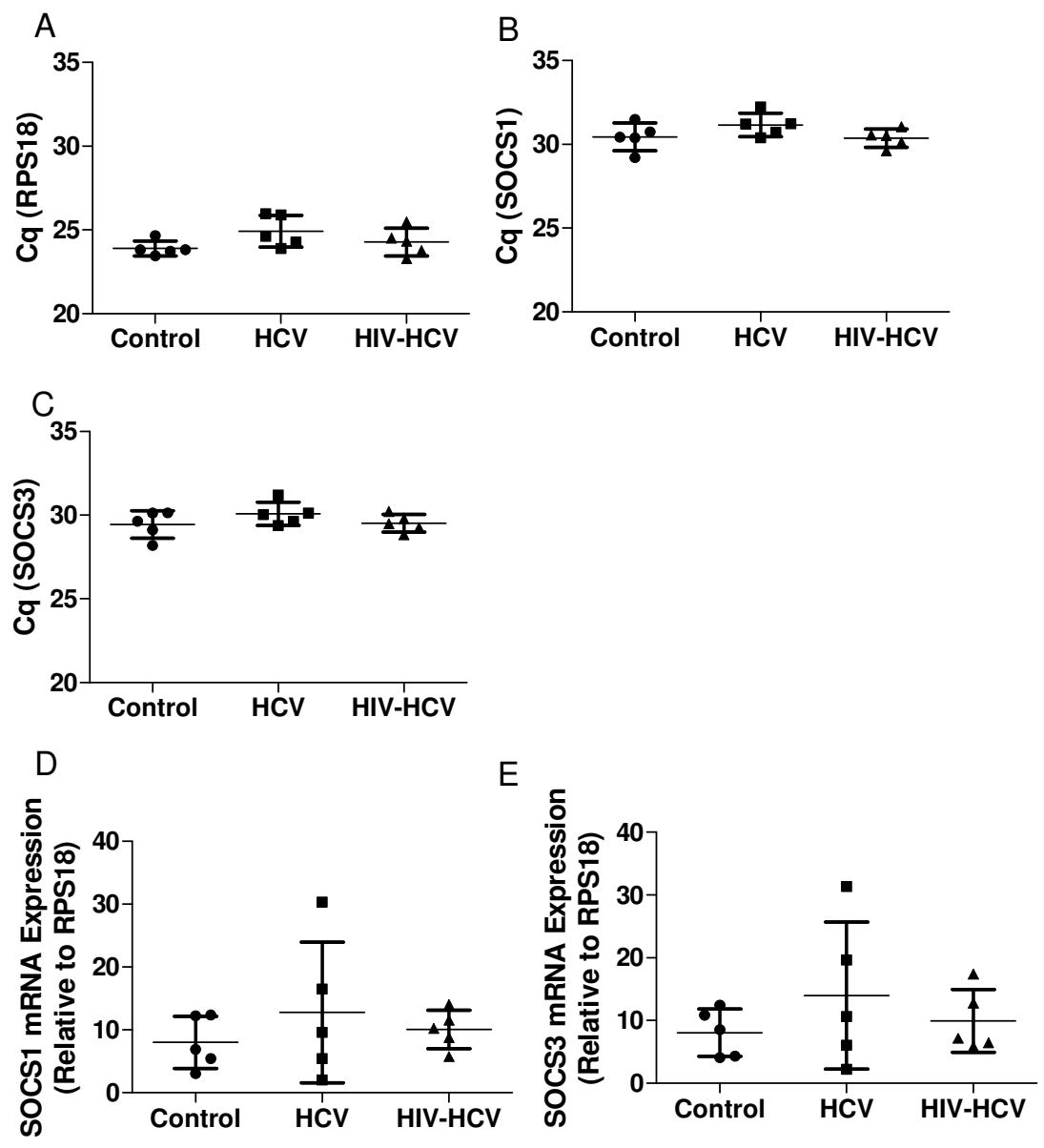
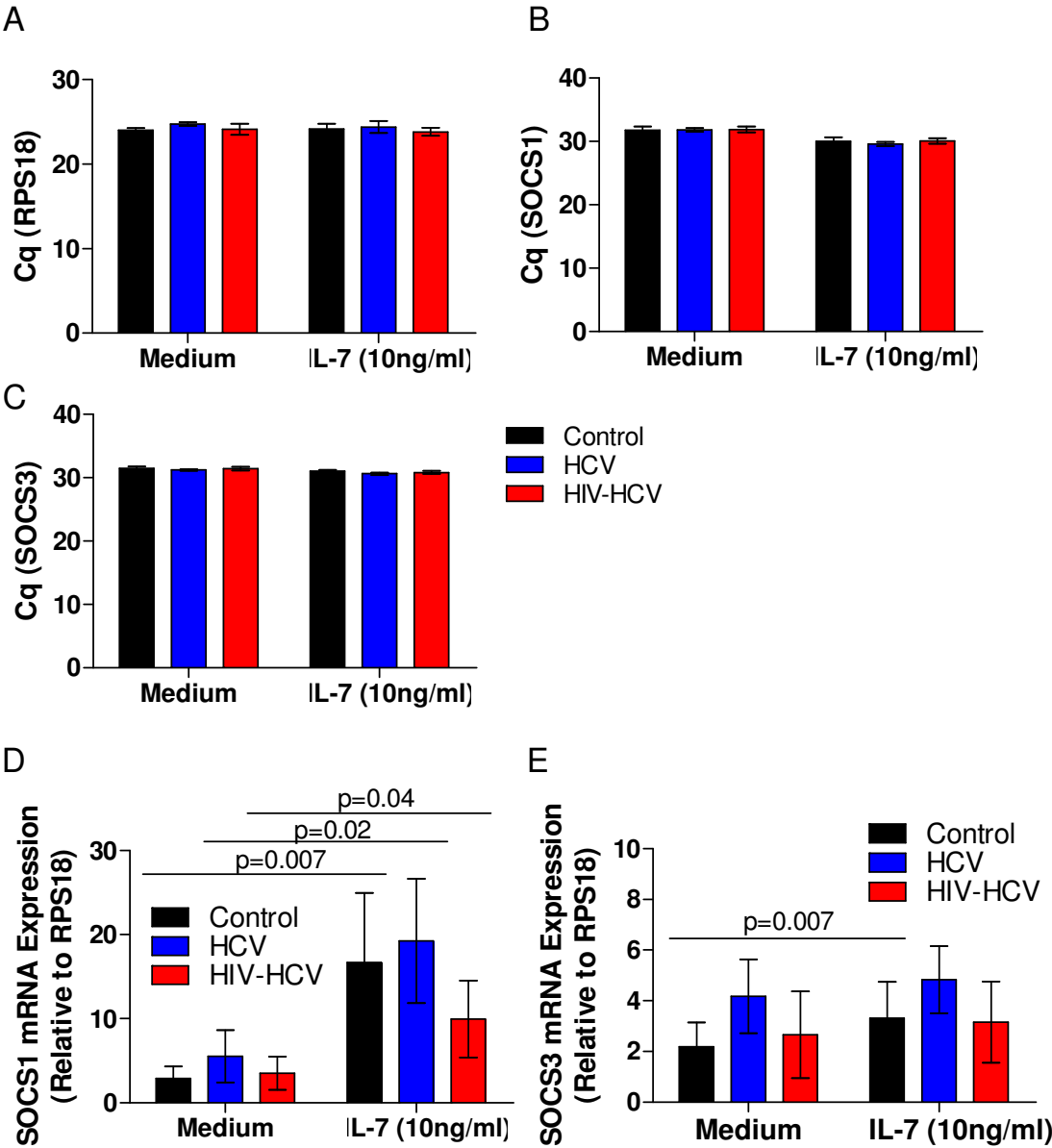


Figure 11. Basal gene expression of SOCS1 and SOCS3 did not differ between controls, HCV mono-, or HIV-HCV co-infection. The basal expression of SOCS genes in blood-derived CD8⁺ T-cells was measured by RT-qPCR. The Cq values for **A)** SOCS1, **B)** SOCS3, and **C)** RPS18 are shown, and **D)** SOCS1 and **E)** SOCS3 expression summarized as fold change from reference gene RPS18 (Control n=6, HCV n=5, HIV-HCV n=5).



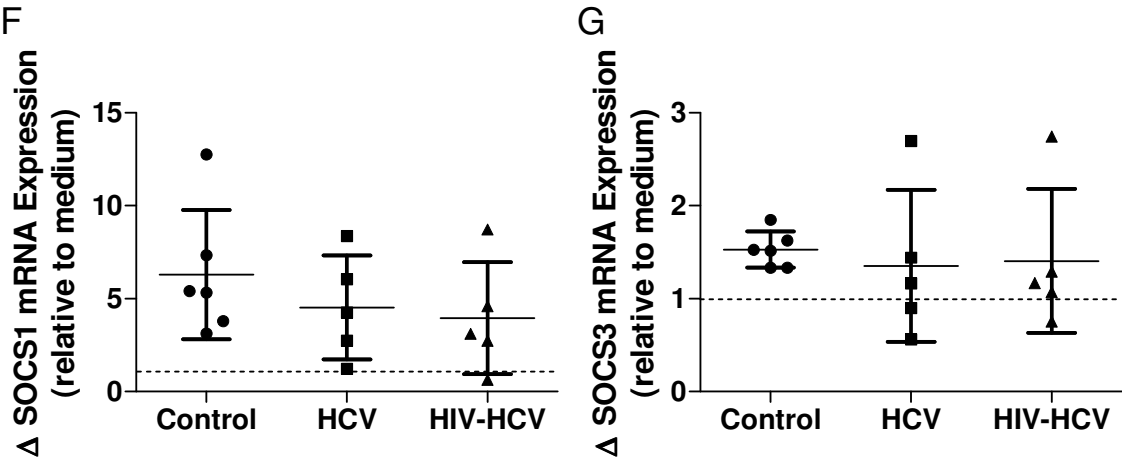


Figure 12. Gene expression of SOCS1 was upregulated in response to IL-7 in all 3 groups, while SOCS3 mRNA expression remained unchanged. CD8⁺ T-cells were cultured with medium or IL-7 (10ng/ml) for 3 hours, and mRNA expression of **A)** SOCS1, **B)** SOCS3, and **C)** RPS18 were measured by qPCR. Expression is summarized as fold change from reference gene RPS18 for **D)** SOCS1 and **E)** SOCS3 and fold change from medium alone for **F)** SOCS1 and **G)** SOCS3 with IL-7 stimulation (Student's paired *t*-test) (Control n=6, HCV n=5, HIV-HCV n=5).

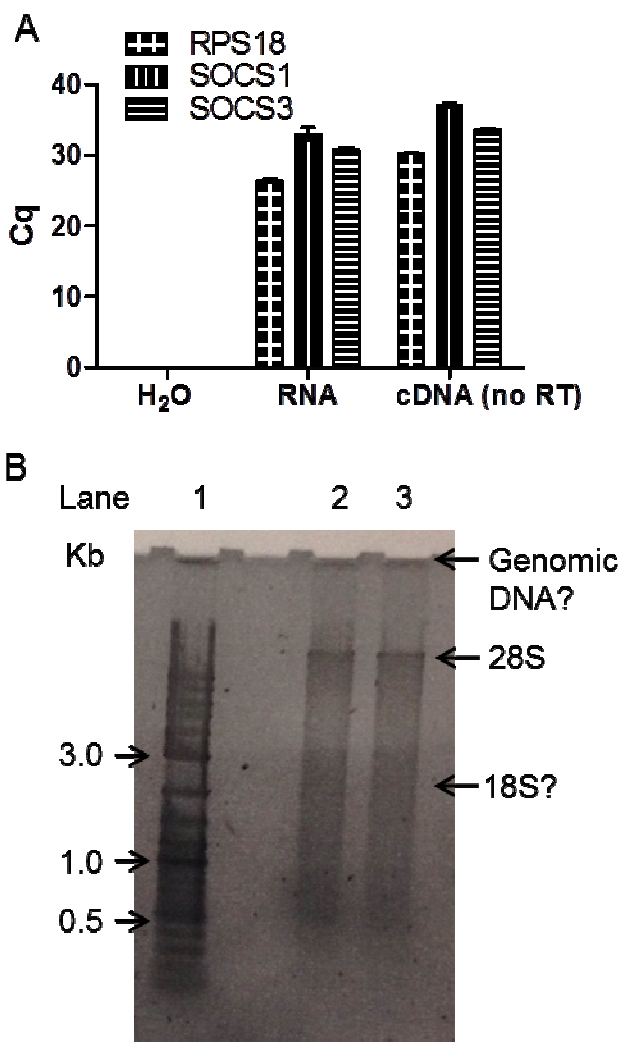


Figure 13. Controls for qPCR measurement of SOCS gene expression. **A)** Gene expression in RNA and water were included as negative controls in the qPCR reaction (n=2). **B)** Two separate pools of RNA (lanes 2 and 3) were assessed against the TriDye 2-log DNA Ladder (lane 1) by gel electrophoresis.

3.6. Phenotype of IH-CD8⁺ T-cells in HCV mono-infection

HCV infection is largely confined to the liver. Most studies of CD8⁺ T-cells in HCV infection are completed on blood-derived cells, but those present in the liver are of utmost interest. In this study, IH-lymphocytes were isolated from liver biopsy samples obtained from 4 HCV-infected individuals and one HIV-HCV co-infected individual. On average, 16.4% of isolated liver cells were CD8⁺ T-cells (± 13.7). To describe phenotypic differences in CD8⁺ T-cells present in the liver vs. blood, IH-lymphocytes were stained with anti-CD8, anti-CD45RA and anti-CCR7 antibodies and evaluated by flow cytometry (Figures 14A and B). In the same donors, the distribution of IH-CD8⁺ T-cell subsets were compared to those present in the blood and found to differ significantly. IH-CD8⁺ T-cells had a higher proportion of IH-T_{CM} cells ($39.3\% \pm 5.3$ vs. $23.5\% \pm 3.1$, $p=0.03$, unpaired Student's *t*-test), and a lower proportion of IH-T_N cells ($11.2\% \pm 6.2$ vs. $28.6\% \pm 5.6$, $p=0.01$) and IH-T_{EMRA} cells ($7.08\% \pm 3.0$ vs. $21.07\% \pm 5.2$, $p=0.03$) compared to blood-derived CD8⁺ T-cells. There was also a trend of more IH-T_{EM} cells than CD8⁺ T-cells in the blood ($42.64\% \pm 5.7$ vs. $26.8\% \pm 6.7$, $p=0.06$) (Figures 14C and D).

3.7. CD127 expression of IH-CD8⁺ T-cells

The expression level of CD127 was equivalent between blood-derived and IH-CD8⁺ T-cells ($27.8\% \pm 3.8$ and $23.6\% \pm 6.0$, respectively) (Figure 15B). Similarly, CD127 expression among CD8⁺ T-cell subsets was the same between blood-derived and IH cells, and followed the same hierarchical pattern: T_N ($66.0\% \pm 8.8$ and $81.3\% \pm 13.9$) > T_{CM} ($38.2\% \pm 17.1$ and $42.6\% \pm 21.8$) > T_{EM} ($27.6\% \pm 6.6$ and $22.3\% \pm 14.3$) $\approx$ T_{EMRA} ($10.7\% \pm 0.7$ and $23.5\% \pm 11.0$) (Figures 15A and C).

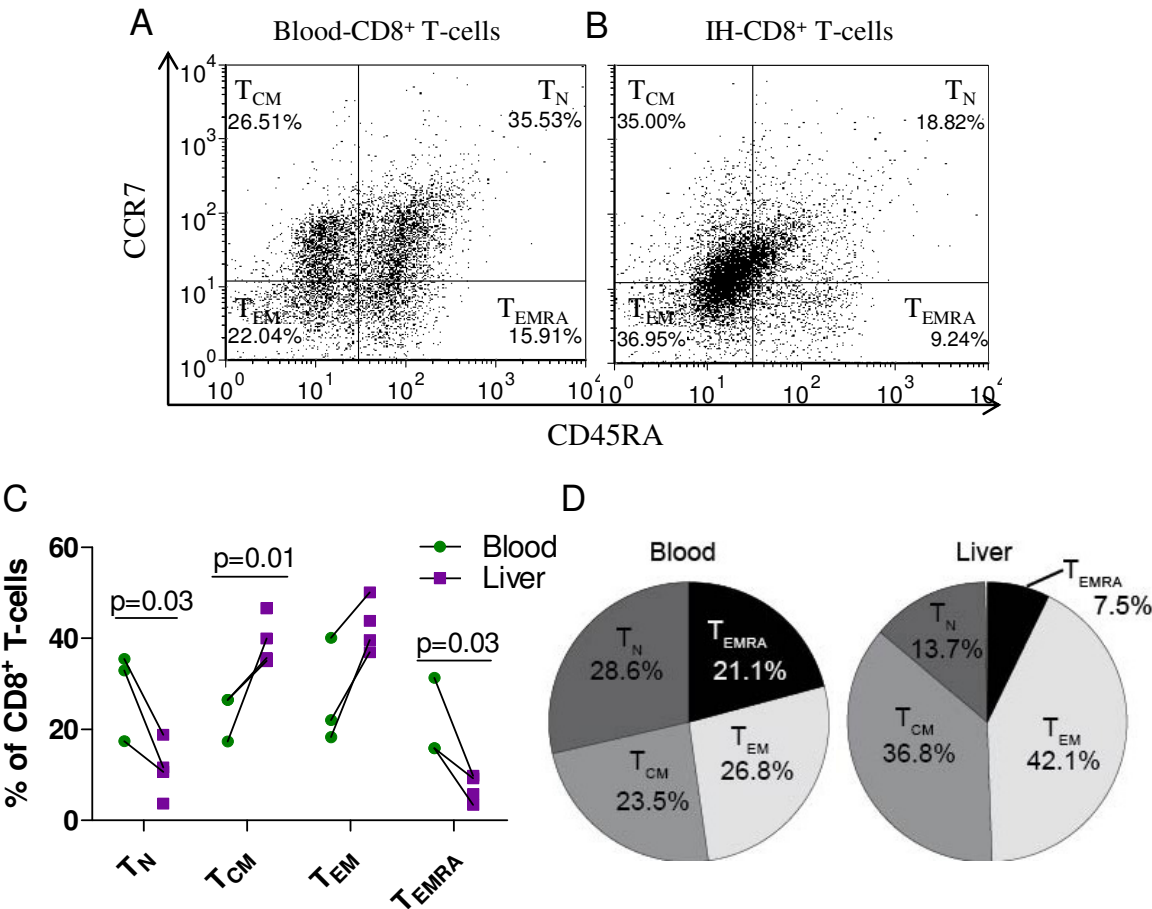


Figure 14. The proportion of CD8⁺ T_{CM} cells is increased, while T_N and T_{EMRA} cells are decreased in the liver in HCV infection. CD8⁺ T-cell subsets were distinguished by flow cytometry on the basis of CD45RA and CCR7 expression. Representative dot plots of subset distribution for **A)** blood-derived and **B)** IH-CD8⁺ T-cells from HCV⁺ individuals are shown, and the mean of these observations is summarized in **C)** a bar graph and **D)** a pie graph (blood n = 3, IH n = 4).

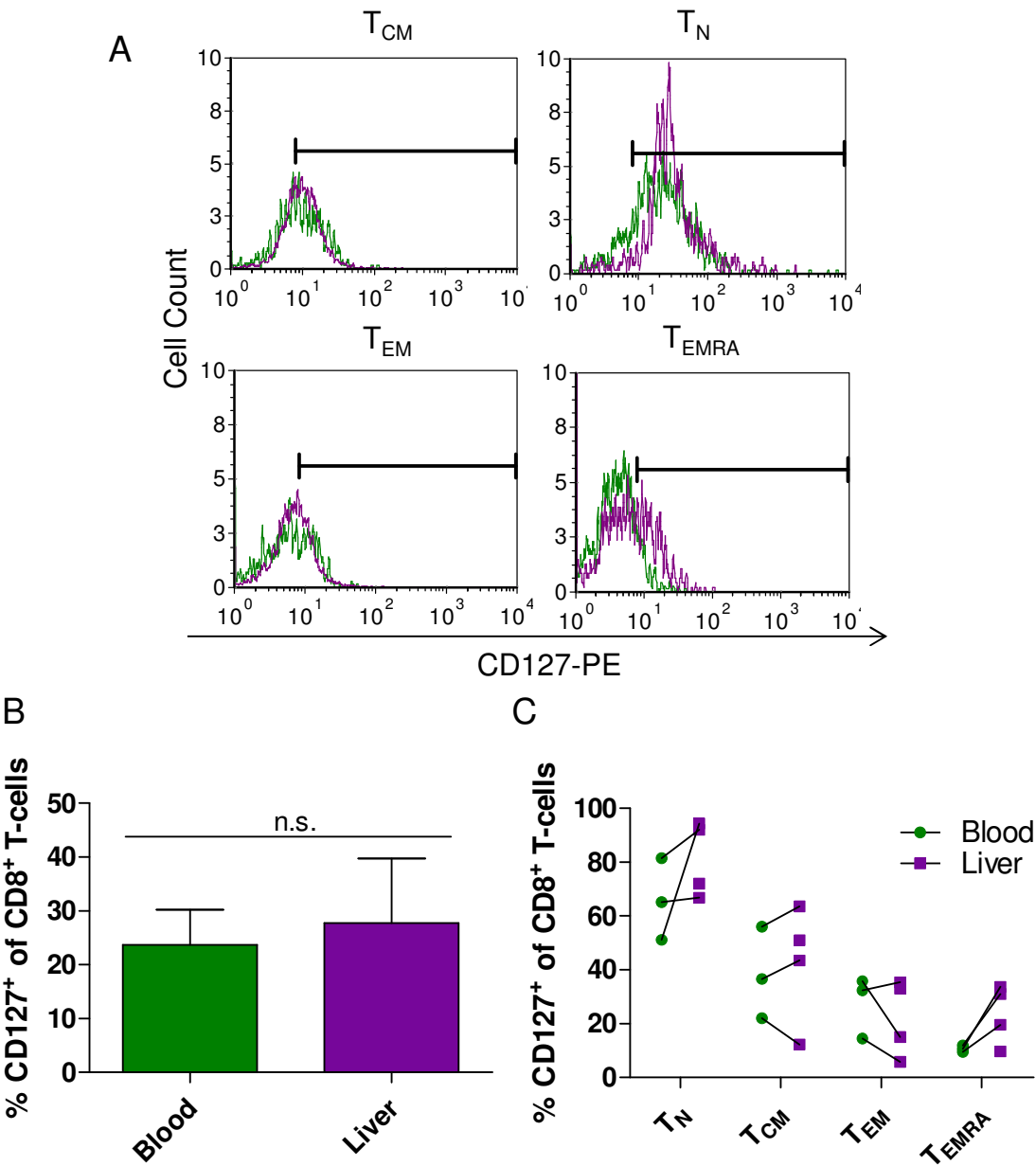


Figure 15. The expression of CD127 does not differ between IH- and blood-derived CD8⁺ T-cells. A) Membrane expression of CD127 was measured on blood-derived and IH-CD8⁺ T-cells, as shown in these representative histograms. Receptor expression on B) bulk CD8⁺ T-cells and C) CD8⁺ T-cell subsets in blood-derived or IH-CD8⁺ T-cells is represented in graphs (blood n = 3, IH n = 4).

3.8. STAT5 activation of IH-CD8⁺ T-cells

In blood-derived CD8⁺ T-cells, in all groups tested, little to no pSTAT5 was observed without cytokine stimulation (Figure 2B). In contrast, IH-CD8⁺ T-cells expressed high basal levels of pSTAT5. These levels were equivalent to the degree of signaling observed in blood CD8⁺ T-cells stimulated with a high concentration of IL-7 (10 ng/ml). To determine the ability of IH CD8⁺ T-cells to respond to IL-7 and other common gamma chain (γ_c) cytokines (e.g. IL-2 and IL-15), STAT5 activation after stimulation was investigated (Figures 16A and B). However, these γ_c cytokines did not further increase pSTAT5 levels in IH-CD8⁺ T-cells of the 2 donors tested (Figures 16C and D).

3.9. Bcl-2 expression of IH-CD8⁺ T-cells

As a measure of survival potential, the basal expression of Bcl-2 was determined after overnight culture (Figure 17A). The autofluorescence of IH-CD8⁺ T-cells was higher than blood-derived cells, and differed between the HCV mono-infected and HIV-HCV co-infected individual (Figure 17B). Once basal Bcl-2 expression was normalized to autofluorescence, Bcl-2 expression in IH-CD8⁺ T-cells from both sets of donors was lower than that of blood-derived CD8⁺ T-cells in HCV mono-infection (Figure 17C).

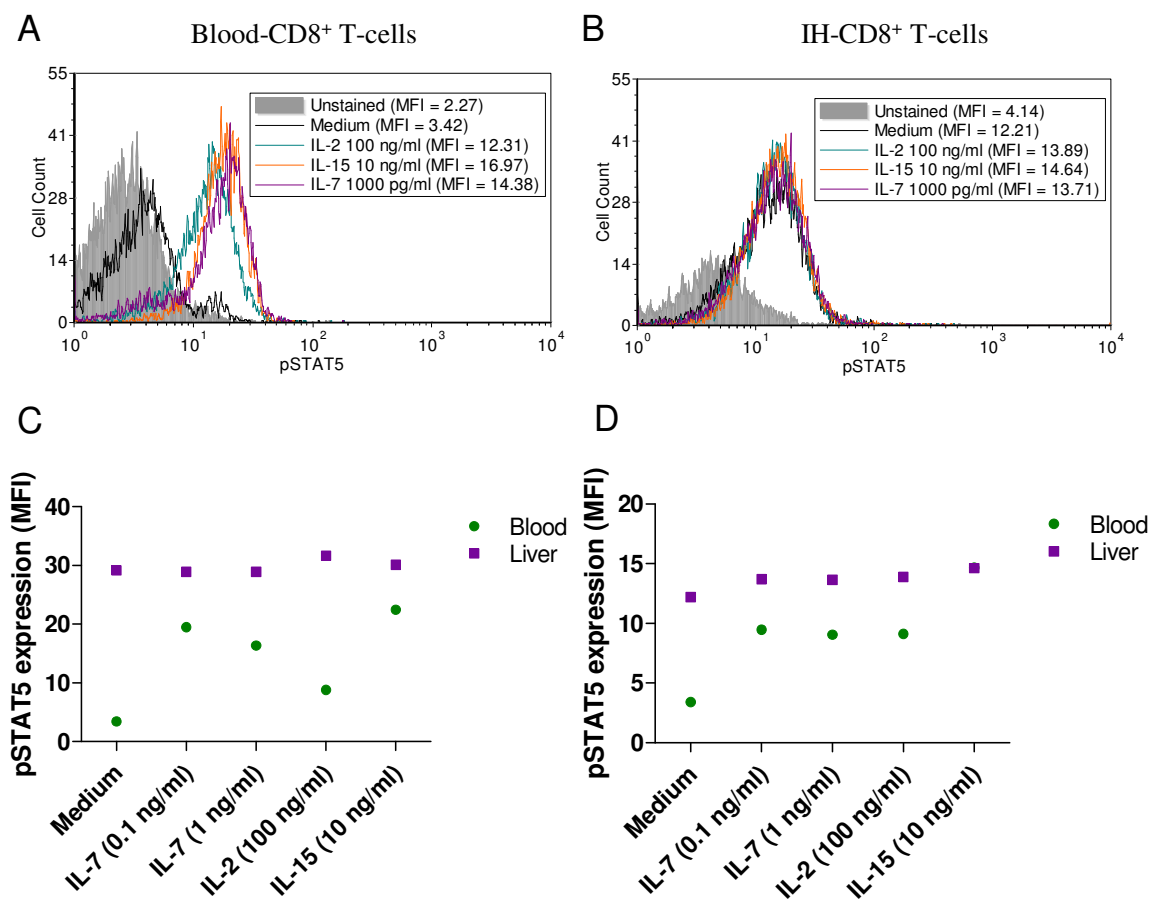


Figure 16. Intrahepatic-CD8⁺ T-cells exhibit high basal activation of STAT5 with no further activation with γ_c cytokines. PBMC and IH-lymphocytes from liver biopsy tissue were cultured with STAT5-activating γ_c cytokines (IL-7 (0.1 or 1ng/ml), IL-2 (100ng/ml), or IL-15 (10ng/ml)) for 15 minutes and pSTAT5 expression of CD8⁺ T-cells was measured by flow cytometry. Representative histograms of pSTAT5 expression in **A)** blood-derived and **B)** IH-CD8⁺ T-cells are shown and **C & D)** data for pSTAT5 expression are shown for each donor with matching blood and liver samples, summarized as MFI values, with no significant differences between unstimulated and stimulated IH-CD8⁺ T-cells detected (blood n=2, liver n=3, paired Student's *t*-test).

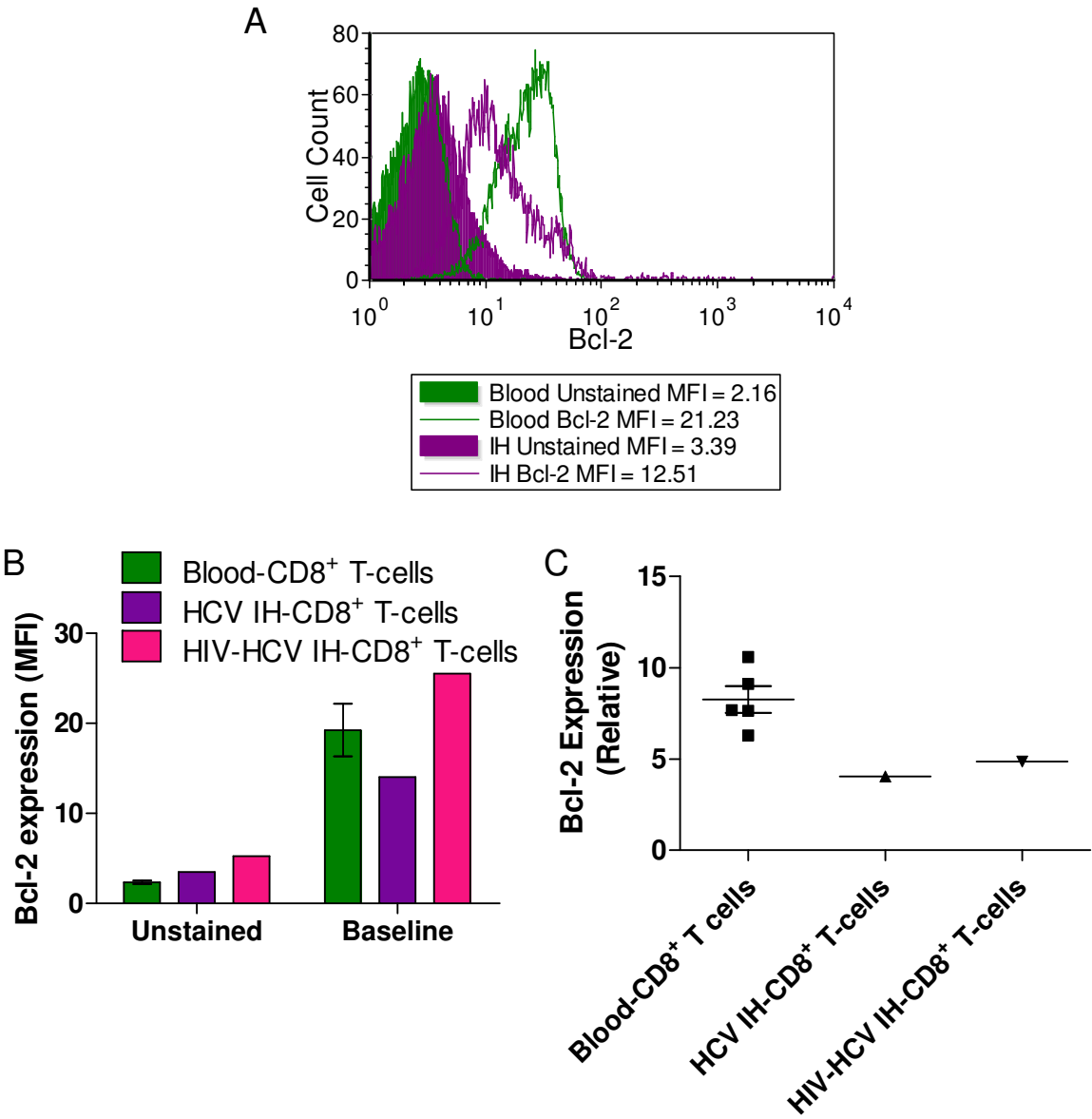


Figure 17. Basal Bcl-2 expression in IH-CD8⁺ T-cells is lower than blood-derived CD8⁺ T cells. **A)** The expression of Bcl-2 in blood-derived CD8⁺ T cells and IH-lymphocytes from liver biopsy tissue was measured without cytokine stimulation after overnight rest at 37°C (basal) and **B)** is summarized (MFI). **C)** Baseline Bcl-2 is also presented relative to unstained cells due to differing degrees of autofluorescence (blood n=5, IH n=1 each).

Chapter 4: Discussion

In this thesis, data are presented which demonstrate the impaired activity of CD8⁺ T-cells isolated from individuals with chronic HCV mono- and HIV-HCV co-infection. This was characterized by an impaired responsiveness to IL-7 in the absence of any observed changes in IL-7 receptor expression and was independent of antigen specificity. This impairment was found to be more pronounced in HIV-HCV co-infection compared to HCV mono-infection. These findings differ from what has been observed in HIV mono-infection, another chronic viral infection mediated in part by impaired CD8⁺ T-cell function, where IL-7 impairment is mainly attributed to decreases in CD127 expression. Also, IH-CD8⁺ T-cells were found to have decreased Bcl-2 expression and were unable to increase activation of STAT5 with cytokine compared to blood-derived cells. These findings are novel as most studies of CD8⁺ T-cells in HCV infection have examined HCV-specific cells while bulk CD8⁺ T-cell activity is less well understood.

4.1. Differences in CD8⁺ T-cell phenotype in chronic HCV infection

Before assessing the activity of bulk CD8⁺ T-cells in HCV infection, the distribution of cell subsets was first described. In this thesis, CD8⁺ T-cell subsets were distinguished by expression of CD45RA and CCR7. These markers have been used and well characterized by others (Carrasco et al., 2006; O'Connor et al., 2010; Sallusto et al., 1999). The proportions of blood-derived CD8⁺ T-cell subsets in chronic HCV infection were slightly different than those in HCV⁻ controls. Fewer T_N cells were found in chronic HCV infection (Figures 1D and E) and this is consistent with a previous report (Hartling et al., 2013). An increased proportion of T_{EMRA} cells was observed in the older age group (Figure 1F). Increasing age

has been associated with an increased proportion of T_{EMRA} cells, especially in individuals who are CMV positive. The number of CMV-specific CD8⁺ T-cells can drastically increase with age, and many of these CMV-specific cells are T_{EMRA} cells (Faist et al., 2010). In the present study, it is unknown which of the individuals may have been CMV positive, or the percentage of cells that were CMV-specific. However, no differences in STAT5 activation in T_{EMRA} cells (Figure 2C) and no other inter-group age associated differences in IL-7 responses were found throughout the study (Supplemental Tables), suggesting age and CMV-specific CD8⁺ T_{EMRA} cells did not account for any of the observed changes.

Where possible, pertinent clinical information about study subjects, such as HCV genotype and stage of fibrosis, was compared to IL-7 responses to test for any potential correlations. The small size of this study did not allow for a wide distribution of characteristics, hence, could not evaluate the association of clinical measures with data in all experiments. Heterogeneity of age, viral genotype, CMV status, etc., among individuals tested may have also contributed to observed variability. Any future studies will recruit a larger number of subjects to allow for more equal distribution of characteristics. The association of fibrosis score with CD8⁺ T-cell activity would be the most interesting to evaluate given observations linking CD8⁺ T-cell function to liver fibrosis. This would support previous studies linking liver disease progression (as measured by fibrosis) with changes in CD8⁺ T-cell function (Feuth et al., 2013; Hartling et al., 2013; Hoare et al., 2013).

4.2. Effect of chronic HCV infection on IL-7 Signaling

In chronic HCV infection, CD8⁺ T-cells have been described to have impaired function in response to HCV peptides, but their responsiveness to cytokine is less well

described. This study assessed responses to IL-7 because it is a crucial cytokine for T-cell development and is important for memory T-cell function and homeostasis. Also, in HIV infection, impaired CD8⁺ T-cell responses to IL-7 are well described, and occur by both receptor-mediated and cell intrinsic mechanisms (Juffroy et al., 2010; Vranjkovic et al., 2011). This is similar to what was observed in this study, where there were no changes in CD127 expression detected and changes in IL-7-induced activities were independent of CD127 expression and thus are potentially cell intrinsic (Figure 5). IL-7 initiates signaling in the cell, and phosphorylation of STAT5 leads to dimerization with another pSTAT5 and initiation of gene transcription (e.g. Bcl-2, growth factor, and SOCS genes) that leads to IL-7 induced functions (Palmer et al., 2008). In this study, in HCV infection, less IL-7-induced signaling was initiated in blood-derived CD8⁺ T-cells compared to controls. This was observed in bulk CD8⁺ T-cells (Figure 2B) with a distinct impairment in T_N and T_{CM} cells (Figure 2C).

Once activated, STAT5 induces the transcription of several target genes including those which regulate proliferation, ion channels, DNA repair, apoptosis, and neuronal development (Kanai et al., 2014). The two most common activities associated with pSTAT5 expression in T-cells are proliferation and anti-apoptotic protein production, and hence were evaluated here.

4.3. IL-7-induced CD8⁺ T-cell function is impaired in chronic HCV infection

It has been shown that production of Bcl-2 is dependent on pSTAT5 expression (Crawley et al., 2014; Tripathi et al., 2010). Along with decreased pSTAT5 in response to IL-7, there was also decreased Bcl-2 expression by CD8⁺ T-cells in chronic HCV infection compared to controls (Figure 4D). Bcl-2 is important for preventing apoptosis by

neutralizing pro-apoptotic proteins that induce mitochondrial membrane permeability, and by inhibiting caspases (Czabotar et al., 2014; Tsujimoto, 1998). In CD8⁺ T-cells, decreased Bcl-2 expression has been associated with more apoptosis (Cheng et al., 2004) and CD8⁺ T-cells have been found to be more susceptible to apoptosis in response to α CD3/28 (TCR) stimulation in HCV infection (Zhao et al., 2013). In Jurkat cells, a T-cell line frequently used to study T-cells, knockdown of Bcl-2 leads to increased activation-induced cell death and caspase 3 expression, directly linking reduced Bcl-2 expression with cell death (Lee et al., 2014). Bcl-2 isn't the only anti-apoptotic protein that may be affecting CD8⁺ T-cell susceptibility to apoptosis in HCV infection, as another member of the Bcl-2 anti-apoptotic family, Mcl-1, is also reduced in both CD127⁺ and CD127⁻ HCV-specific CD8⁺ T-cells (Larrubia et al., 2013). The effect of small decreases in Bcl-2 on CD8⁺ T-cell life span, and function *in vivo* can only be hypothesized here as decreases in Bcl-2 indicate the cells are more susceptible to apoptosis, and do not guarantee subsequent apoptosis. In the future, apoptosis of CD8⁺ T-cells could be measured to understand the consequences of impaired basal and cytokine-induced Bcl-2 production.

The proliferation of CD8⁺ T-cells in response to a mitogen (PHA) and IL-7 was similar between controls and HCV infection (Figure 3C). This differs from observations in HIV infection where IL-7-induced proliferation is impaired (Vranjkovic et al., 2012). While proliferation in response to IL-7 has not been evaluated in HCV infection previously, no deficiency in anti-CD3/CD28-mediated CD8⁺ T-cell proliferation has been observed (Zhao et al., 2013), though decreased proliferation in response to HCV peptide has been reported (Wedemeyer et al., 2002). IL-7-induced proliferation may be mediated through the JAK/STAT and PI3K pathways. The PI3K pathway activity may have compensated for

reduced STAT5 activation (Crawley et al., 2014; Swainson et al., 2007), although in this study, the pathway could not be reliably evaluated.

Production of Bcl-2 and proliferation are only 2 of the many IL-7-induced CD8⁺ T-cell functions that have been evaluated by others in other contexts (Crawley et al., 2014; Smyth et al., 1991). Examination of cytotoxic function, such as production of IFN- γ , CD107a (degranulation marker), and perforin, could reveal functional deficits with potential implications in immune response. Direct cytolytic assays, such as a mixed lymphocyte reaction, would indicate the ability of CD8⁺ T-cells to directly kill cellular targets in chronic HCV infection. The ability of HCV-specific CD8⁺ T-cells to perform many of these functions in response to HCV peptide has been studied (less production of perforin, reduced CTL function, and reduced ability to produce IL-2, TNF- α and IFN- γ) (Rehermann, 2007) but have not been evaluated on a bulk CD8⁺ T-cell platform. This could be studied through T-cell response to cytokine or activity in a mixed lymphocyte reaction as antigen-specificity would not be a concern.

4.4. Further impairment in HIV-HCV co-infection

As co-infection with HIV is very common in HCV infection, it was of great interest to understand the immunological burden of these concurrent chronic viral infections. The activity of CD8⁺ T-cells isolated from HIV-HCV co-infected individuals was assessed. The study focused on individuals with successful HAART, the standard of care for co-infected individuals. Phenotypically, subpopulations of CD8⁺ T-cells in HIV-HCV co-infection were similar to those in HCV mono-infection and HCV⁻ controls, with only a higher proportion of T_{EM} cells detected (Figure 1D). Only an increase in effector cells has been observed and reported in HIV-HCV co-infection (Feuth et al., 2013). After evaluation of CD8⁺ T-cell

responses to IL-7 in HIV-HCV co-infection, it was found that the degree of STAT5 activation was lower than in HCV mono-infection or HCV⁻ controls, in all cell subsets that produce detectable IL-7-induced pSTAT5 expression (Figure 2C). A high level of individual variation in responses prevented any conclusion on impairment of IL-7-induced pSTAT5 expression in bulk CD8⁺ T-cells in HIV-HCV co-infection (Figure 2B). In HAART-treated HIV mono-infection, IL-7-induced activation of STAT5 by CD8⁺ T-cells is impaired (Juffroy et al., 2010; Vranjkovic et al., 2011), but it is not known how the magnitude of the impairment in HIV-HCV co-infection compares to that observed in HIV mono-infection.

Of the two IL-7-induced functions measured here, it was found that Bcl-2 expression of CD8⁺ T-cells is similarly impaired in HCV mono- and HIV-HCV co-infection (Figure 3D), while IL-7-induced proliferation is significantly impaired only in HIV-HCV co-infection (Figure 5D). In HIV mono-infection, CD8⁺ T-cells produce less Bcl-2 and undergo less proliferation in response to IL-7 than healthy controls (Vranjkovic et al., 2011). As Bcl-2 is directly dependent on pSTAT5, one might expect it to be further impaired in HIV-HCV co-infection as pSTAT5 responses to IL-7 were lower than in HCV mono-infection (Figure 2C). While it is known that Bcl-2 production in CD8⁺ T-cells requires STAT5 activation (Crawley et al., 2014; Tripathi et al., 2010), the direct relationship between the degree of STAT5 activation and the amount of Bcl-2 production is unknown. In HIV-HCV co-infection, CD8⁺ T-cells are pre-disposed to activation-induced cell death (AICD), which could be explained by the reduced Bcl-2 expression observed, as prevention of Bcl-2 production has been found to increase AICD (Harcourt et al., 2005; Lee et al., 2014).

The proliferation of CD8⁺ T-cells cultured with IL-7 alone or IL-7 + PHA was significantly decreased in HIV-HCV co-infection, yet remain unchanged in HCV mono-

infection compared to HCV⁻ controls (Figure 3D). IL-7-induced proliferation is known to be impaired in HIV mono-infection and both phosphorylation of STAT5 and Akt signaling pathways are also impeded (Vranjkovic et al., 2011). Perhaps there is no compensatory proliferation support by the PI3K pathway, as suggested here to occur in HCV mono-infection. Therefore, it appears that in this case, HIV infection determines the extent of IL-7-induced proliferation in HCV infection, potentially by impairing both elements of the IL-7 signaling pathway involved in cell proliferation.

The underlying mechanisms of the CD8⁺ T-cell impairment observed in HIV-HCV co-infection and HCV mono-infection are unknown but it is possible that either similar influences or disease-specific causes may be involved. HIV-HCV co-infection is a complex issue as age at infection, the order of infection, and time between infections may impact the mechanisms of impairment. In the case of STAT5 and proliferation, CD8⁺ T-cells were even more impaired than in HCV mono-infection, which could be attributed to HIV infection. With Bcl-2 production, where there was no further impairment, HCV infection may have been the primary determinant, though without an HIV mono-infected control this cannot be definitively stated. This is consistent with others who found HCV infection to be the determining variable in rate of T-cell apoptosis in HIV disease (Laskus et al., 2013).

This study did not include an HIV mono-infected, HAART-treated control group. The effect of HIV on IL-7 signaling is well described and was determined to be outside the scope of this study. In the future, the inclusion of HIV mono-infected individuals would be of value to directly compare such immunological outcomes as those measured here, and to determine the potential additive effects of 2 ongoing chronic infections.

4.5. Associations with fibrosis

Although this study was not designed to test associations with functional outcomes of IL-7 stimulation in HCV infection, it is possible to draw certain conclusions using the clinical data available for some of the individuals tested herein. In HCV infection, the potential to produce Bcl-2 in response to high concentrations of IL-7 (10ng/ml) was correlated with the degree of liver fibrosis (i.e. lower Bcl-2 production in those with fibrosis (F3-F4) compared to those with little to none (F0-2) (Figure 4E). This indicates there may be an association between the degree of fibrosis and susceptibility of CD8⁺ T-cells to apoptosis. With increased fibrosis, CD8⁺ T-cells have been found to have more double strand DNA breaks, which are known markers of apoptosis in T-cells (Hoare et al., 2013; Kaina, 2003). In this study, fibrosis was not associated with any changes in CD8⁺ T-cell subsets, CD127 expression, pSTAT5 expression or proliferation. This is in contrast to other studies where CD127 expression on CD4⁺ T-cells was inversely correlated with fibrosis (Kared et al., 2014), however, CD4⁺ T cells may be regulated differently than CD8⁺ T-cells in HCV infection. In chronic HCV, increasing fibrosis is negatively correlated with the number of recent thymic emigrants in circulation (CD4⁺ and CD8⁺ T-cells) (Hartling et al., 2013), which could be explained by the decreased response to IL-7 detected here since IL-7 is so critical for T-cell development in the thymus. In the individuals tested in this study, there was not a wide range of fibrosis scores; in some experiments there may have only been one, or no, individuals with a score > F0-1. In the future, a balanced recruitment of individuals across the fibrosis score spectrum may offer greater insight in the determinations of how fibrosis may contribute to CD8⁺ T-cell activity.

4.6. Intrahepatic-CD8⁺ T-cells in chronic HCV infection

The collection of lymphocytes from liver biopsies is a valuable resource from which to seek insight into what is occurring *in vivo* in the liver during chronic HCV infection. Some studies of IH-CD8⁺ T-cells have been reported, but they are limited to either the description of *ex vivo* characteristics or assays of long-term *in vitro*-expanded IH-CD8⁺ T-cells (Abel et al., 2006; Barrett et al., 2014; Spangenberg et al., 2005). In this study, while rare, sufficient CD8⁺ T-cells were collected from liver biopsies to allow for evaluation of cell surface marker-based phenotypes, activation of STAT5, and basal expression of Bcl-2. Biopsies collected from four individuals with HCV mono-infection and one from an HIV-HCV co-infected individual were analyzed in this research. No biopsy tissues were collected from control individuals, but IH-CD8⁺ T-cells were compared to blood-derived CD8⁺ T-cells from the same individual for internal comparisons. Unfortunately, a matching blood sample was not available in one HCV mono-infection case, and the blood samples were not always collected on the same day as the biopsy. In each experiment using liver-derived cells, the flow cytometer settings were calibrated using blood-derived cells in order to maximize the number of IH-CD8⁺ T-cells that could be used for the experiments.

Phenotypically, IH-CD8⁺ T-cells are almost completely T_{EM} and T_{CM} cells (Figures 14C and D). This is in contrast to blood-derived where there is a more even distribution of the four subsets (Figure 1). It is to be expected that the cells infiltrating the liver tissue are antigen experienced. It is known that liver infiltrating CD8⁺ T-cells in both health and infection are of a memory (CD45RA-) phenotype (Heydtmann et al., 2006; Kroy et al., 2014; Sumida et al., 2013), and a greater proportion of IH-CD8⁺ T-cells can respond to antigen than blood-derived CD8⁺ T-cells (Vali et al., 2008).

IH-CD8⁺ T-cells were found to have a high expression of pSTAT5 and no ability to produce more pSTAT5 with γ_c cytokine stimulation (Figures 16C and D). In viral hepatitis (HCV or HBV), IH-CD8⁺ T-cells frequently have an activated phenotype (i.e. decreased CD28 and increased IFN- γ expression) (Valiante et al., 2000). The elevated level of pSTAT5 in unstimulated cells may be due to the inherent activation of liver-infiltrating cells during hepatic viral infection, in contrast to the activity level of their counterparts in the circulation. In addition, due to the low number of CD8⁺ T-cells in each biopsy, they were not separated from other lymphocytes and contaminating hepatocytes, and were identified by flow cytometry on the basis of CD8 expression. The non-CD8⁺ T-cells present in the culture may have helped maintain the high STAT5 activation observed in the CD8⁺ T-cells, particularly IL-7 cytokine secretion by hepatocytes (Larrea et al., 2014). This was not the case in blood-derived CD8⁺ T-cells, where they were not isolated from PBMC yet CD8⁺ T-cells in unstimulated cultures expressed little or no STAT5 activation.

Despite a high level of STAT5 activation, the inability to activate STAT5 further with STAT5-signaling cytokines suggests that IH-CD8⁺ T-cells may be unresponsive to IL-7. This could be due to close proximity to the ongoing infection or to factors specific to the liver itself. Liver dendritic cells, hepatocytes, and Kupffer cells expressing co-stimulatory molecules can activate CD8⁺ T-cells in inflammatory conditions (Chen et al., 2005; Huang et al., 2013; Jenne and Kubes, 2013; Tokita et al., 2008), however, T-cells activated in the liver are more prone to apoptosis and produce less IFN- γ and IL-2 (Bertolino et al., 2002; Holz et al., 2012; Tokita et al., 2008). This may contribute to the lower Bcl-2 expression found in IH-CD8⁺ T-cells compared to blood-derived cells (Figure 17C), along with the differences in CD8⁺ T-cell subsets observed (Figure 14C). It is not known whether these

differences in pSTAT5 and Bcl-2 expression are due to the higher proportions of T_{CM} and T_{EM}, and fewer T_N and T_{EMRA} cells that we, and others, have observed in the liver, compared to blood-derived cells (Figure 15C) (Kroy et al., 2014; Sumida et al., 2013).

Due to the limited access of clinical samples, only one liver biopsy was obtained from an HIV-HCV co-infected individual. The data for this sample were very similar to that observed in the other biopsies from HCV mono-infected individuals. A lack of CD4⁺ T-cell help, higher apoptosis (Canchis et al., 2004; Vali et al., 2008), and high number of regulatory cells (Barrett et al., 2014) has been reported in the liver in HIV-HCV co-infection compared to HCV mono-infection. Therefore there may have been differences between IH-CD8⁺ T-cells in HCV mono- and HIV-HCV co-infection if the sample size was increased.

CD8⁺ T-cells are hypothesized to be a driving force of the apoptosis and subsequent fibrosis seen in the liver in chronic HCV infection. During infection with dengue virus, a member of the flaviviridae family, CD8⁺ T-cells infiltrate into the liver, directly leading to increased cleavage of caspase 3 and increased proportions of TUNNEL⁺ hepatocytes, indicative of apoptosis, which was prevented by depleting CD8⁺ T-cells (Sung et al., 2012). It has also been suggested that much of the liver pathology of hepatic infections is due to liver infiltration of non-virus specific CD8⁺ T-cells and their inappropriate activation and killing. In HBV infection, individuals with no liver pathology have very high proportions of HBV-specific CD8⁺ T-cells infiltrating into the liver, while those exhibiting liver damage have a greater proportion of HBV non-specific CD8⁺ T-cells in the liver (Maini et al., 2000). In other non-infectious liver diseases, CD8⁺ T-cells are also implicated as the driving factor of hepatocyte damage and eventual hepatocellular carcinoma development. In a mouse model of nonalcoholic steatohepatitis (NASH), development of NASH lead to activation of

CD8⁺ T-cells and NKT-cells, and production of cytokines, inducing hepatocyte death and liver damage (Wolf et al., 2014). It may be the inappropriately high activation of CD8⁺ T-cells, of bulk cells or cells that are not required for immune response in the liver, which may contribute to observed bystander death of hepatocytes and liver damage.

Studies investigating IH-CD8⁺ T-cells by flow cytometry are challenging due to the high autofluorescence of liver cells (Sheahan and Rice, 2013). Also, HCV infection in the liver is very focal, occurring in clusters (Stiffler et al., 2009), and if the biopsies collected were not from an area near a focus of infection, the IH-CD8⁺ T-cells may be very different, or not even present to the same extent. In addition, it is unknown how many contaminating hepatocytes were present in these experiments as a hepatocyte marker was not found to use for flow cytometry. In the future, further mechanical disruption may increase the number of lymphocytes isolated, and histological staining of a portion of a biopsy may provide an appreciation of the composition and quantities of cells present.

4.7. Potential contributors to impairment – CD127, IL-7 specificity, and SOCS

As CD127 is the first point at which IL-7 interacts with the cell, it is an obvious potential culprit for observations of reduced downstream signaling and function, particularly given its reported role in decreased IL-7 response in HIV infection. One might expect that the reduced T_N cell population might account for the lower pSTAT5 signaling and Bcl-2 expression observed in HCV infection, as they are the cell type with the highest expression of CD127 and the most responsive to IL-7. This was not the case; as overall, CD127 expression on bulk CD8⁺ T-cells was not different between controls and HCV-infected individuals (Figure 5B).

In HIV infection, decreased CD127 expression and decreased response to IL-7 are well established, and the latter is not entirely dependent on the former. In response to IL-7, there is reduced signaling, Bcl-2 production, and proliferation of CD127⁺CD8⁺ T-cells compared to controls, indicating there may be inherent cell deficiencies in HIV infection (Vranjkovic et al., 2011). This is similar to what has been found here, in that there was no change in CD127 expression on bulk CD8⁺ T-cells in HCV infection (Figure 5B), but there were decreases in responses when stimulated with IL-7. The only CD8⁺ T-cell subset to exhibit a lower expression of CD127 in HCV infection, compared to controls, was T_{CM} cells (Figure 5C). But, signaling of T_N cells, whose CD127 expression was unaltered with infection, had an even lower production of pSTAT5 in response to IL-7 than T_{CM} cells, so the CD127 expression of T_{CM} cells may not have played an important role in their impairment (Figure 2C).

The lack of change in CD127 expression is not unique to this study. While CD127 has been used as a predictor for clearance of HCV during the acute stage (Golden-Mason et al., 2006), others have found no difference in CD127 expression on bulk CD8⁺ T-cells in chronic HCV infection (Hartling et al., 2013), nor between patient age, gender, or ethnicity (Larrubia et al., 2013). A decrease in CD127 expression on bulk CD8⁺ T-cells was observed in HIV-HCV co-infection (Figure 5B), though there were no statistically significant differences in any of the subsets (Figure 5C). This decreased CD127 expression may contribute to the even lower level of pSTAT5 expression and lower proliferation seen in co-infection compared to HCV mono-infection (Figures 2C and 3D). The lower CD127 expression is consistent with other studies of HIV⁺, HAART-treated patients, where CD127

is not as low as in untreated HIV infection, but recovery of receptor expression does not return to the levels observed in controls (MacPherson et al., 2001).

As the dysfunction observed here was independent of CD127 expression, it could be due to a change in CD132 expression, the other component of the IL-7 receptor complex. To determine this, the response to other cytokines which share this receptor chain and signal through STAT5, namely IL-2 and IL-15, were evaluated. It was found that there was no difference in IL-2- or IL-15-induced pSTAT5 or Bcl-2 expression, across a range of doses, in bulk CD8⁺ T-cells isolated from HCV-infected individuals compared to controls (Figures 8 and 9). As no differences in STAT5 activation in response to IL-2 and IL-15 were observed in bulk CD8⁺ T-cells, this was not evaluated in CD8⁺ T-cell subsets. However, it is possible that subset-specific differences could have been detected, hence it remains uncertain whether these findings are IL-7 specific. Measurement of proliferation in response to IL-2 and IL-15 was also attempted, but even very low concentrations of these cytokines stimulated a significant amount of proliferation, and so we were unable to test differences between HCV infection and controls. Also, chronic viral infection can increase the proportion of cells expressing CD132 (Faller et al., 2006; Sasson et al., 2006), so it is concluded that changes in CD132 are not the cause of lower IL-7 responses in HCV mono- and HIV-HCV co-infection, nor impairment of STAT5 activation. If the receptor complex and downstream signaling is not to blame for changes in IL-7 responsiveness, cell intrinsic changes in signal initiation may be the cause.

Intracellular proteins known as suppressors of cytokine signaling (SOCS) regulate multiple steps of JAK/STAT signaling; phosphorylation of JAK and TYK on receptor complexes, recruitment of STAT molecules to receptor complexes, and ubiquitination of

STAT molecules, and their expression changes throughout T-cell differentiation (Palmer and Restifo, 2009). An increase in the expression of mRNA transcripts encoding SOCS proteins at basal levels or with cytokine stimulation in disease vs. health may have explained some of the IL-7 signaling and response deficiencies observed here. Only gene expression of SOCS1 was clearly upregulated in all groups with IL-7 stimulation, as expected (Figure 12) (Palmer and Restifo, 2009). However, there were no significant differences in SOCS1 mRNA expression levels between groups in either unstimulated or IL-7-stimulated cells (Figure 11D, 12D and F). In contrast, while not significantly changed at baseline (Figure 11E), the expression of SOCS3 was increased in HCV infection compared to controls in unstimulated cells after overnight rest at 37°C (Figure 12EB). This is consistent with another study that found endogenous levels of SOCS3 mRNA upregulated in HCV infection (Collins et al., 2014). While results observed here are similar to others, there were some notable technical problems with the associated experiments herein. The total RNA isolated from CD8⁺ T-cells used for the measurement of SOCS expression was not pure and was degraded (Figure 13). It is known that in certain cases freezing and thawing T-cells can degrade RNA (Mallone et al., 2011), and the current experiment should be repeated on cells freshly isolated from blood or cells frozen with an RNA stabilizing agent. In addition, a DNase step should be added to the RNA isolation procedure to remove any contaminating genomic DNA.

The cause of changes in SOCS expression has been suggested to be due to the effects of circulating HCV core protein (Yao et al., 2008), and further study into this may prove promising in understanding CD8⁺ T-cell dysfunction in chronic HCV infection. Another mechanism of signaling regulation could have been changes in the activation of JAK molecules. While it is unknown if there are any effects of HCV on JAK proteins, changes in

pSTAT5 expression levels have been used as indicators of mutations or alterations in JAK2 (Aboudola et al., 2007), and STAT5 expression was observed to be reduced here.

It is concluded that the impaired response of CD8⁺ T-cells to IL-7 in HCV mono- and HIV-HCV co-infection is not due to differences in CD127 expression. It could be due to impediments to the transphosphorylation of JAK1, phosphorylation of CD127 by JAK1, docking of STAT5 to the phosphorylated JAK/CD127 complex, or phosphorylation of STAT5 by the latter complex (Figure 19). Therefore, further avenues of research would include an evaluation of JAK1 expression and phosphorylation, CD127 phosphorylation and differential SOCS gene expression. Alterations in this impairment could also be dependent or independent of effects of HCV proteins such as core. Overall, these may lead to differences in the transcription of genes related to the Jak1/STAT5 signaling pathway.

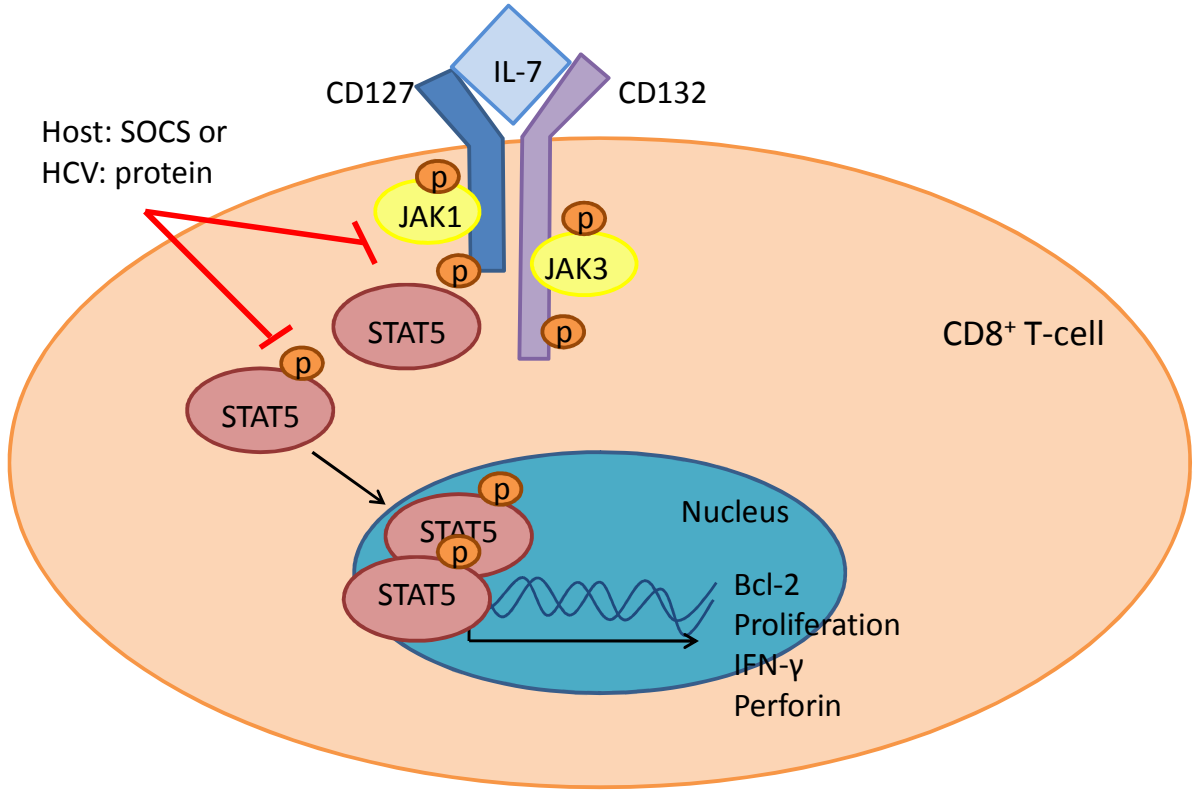


Figure 19. Potential mechanism of impaired CD8⁺ T-cell responses to IL-7 in HCV infection.

4.8. Summary and relevance

This study demonstrates that CD8⁺ T-cells are globally impaired in response to IL-7 in chronic HCV infection, independent of antigen specificity. This has implications for vaccine immunogenicity (e.g. influenza, HBV) and for T-cell based therapies for HCV infection. Preliminary trials of T-cell-mediated HCV vaccination of individuals with chronic HCV infection showed weaker T-cell responses than controls, suggesting that CD8⁺ T-cell dysfunction may be a challenge for the development of an effective therapeutic HCV vaccine (Houghton, 2011). The pronounced impairment of CD8⁺ T-cells in the liver in response to survival cytokines and potential susceptibility to apoptosis may contribute to the high levels of apoptosis and necrosis that occur in that organ, and may contribute to subsequent fibrosis and HCC. Ongoing research in this area, particularly into the mechanisms of impairment, may provide insights into the design of immune therapeutics to boost CD8⁺ T-cell function. Improvement or reversal of CD8⁺ T-cell impairment could complement existing DAA therapies and assist in the design of future treatments such as therapeutic vaccines. An important question is if the observations of CD8⁺ T-cell dysfunction, and that of others, are reversed by current successful DAA treatments, or if dysfunction persists. DAA treatment is so new that its long lasting effects and the effect of subsequent reversal of liver fibrosis on CD8⁺ T-cells is unknown, in which case, a treatment for the dysfunction itself may become an important complement to treatment. The questions remaining after this study may include the following: (1) Are cytotoxic and killing functions of bulk CD8⁺ T-cells also impaired in response to IL-7? (2) What is the mechanism(s) of this IL-7-specific, CD127- and antigen-independent impairment? and (3) Is this impairment reversed or recovered with successful DAA treatment?

References

- Abel, M., Sene, D., Pol, S., Bourliere, M., Poynard, T., Charlotte, F., Cacoub, P., and Caillat-Zucman, S. (2006). Intrahepatic virus-specific IL-10-producing CD8 T cells prevent liver damage during chronic hepatitis C virus infection. *Hepatology* 44, 1607-1616.
- Aboudola, S., Murugesan G Fau - Szpurka, H., Szpurka H Fau - Ramsingh, G., Ramsingh G Fau - Zhao, X., Zhao X Fau - Prescott, N., Prescott N Fau - Tubbs, R.R., Tubbs Rr Fau - Maciejewski, J.P., Maciejewski Jp Fau - Hsi, E.D., and Hsi, E.D. (2007). Bone marrow phospho-STAT5 expression in non-CML chronic myeloproliferative disorders correlates with JAK2 V617F mutation and provides evidence of in vivo JAK2 activation. *Am J Surg Pathol* 31, 7.
- Akkina, R. (2013). New generation humanized mice for virus research: comparative aspects and future prospects. *Virology* 435, 14-28.
- Al-Ghazawi, F. (2013). Understanding the mechanisms by which interleukin (IL)-7 down-regulates expression of the IL-7 receptor alpha-chain (CD127) in human CD8 T cells. In *Faculty of Graduate and Postdoctoral Studies* (Ottawa: University of Ottawa).
- Badr, G., Bedard, N., Abdel-Hakeem, M.S., Trautmann, L., Willems, B., Villeneuve, J.P., Haddad, E.K., Sekaly, R.P., Bruneau, J., and Shoukry, N.H. (2008). Early interferon therapy for hepatitis C virus infection rescues polyfunctional, long-lived CD8+ memory T cells. *Journal of virology* 82, 10017-10031.
- Bansal, S., Singal, A.K., McGuire, B.M., and Anand, B.S. (2015). Impact of all oral anti-hepatitis C virus therapy: A meta-analysis. *World J Hepatol* 7, 806-813.
- Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., Freeman, G.J., and Ahmed, R. (2006). Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439, 682-687.
- Barrett, L., Gallant, M., Howley, C., Bowmer, M.I., Hirsch, G., Peltekian, K., and Grant, M. (2008). Enhanced IL-10 production in response to hepatitis C virus proteins by peripheral blood mononuclear cells from human immunodeficiency virus-monoinfected individuals. *BMC immunology* 9, 28.
- Barrett, L., Gallant, M., Howley, C., Ian Bowmer, M., Hirsch, G., Peltekian, K., and Grant, M. (2011). Stronger hepatitis C virus-specific CD8+ T-cell responses in HIV coinfection. *Journal of viral hepatitis* 18, 170-180.
- Barrett, L., Trehanpati, N., Poonia, S., Daigh, L., Sarin, S.K., Masur, H., and Kottlilil, S. (2014). Hepatic compartmentalization of exhausted and regulatory cells in HIV/HCV-coinfected patients. *Journal of viral hepatitis*.

- Bengsch, B., Spangenberg, H.C., Kersting, N., Neumann-Haefelin, C., Panther, E., von Weizsacker, F., Blum, H.E., Pircher, H., and Thimme, R. (2007). Analysis of CD127 and KLRG1 expression on hepatitis C virus-specific CD8+ T cells reveals the existence of different memory T-cell subsets in the peripheral blood and liver. *Journal of virology* 81, 945-953.
- Bertolino, P., McCaughan, G.W., and Bowen, D.G. (2002). Role of primary intrahepatic T-cell activation in the 'liver tolerance effect'. *Immunology and cell biology* 80, 84-92.
- Bertolino, P., Trescol-Biemont, M.C., Thomas, J., Fazekas de St Groth, B., Pihlgren, M., Marvel, J., and Rouboudin-Combe, C. (1999). Death by neglect as a deletional mechanism of peripheral tolerance. *International immunology* 11, 1225-1238.
- Bio-rad. SsoAdvanced Universal SYBR Green Supermix.
- Boutboul, F., Puthier, D., Appay, V., Pelle, O., Ait-Mohand, H., Combadiere, B., Carcelain, G., Katlama, C., Rowland-Jones, S.L., Debre, P., *et al.* (2005). Modulation of interleukin-7 receptor expression characterizes differentiation of CD8 T cells specific for HIV, EBV and CMV. *AIDS* 19, 1981-1986.
- Bull, R.A., Leung, P., Gaudieri, S., Deshpande, P., Cameron, B., Walker, M., Chopra, A., Lloyd, A.R., and Luciani, F. (2015). Transmitted/Founder Viruses Rapidly Escape from CD8+ T Cell Responses in Acute Hepatitis C Virus Infection. *Journal of virology* 89, 5478-5490.
- Canchis, P.W., Yee, H.T., Fiel, M.I., Dieterich, D.T., Liu, R.C., Chiriboga, L., Jacobson, I.M., Edlin, B.R., and Talal, A.H. (2004). Intrahepatic CD4+ cell depletion in hepatitis C virus/HIV-coinfected patients. *Journal of acquired immune deficiency syndromes* (1999) 37, 1125-1131.
- Carrasco, J., Godelaine, D., Van Pel, A., Boon, T., and van der Bruggen, P. (2006). CD45RA on human CD8 T cells is sensitive to the time elapsed since the last antigenic stimulation. *Blood* 108, 2897-2905.
- CDC (2013). National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention. HIV and viral hepatitis.
- Chen, M., Tabaczewski, P., Truscott, S.M., Van Kaer, L., and Stroynowski, I. (2005). Hepatocytes Express Abundant Surface Class I MHC and Efficiently Use Transporter Associated with Antigen Processing, Tapasin, and Low Molecular Weight Polypeptide Proteasome Subunit Components of Antigen Processing and Presentation Pathway. *The Journal of Immunology* 175, 1047-1055.
- Cheng, N., Janumyan, Y.M., Didion, L., Van Hofwegen, C., Yang, E., and Knudson, C.M. (2004). Bcl-2 inhibition of T-cell proliferation is related to prolonged T-cell survival. *Oncogene* 23, 3770-3780.

- Collins, A.S., Ahmed, S., Napoletano, S., Schroeder, M., Johnston, J.A., Hegarty, J.E., O'Farrelly, C., and Stevenson, N.J. (2014). Hepatitis C virus (HCV)-induced suppressor of cytokine signaling (SOCS) 3 regulates proinflammatory TNF- α responses. *Journal of leukocyte biology* 96, 255-263.
- Cox, M.A., Kahan, S.M., and Zajac, A.J. (2013). Anti-viral CD8 T cells and the cytokines that they love. *Virology* 435, 157-169.
- Crawley, A.M., Vranjkovic, A., Faller, E., McGuinty, M., Busca, A., Burke, S.C., Cousineau, S., Kumar, A., Macpherson, P.A., and Angel, J.B. (2014). Jak/STAT and PI3K signaling pathways have both common and distinct roles in IL-7-mediated activities in human CD8⁺ T cells. *Journal of leukocyte biology* 95, 117-127.
- Crawley, A.M., Vranjkovic, A., Young, C., and Angel, J.B. (2010). Interleukin-4 downregulates CD127 expression and activity on human thymocytes and mature CD8⁺ T cells. *European journal of immunology* 40, 1396-1407.
- Czabotar, P.E., Lessene, G., Strasser, A., and Adams, J.M. (2014). Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol* 15, 49-63.
- Dorner, M., Horwitz, J.A., Robbins, J.B., Barry, W.T., Feng, Q., Mu, K., Jones, C.T., Schoggins, J.W., Catanese, M.T., Burton, D.R., *et al.* (2011). A genetically humanized mouse model for hepatitis C virus infection. *Nature* 474, 208-211.
- Douam, F., Lavillette, D., and Cosset, F.L. (2015). The mechanism of HCV entry into host cells. *Progress in Molecular Biology and Translational Science* 129, 63-107.
- Faist, B., Fleischer, B., and Jacobsen, M. (2010). Cytomegalovirus infection- and age-dependent changes in human CD8⁺ T-cell cytokine expression patterns. *Clin Vaccine Immunol* 17, 986-992.
- Faller, E.M., McVey, M.J., Kakal, J.A., and MacPherson, P.A. (2006). Interleukin-7 receptor expression on CD8 T-cells is downregulated by the HIV Tat protein. *Journal of acquired immune deficiency syndromes (1999)* 43, 257-269.
- Faller, E.M., Sugden, S.M., McVey, M.J., Kakal, J.A., and MacPherson, P.A. (2010). Soluble HIV Tat protein removes the IL-7 receptor alpha-chain from the surface of resting CD8 T cells and targets it for degradation. *Journal of immunology* 185, 2854-2866.
- Feuth, T., Arends, J.E., Fransen, J.H., Nanlohy, N.M., van Erpecum, K.J., Siersema, P.D., Hoepelman, A.I., and van Baarle, D. (2013). Complementary role of HCV and HIV in T-cell activation and exhaustion in HIV/HCV coinfection. *PloS one* 8, e59302.
- Ghazawi, F.M., Faller, E.M., Sugden, S.M., Kakal, J.A., and MacPherson, P.A. (2013). IL-7 downregulates IL-7R α expression in human CD8 T cells by two independent mechanisms. *Immunology and cell biology* 91, 149-158.

- Golden-Mason, L., Burton, J.R., Jr., Castelblanco, N., Klarquist, J., Benlloch, S., Wang, C., and Rosen, H.R. (2006). Loss of IL-7 receptor alpha-chain (CD127) expression in acute HCV infection associated with viral persistence. *Hepatology* 44, 1098-1109.
- Golden-Mason, L., Palmer, B., Klarquist, J., Mengshol, J.A., Castelblanco, N., and Rosen, H.R. (2007). Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. *Journal of virology* 81, 9249-9258.
- Golden-Mason, L., Palmer, B.E., Kassam, N., Townshend-Bulson, L., Livingston, S., McMahon, B.J., Castelblanco, N., Kuchroo, V., Gretch, D.R., and Rosen, H.R. (2009). Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *Journal of virology* 83, 9122-9130.
- Greenbaum, E., Nir-Paz, R., Linton, D.M., Ben-Hur, T., Meirovitz, A., and Zakay-Rones, Z. (2004). Severe influenza infection in a chronic hepatitis C carrier: failure of protective serum HI antibodies after IM vaccination. *J Clin Virol* 29, 23-26.
- Gruener, N.H., Lechner, F., Jung, M.C., Diepolder, H., Gerlach, T., Lauer, G., Walker, B., Sullivan, J., Phillips, R., Pape, G.R., *et al.* (2001). Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *Journal of virology* 75, 5550-5558.
- Harcourt, G.C., Donfield, S., Gomperts, E., Daar, E.S., Goulder, P., Jr., Phillips, R.E., Klenerman, P., Hemophilia, G., and Development, S. (2005). Longitudinal analysis of CD8 T-cell responses to HIV and hepatitis C virus in a cohort of co-infected haemophiliacs. *AIDS* 19, 1135-1143.
- Hartling, H.J., Gaardbo, J.C., Ronit, A., Salem, M., Laye, M., Clausen, M.R., Skogstrand, K., Gerstoft, J., Ullum, H., and Nielsen, S.D. (2013). Impaired thymic output in patients with chronic hepatitis C virus infection. *Scandinavian journal of immunology* 78, 378-386.
- Heydtmann, M., Hardie, D., Shields, P.L., Faint, J., Buckley, C.D., Campbell, J.J., Salmon, M., and Adams, D.H. (2006). Detailed Analysis of Intrahepatic CD8 T Cells in the Normal and Hepatitis C-Infected Liver Reveals Differences in Specific Populations of Memory Cells with Distinct Homing Phenotypes. *The Journal of Immunology* 177, 729-738.
- Hoare, M., Shankar, A., Shah, M., Rushbrook, S., Gelson, W., Davies, S., Akbar, A., and Alexander, G.J. (2013). gamma-H2AX+CD8+ T lymphocytes cannot respond to IFN-alpha, IL-2 or IL-6 in chronic hepatitis C virus infection. *Journal of hepatology* 58, 868-874.
- Hofmeister, R., Khaled, A.R., Benbernou, N., Rajnavolgyi, E., Muegge, K., and Durum, S.K. (1999). Interleukin-7: physiological roles and mechanisms of action. *Cytokine & growth factor reviews* 10, 41-60.

- Holz, L.E., Benseler, V., Vo, M., McGuffog, C., Van Rooijen, N., McCaughan, G.W., Bowen, D.G., and Bertolino, P. (2012). Naive CD8 T cell activation by liver bone marrow-derived cells leads to a "neglected" IL-2low Bimhigh phenotype, poor CTL function and cell death. *Journal of hepatology* 57, 830-836.
- Houghton, M. (2011). Prospects for prophylactic and therapeutic vaccines against the hepatitis C viruses. *Immunological reviews* 239, 99-108.
- Huang, L.R., Wohlleber, D., Reisinger, F., Jenne, C.N., Cheng, R.L., Abdullah, Z., Schildberg, F.A., Odenthal, M., Dienes, H.P., van Rooijen, N., *et al.* (2013). Intrahepatic myeloid-cell aggregates enable local proliferation of CD8(+) T cells and successful immunotherapy against chronic viral liver infection. *Nature immunology* 14, 574-583.
- Ito, M., Kusunoki, H., Mochida, K., Yamaguchi, K., and Mizuochi, T. (2011). HCV infection and B-cell lymphomagenesis. *Adv Hematol* 2011, 835314.
- Ivashkiv, L.B., and Donlin, L.T. (2014). Regulation of type I interferon responses. *Nature reviews Immunology* 14, 36-49.
- Jenne, C.N., and Kubes, P. (2013). Immune surveillance by the liver. *Nature immunology* 14, 996-1006.
- Jones, D.M., and McLauchlan, J. (2010). Hepatitis C virus: assembly and release of virus particles. *J Biol Chem* 285, 22733-22739.
- Juffroy, O., Bugault, F., Lambotte, O., Landires, I., Viard, J.P., Niel, L., Fontanet, A., Delfraissy, J.F., Theze, J., and Chakrabarti, L.A. (2010). Dual mechanism of impairment of interleukin-7 (IL-7) responses in human immunodeficiency virus infection: decreased IL-7 binding and abnormal activation of the JAK/STAT5 pathway. *Journal of virology* 84, 96-108.
- Kaina, B. (2003). DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation and signaling. *Biochemical Pharmacology* 66, 1547-1554.
- Kanai, T., Seki, S., Jenks, J.A., Kohli, A., Kawli, T., Martin, D.P., Snyder, M., Bacchetta, R., and Nadeau, K.C. (2014). Identification of STAT5A and STAT5B target genes in human T cells. *PloS one* 9, e86790.
- Kaplan, D.E., Sugimoto, K., Newton, K., Valiga, M.E., Ikeda, F., Aytaman, A., Nunes, F.A., Lucey, M.R., Vance, B.A., Vonderheide, R.H., *et al.* (2007). Discordant role of CD4 T-cell response relative to neutralizing antibody and CD8 T-cell responses in acute hepatitis C. *Gastroenterology* 132, 654-666.
- Kared, H., Saeed, S., Klein, M.B., and Shoukry, N.H. (2014). CD127 expression, exhaustion status and antigen specific proliferation predict sustained virologic response to IFN in HCV/HIV co-infected individuals. *PloS one* 9, e101441.

- Kim, A.Y., Schulze zur Wiesch, J., Kuntzen, T., Timm, J., Kaufmann, D.E., Duncan, J.E., Jones, A.M., Wurcel, A.G., Davis, B.T., Gandhi, R.T., *et al.* (2006). Impaired hepatitis C virus-specific T cell responses and recurrent hepatitis C virus in HIV coinfection. *PLoS medicine* 3, e492.
- Kimura, M.Y., Pobezinsky, L.A., Guinter, T.I., Thomas, J., Adams, A., Park, J.H., Tai, X., and Singer, A. (2013). IL-7 signaling must be intermittent, not continuous, during CD8(+) T cell homeostasis to promote cell survival instead of cell death. *Nature immunology* 14, 143-151.
- Kittlesen, D.J., Chianese-Bullock, K.A., Yao, Z.Q., Braciale, T.J., and Hahn, Y.S. (2000). Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation. *The Journal of clinical investigation* 106, 1239-1249.
- Kroy, D.C., Ciuffreda, D., Cooperrider, J.H., Tomlinson, M., Hauck, G.D., Aneja, J., Berger, C., Wolski, D., Carrington, M., Wherry, E.J., *et al.* (2014). Liver environment and HCV replication affect human T-cell phenotype and expression of inhibitory receptors. *Gastroenterology* 146, 550-561.
- Larrea, E., Riezu-Boj, J.I., Aldabe, R., Guembe, L., Echeverria, I., Balasiddaiah, A., Gastaminza, P., Civeira, M.P., Sarobe, P., and Prieto, J. (2014). Dysregulation of interferon regulatory factors impairs the expression of immunostimulatory molecules in hepatitis C virus genotype 1-infected hepatocytes. *Gut* 63, 665-673.
- Larrubia, J.R., Lokhande, M.U., Garcia-Garzon, S., Miquel, J., Gonzalez-Praetorius, A., Parra-Cid, T., and Sanz-de-Villalobos, E. (2013). Persistent hepatitis C virus (HCV) infection impairs HCV-specific cytotoxic T cell reactivity through Mcl-1/Bim imbalance due to CD127 down-regulation. *Journal of viral hepatitis* 20, 85-94.
- Laskus, T., Kibler, K.V., Chmielewski, M., Wilkinson, J., Adair, D., Horban, A., Stanczak, G., and Radkowski, M. (2013). Effect of hepatitis C infection on HIV-induced apoptosis. *PloS one* 8, e75921.
- Lechner, F., Wong, D.K., Dunbar, P.R., Chapman, R., Chung, R.T., Dohrenwend, P., Robbins, G., Phillips, R., Klenerman, P., and Walker, B.D. (2000). Analysis of successful immune responses in persons infected with hepatitis C virus. *The Journal of experimental medicine* 191, 1499-1512.
- Lee, Y.J., Won, T.J., Hyung, K.E., Lee, M.J., Moon, Y.H., Lee, I.H., Go, B.S., and Hwang, K.W. (2014). Bcl-2 knockdown accelerates T cell receptor-triggered activation-induced cell death in jurkat T cells. *Korean J Physiol Pharmacol* 18, 73-78.
- Levy, Y., Lacabaratz, C., Weiss, L., Viard, J.P., Goujard, C., Lelievre, J.D., Boue, F., Molina, J.M., Rouzioux, C., Avettand-Fenoel, V., *et al.* (2009). Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment. *The Journal of clinical investigation* 119, 997-1007.

- Li, H.C., and Lo, S.Y. (2015). Hepatitis C virus: Virology, diagnosis and treatment. *World J Hepatol* 7, 1377-1389.
- Li, Y., Wang, X., Douglas, S.D., Metzger, D.S., Woody, G., Zhang, T., Song, L., and Ho, W.Z. (2005). CD8+ T cell depletion amplifies hepatitis C virus replication in peripheral blood mononuclear cells. *The Journal of infectious diseases* 192, 1093-1101.
- Lin, W., Kim, S.S., Yeung, E., Kamegaya, Y., Blackard, J.T., Kim, K.A., Holtzman, M.J., and Chung, R.T. (2006). Hepatitis C virus core protein blocks interferon signaling by interaction with the STAT1 SH2 domain. *Journal of virology* 80, 9226-9235.
- Lucas, M., Vargas-Cuero, A.L., Lauer, G.M., Barnes, E., Willberg, C.B., Semmo, N., Walker, B.D., Phillips, R., and Klenerman, P. (2004). Pervasive influence of hepatitis C virus on the phenotype of antiviral CD8+ T cells. *Journal of immunology* 172, 1744-1753.
- Maasoumy, B., and Wedemeyer, H. (2012). Natural history of acute and chronic hepatitis C. *Best practice & research Clinical gastroenterology* 26, 401-412.
- MacPherson, P.A., Fex, C., Sanchez-Dardon, J., Hawley-Foss, N., and Angel, J.B. (2001). Interleukin-7 receptor expression on CD8(+) T cells is reduced in HIV infection and partially restored with effective antiretroviral therapy. *Journal of acquired immune deficiency syndromes* (1999) 28, 454-457.
- Maillard, P., Krawczynski, K., Nitkiewicz, J., Bronnert, C., Sidorkiewicz, M., Gounon, P., Dubuisson, J., Faure, G., Crainic, R., and Budkowska, A. (2001). Nonenveloped Nucleocapsids of Hepatitis C Virus in the Serum of Infected Patients. *Journal of virology* 75, 8240-8250.
- Maini, M.K., Boni, C., Lee, C.K., Larrubia, J.R., Reignat, S., Ogg, G.S., King, A.S., Herberg, J., Gilson, R., Alisa, A., *et al.* (2000). The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. *The Journal of experimental medicine* 191, 1269-1280.
- Mallone, R., Mannering, S.I., Brooks-Worrell, B.M., Durinovic-Belló, I., Cilio, C.M., Wong, F.S., Schloot, N.C., and on behalf of the Immunology of Diabetes Society, T.C.W.C. (2011). Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. *Clinical and experimental immunology* 163, 33-49.
- Mazzucchelli, R., and Durum, S.K. (2007). Interleukin-7 receptor expression: intelligent design. *Nature reviews Immunology* 7, 144-154.
- McMahan, R.H., Golden-Mason, L., Nishimura, M.I., McMahon, B.J., Kemper, M., Allen, T.M., Gretch, D.R., and Rosen, H.R. (2010). Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores

- hepatocyte-directed in vitro cytotoxicity. *The Journal of clinical investigation* 120, 4546-4557.
- Meyer-Olson, D., Shoukry, N.H., Brady, K.W., Kim, H., Olson, D.P., Hartman, K., Shintani, A.K., Walker, C.M., and Kalams, S.A. (2004). Limited T cell receptor diversity of HCV-specific T cell responses is associated with CTL escape. *The Journal of experimental medicine* 200, 307-319.
- Moodie, E.E., Pant Pai, N., and Klein, M.B. (2009). Is antiretroviral therapy causing long-term liver damage? A comparative analysis of HIV-mono-infected and HIV/hepatitis C co-infected cohorts. *PloS one* 4, e4517.
- Moorman, J.P., Zhang, C.L., Ni, L., Ma, C.J., Zhang, Y., Wu, X.Y., Thayer, P., Islam, T.M., Borthwick, T., and Yao, Z.Q. (2011). Impaired hepatitis B vaccine responses during chronic hepatitis C infection: involvement of the PD-1 pathway in regulating CD4(+) T cell responses. *Vaccine* 29, 3169-3176.
- Nascimbeni, M., and Rehermann, B. (2004). Determination of hepatitis B virus-specific CD8+ T-cell activity in the liver. *Methods in molecular medicine* 96, 65-83.
- Nuti, S., Rosa, D., Valiante, N.M., Saletti, G., Caratozzolo, M., Dellabona, P., Barnaba, V., and Abrignani, S. (1998). Dynamics of intra-hepatic lymphocytes in chronic hepatitis C: enrichment for Valpha24+ T cells and rapid elimination of effector cells by apoptosis. *European journal of immunology* 28, 3448-3455.
- O'Connor, A.M., Crawley, A.M., and Angel, J.B. (2010). Interleukin-7 enhances memory CD8(+) T-cell recall responses in health but its activity is impaired in human immunodeficiency virus infection. *Immunology* 131, 525-536.
- Owusu Sekyere, S., Suneetha, P.V., Kraft, A.R., Zhang, S., Dietz, J., Sarrazin, C., Manns, M.P., Schlaphoff, V., Cornberg, M., and Wedemeyer, H. (2015). A heterogeneous hierarchy of co-regulatory receptors regulates exhaustion of HCV-specific CD8 T cells in patients with chronic hepatitis C. *Journal of hepatology* 62, 31-40.
- Palmer, D.C., and Restifo, N.P. (2009). Suppressors of cytokine signaling (SOCS) in T cell differentiation, maturation, and function. *Trends in immunology* 30, 592-602.
- Palmer, M.J., Mahajan, V.S., Trajman, L.C., Irvine, D.J., and Lauffenburger, D.A. (2008). Interleukin-7 Receptor Signaling Network: An Integrated Systems Perspective. *Cell Mol Immunol* 5, 79-89.
- Park, S.H., and Rehermann, B. (2014). Immune responses to HCV and other hepatitis viruses. *Immunity* 40, 13-24.
- Ploss, A., and Rice, C.M. (2009). Towards a small animal model for hepatitis C. *EMBO reports* 10, 1220-1227.

- Pol, S., Lamorthe, B., Thi, N.T., Thiers, V., Carnot, F., Zylberberg, H., Berthelot, P., Brechot, C., and Nalpas, B. (1998). Retrospective analysis of the impact of HIV infection and alcohol use on chronic hepatitis C in a large cohort of drug users. *Journal of hepatology* 28, 945-950.
- Public Health Agency of Canada (2010). Epidemiology of acute hepatitis C infection in Canada results from the enhanced hepatitis strain surveillance system (EHSSS).
- Qiagen (2012). RNeasy Mini Handbook.
- Ramadori, G., Moriconi, F., Malik, I., and Dudas, J. (2008). Physiology and pathophysiology of liver inflammation, damage and repair. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society* 59 Suppl 1, 107-117.
- Rehermann, B. (2007). Chronic infections with hepatotropic viruses: mechanisms of impairment of cellular immune responses. *Seminars in liver disease* 27, 152-160.
- Rehermann, B. (2013). Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. *Nature medicine* 19, 859-868.
- Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401, 708-712.
- Sarhan, M.A., Chen, A.Y., Russell, R.S., and Michalak, T.I. (2012). Patient-derived hepatitis C virus and JFH-1 clones differ in their ability to infect human hepatoma cells and lymphocytes. *J Gen Virol* 93, 2399-2407.
- Sasson, S.C., Zaunders, J.J., Zanetti, G., King, E.M., Merlin, K.M., Smith, D.E., Stanley, K.K., Cooper, D.A., and Kelleher, A.D. (2006). Increased plasma interleukin-7 level correlates with decreased CD127 and Increased CD132 extracellular expression on T cell subsets in patients with HIV-1 infection. *The Journal of infectious diseases* 193, 505-514.
- Sawa, Y., Arima, Y., Ogura, H., Kitabayashi, C., Jiang, J.J., Fukushima, T., Kamimura, D., Hirano, T., and Murakami, M. (2009). Hepatic interleukin-7 expression regulates T cell responses. *Immunity* 30, 447-457.
- Schluns, K.S., and Lefrancois, L. (2003). Cytokine control of memory T-cell development and survival. *Nature reviews Immunology* 3, 269-279.
- Sheahan, T., and Rice, C. (2013). Single cell analysis of HCV infected patient hepatocytes: The science is no longer science fiction. *Gastroenterology* 145, 10.1053/j.gastro.2013.1010.1035.
- Sherman, M., Bilodeau, M., Cooper, C., Mackie, D., Depew, W., Villeneuve, J.-P., and Bain, V. (2013). Liver Disease in Canada.

- Shin, E.C., Park, S.H., Nascimbeni, M., Major, M., Caggiari, L., de Re, V., Feinstone, S.M., Rice, C.M., and Rehermann, B. (2013). The frequency of CD127(+) hepatitis C virus (HCV)-specific T cells but not the expression of exhaustion markers predicts the outcome of acute HCV infection. *Journal of virology* 87, 4772-4777.
- Shoukry, N.H., Grakoui, A., Houghton, M., Chien, D.Y., Ghayeb, J., Reimann, K.A., and Walker, C.M. (2003). Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. *The Journal of experimental medicine* 197, 1645-1655.
- Simeonova, P.P., Gallucci, R.M., Hulderman, T., Wilson, R., Kommineni, C., Rao, M., and Luster, M.I. (2001). The role of tumor necrosis factor-alpha in liver toxicity, inflammation, and fibrosis induced by carbon tetrachloride. *Toxicol Appl Pharmacol* 177, 112-120.
- Sin, J.I., Kim, J., Pachuk, C., and Weiner, D.B. (2000). Interleukin 7 can enhance antigen-specific cytotoxic-T-lymphocyte and/or Th2-type immune responses in vivo. *Clin Diagn Lab Immunol* 7, 751-758.
- Smyth, M.J., Norihisa, Y., Gerard, J.R., Young, H.A., and Ortaldo, J.R. (1991). IL-7 regulation of cytotoxic lymphocytes: pore-forming protein gene expression, interferon-gamma production, and cytotoxicity of human peripheral blood lymphocytes subsets. *Cellular immunology* 138, 390-403.
- Sommershof, A., Aichinger, H., Engler, H., Adenauer, H., Catani, C., Boneberg, E.M., Elbert, T., Groettrup, M., and Kolassa, I.T. (2009). Substantial reduction of naive and regulatory T cells following traumatic stress. *Brain, behavior, and immunity* 23, 1117-1124.
- Spangenberg, H.C., Viazov, S., Kersting, N., Neumann-Haefelin, C., McKinney, D., Roggendorf, M., von Weizsacker, F., Blum, H.E., and Thimme, R. (2005). Intrahepatic CD8+ T-cell failure during chronic hepatitis C virus infection. *Hepatology* 42, 828-837.
- Stelekati, E., Shin, H., Doering, T.A., Dolfi, D.V., Ziegler, C.G., Beiting, D.P., Dawson, L., Liboon, J., Wolski, D., Ali, M.A., *et al.* (2014). Bystander chronic infection negatively impacts development of CD8(+) T cell memory. *Immunity* 40, 801-813.
- Stemcell Technologies. Human CD8 Positive Selection Kit.
- Stiffler, J.D., Nguyen M Fau - Sohn, J.A., Sohn Ja Fau - Liu, C., Liu C Fau - Kaplan, D., Kaplan D Fau - Seeger, C., and Seeger, C. (2009). Focal distribution of hepatitis C virus RNA in infected livers. *PloS one*.
- Sulkowski, M.S., and Thomas, D.L. (2003). Hepatitis C in the HIV-Infected Person. *Ann Intern Med* 138, 197-207.

- Sumida, K., Shimoda, S., Iwasaka, S., Hisamoto, S., Kawanaka, H., Akahoshi, T., Ikegami, T., Shirabe, K., Shimono, N., Maehara, Y., *et al.* (2013). Characteristics of splenic CD8+ T cell exhaustion in patients with hepatitis C. *Clinical and experimental immunology* 174, 172-178.
- Sung, J.M., Lee, C.K., and Wu-Hsieh, B.A. (2012). Intrahepatic infiltrating NK and CD8 T cells cause liver cell death in different phases of dengue virus infection. *PloS one* 7, e46292.
- Swainson, L., Kinet, S., Mongellaz, C., Sourisseau, M., Henriques, T., and Taylor, N. (2007). IL-7-induced proliferation of recent thymic emigrants requires activation of the PI3K pathway. *Blood* 109, 1034-1042.
- Thimme, R., Oldach, D., Chang, K.M., Steiger, C., Ray, S.C., and Chisari, F.V. (2001). Determinants of viral clearance and persistence during acute hepatitis C virus infection. *The Journal of experimental medicine* 194, 1395-1406.
- Thorpe, J., Saeed, S., Moodie, E.E., Klein, M.B., and Canadian Co-infection Cohort, S. (2011). Antiretroviral treatment interruption leads to progression of liver fibrosis in HIV-hepatitis C virus co-infection. *AIDS* 25, 967-975.
- Tokita, D., Sumpter, T.L., Raimondi, G., Zahorchak, A.F., Wang, Z., Nakao, A., Mazariegos, G.V., Abe, M., and Thomson, A.W. (2008). Poor allostimulatory function of liver plasmacytoid DC is associated with pro-apoptotic activity, dependent on regulatory T cells. *Journal of hepatology* 49, 1008-1018.
- Tripathi, P., Kurtulus, S., Wojciechowski, S., Sholl, A., Hoebe, K., Morris, S.C., Finkelman, F.D., Grimes, H.L., and Hildeman, D.A. (2010). STAT5 is critical to maintain effector CD8+ T cell responses. *Journal of immunology* 185, 2116-2124.
- Tsujimoto, Y. (1998). Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes Cells* 3, 11.
- Umemura, T., Wang, R.Y., Schechterly, C., Shih, J.W., Kiyosawa, K., and Alter, H.J. (2006). Quantitative analysis of anti-hepatitis C virus antibody-secreting B cells in patients with chronic hepatitis C. *Hepatology* 43, 91-99.
- Urbani, S., Amadei, B., Tola, D., Massari, M., Schivazappa, S., Missale, G., and Ferrari, C. (2006). PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *Journal of virology* 80, 11398-11403.
- Vali, B., Yue, F.Y., Jones, R.B., Sheth, P.M., Kaul, R., Betts, M.R., Wong, D., Kovacs, C., Loutfy, M., Common, A., *et al.* (2008). HIV-specific T-cells accumulate in the liver in HCV/HIV co-infection. *PloS one* 3, e3454.
- Valiante, N.M., D'Andrea, A., Crotta, S., Lechner, F., Klenerman, P., Nuti, S., Wack, A., and Abrignani, S. (2000). Life, activation and death of intrahepatic lymphocytes in chronic hepatitis C. *Immunological reviews* 174, 77-89.

- Vranjkovic, A., Crawley, A.M., and Angel, J.B. (2012). In vitro HIV Type 1 infection indirectly alters CD127 expression on CD8(+) T cells. *AIDS research and human retroviruses* 28, 295-298.
- Vranjkovic, A., Crawley, A.M., Gee, K., Kumar, A., and Angel, J.B. (2007). IL-7 decreases IL-7 receptor alpha (CD127) expression and induces the shedding of CD127 by human CD8+ T cells. *International immunology* 19, 1329-1339.
- Vranjkovic, A., Crawley, A.M., Patey, A., and Angel, J.B. (2011). IL-7-dependent STAT-5 activation and CD8+ T cell proliferation are impaired in HIV infection. *Journal of leukocyte biology* 89, 499-506.
- Wang, Y., Keck, Z.Y., and Fount, S.K. (2011). Neutralizing antibody response to hepatitis C virus. *Viruses* 3, 2127-2145.
- Wedemeyer, H., He, X.S., Nascimbeni, M., Davis, A.R., Greenberg, H.B., Hoofnagle, J.H., Liang, T.J., Alter, H., and Rehermann, B. (2002). Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *Journal of immunology* 169, 3447-3458.
- Wolf, Monika J., Adili, A., Piotrowitz, K., Abdullah, Z., Boege, Y., Stemmer, K., Ringelhan, M., Simonavicius, N., Egger, M., Wohlleber, D., *et al.* (2014). Metabolic Activation of Intrahepatic CD8+ T Cells and NKT Cells Causes Nonalcoholic Steatohepatitis and Liver Cancer via Cross-Talk with Hepatocytes. *Cancer Cell* 26, 549-564.
- Wong, T., and Lee, S.S. (2006). Hepatitis C: a review for primary care physicians. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne* 174, 649-659.
- Yao, Z.Q., Prayther, D., Trabue, C., Dong, Z.P., and Moorman, J. (2008). Differential regulation of SOCS-1 signalling in B and T lymphocytes by hepatitis C virus core protein. *Immunology* 125, 197-207.
- Zhao, B.B., Zheng, S.J., Gong, L.L., Wang, Y., Chen, C.F., Jin, W.J., Zhang, D., Yuan, X.H., Guo, J., Duan, Z.P., *et al.* (2013). T lymphocytes from chronic HCV-infected patients are primed for activation-induced apoptosis and express unique pro-apoptotic gene signature. *PloS one* 8, e77008.

Supplemental Tables**Supplemental Table 1. Subset distribution of CD8⁺ T-cells**

Study ID	Status	Fibrosis	Genotype	Age	% of CD8 ⁺			
					Naïve	CM	EM	EMRA
I089	HCV	4	3	62	32.95	17.34	18.33	31.38
I090	HCV	3	3	55	17.44	26.51	40.13	15.93
I091	HCV	0-1	1A	48	28.37	42.01	14.35	15.27
I092	HCV	1	1A	49	24.21	32.26	33.29	10.25
I093	HCV	0	3	23	35.53	26.51	22.04	15.91
I098	HCV	0-1	1A	55	9.51	9.52	60.16	20.18
I099	HCV	3	1A	58	13.92	34.82	26.63	24.63
I102	HCV	-	1B	40	36.75	10.57	30.09	22.6
I103	HCV	4	1	62	30.47	20.22	26.81	22.5
I105	HCV	0-1	1	62	28.5	9.77	25.82	35.91
I106	HCV	0-1	3A	63	32.89	35.64	14.97	16.51
I107	HCV	0-1	3	52	30.01	16.92	33.84	19.23
I094	HIV-HCV	0-1	1A	45	28.74	19.18	41.07	11.01
I100	HIV-HCV	1	2	54	24.16	12.24	45.85	17.75
I104	HIV-HCV	0-1	1	62	19.16	4.04	53.17	23.64
I108	HIV-HCV	0-1	1	49	38.17	11.53	15.97	34.32
I109	HIV-HCV	0-1	1	52	30.6	11.78	28.92	28.7
I110	HIV-HCV	0-1	3A	59	27.78	33.41	23.6	15.21
I111	HIV-HCV	3	1A	46	33.9	20.63	27.79	17.69

Supplemental Table 2. Expression of CD127 on CD8⁺ T-cell subsets

Study ID	Status	Fibrosis	Genotype	Age	mCD127 Expression (%)			
					Naïve	CM	EM	EMRA
I089	HCV	4	3	62	81.54	56.09	32.44	10.63
I090	HCV	3	3	55	51.15	36.58	35.82	11.89
I091	HCV	0-1	1A	48	66.91	33.03	22.86	7.51
I092	HCV	1	1A	49	74.45	44.53	21.56	15.51
I093	HCV	0	3	23	65.16	22.06	14.45	9.46
I098	HCV	0-1	1A	55	85.54	67.42	41.65	13.82
I099	HCV	3	1A	58	53.75	42.77	24.86	3.06
I102	HCV	-	1B	40	89.18	57.22	38.3	30.71
I103	HCV	4	1	62	86.26	51.31	54.4	21.67
I105	HCV	0-1	1	62	81.9	57.24	40.27	18.15
I106	HCV	0-1	3A	63	83.66	51.41	23.46	6.02
I107	HCV	0-1	3	52	83.47	56.3	32.64	15.81
I094	HIV-HCV	0-1	1A	45	81.95	44.83	4.46	10.8
I100	HIV-HCV	1	2	54	79.78	61.27	12.76	9.18
I104	HIV-HCV	0-1	1	62	82.19	54.83	66.45	38.42
I108	HIV-HCV	0-1	1	49	63.06	49.16	6.4	3.5
I109	HIV-HCV	0-1	1	52	79.66	57.23	39.51	18.04
I110	HIV-HCV	0-1	3A	59	57.81	43.97	39.24	18.41
I111	HIV-HCV	3	1A	46	61.48	41.27	18.25	10.2

Supplemental Table 3. Expression of pSTAT5 in response to IL-7

Study ID	Status	Fibrosis	Genotype	Age	MFI					
					Medium	IL-7 (ng/ml)				
						0.01	0.1	1	5	10
I043	HCV	0-1	1	55	6.03	6.16	6.24	7.71		19
I044	HCV	3-4	3	44	5.47	5.1	7.42	9.55	14	13.56
I045	HCV	0-1	1B	52	4.37	4.43	4.46	7.1	10.95	14.51
I046	HCV	1	1A	30	4.29	4.2	4.39	5.69	7.17	8.78
I053	HCV	1	1	62	4.22	10.85	12.37	15.41	13.88	15.91
I054	HCV	0-1	1B	50	4.09	7	15.38	16.45	16.01	14.79
I060	HCV	1-2	1A	48	4.8	4.16	4.48	9.09	12.36	14.44
I061	HCV	0-1	1A	31	3.72	3.52	3.77	7.45	9.74	11.8
I062	HCV	2-3	1B	53	3.04	3.39	3.41	5.66	10.57	11.84
I059	HIV-HCV	0-1	1A	55	5.04	5.7	12.37	16.03	17.24	17.99
I066	HIV-HCV	-	1B	53	4.03	4.34	4.94	6.18	10.92	11.2
I067	HIV-HCV	0-1	4	64	3.49	3.49	3.52	4.25	12.04	10.78
I084	HIV-HCV	0-1	1A	45	3.4	10.16	12	13.74	13.28	13.36

Supplemental Table 4. Expression of pSTAT5 in response to IL-7 in CD8⁺ T-cell subsets

Study ID	Status	Fibrosis	Genotype	Age	Naïve - MFI				
					Medium	IL-7 (pg/ml)			
						0.01	0.1	1	10
I102	HCV	-	1B	40	2.88	10.15	13.9	10.53	9.84
I103	HCV	4	1	62	3.38	9.54	10.2	10.67	15.39
I105	HCV	0-1	1	62	2.96	4.39	6.04	5.99	6.3
I106	HCV	0-1	3A	63	2.82	5.51	9.98	10.98	11.1
I107	HCV	0-1	3	52	3.05	5.4	7.88	8.46	10.3
I104	HIV-HCV	0-1	1	62	2.45	3.89	7.96	7.32	7.65
I108	HIV-HCV	0-1	1	49	2	4.05	7.31	8.7	8.77
I109	HIV-HCV	0-1	1	52	3.57	4.42	4.56	6.07	6.57
I110	HIV-HCV	0-1	3A	59	2.9	5.28	10.02	8.96	10.53
I111	HIV-HCV	3	1A	46	3.19	5.62	8.33	10.64	8.18

CM - MFI					EM - MFI				
Medium	IL-7 (pg/ml)				Medium	IL-7 (pg/ml)			
	0.01	0.1	1	10		0.01	0.1	1	10
3.09	8.33	11.79	9.08	9.08	2.96	6.99	11.42	8.56	7.56
3.61	10.38	10.48	11	14.94	3.39	6.64	7.92	7.7	9.78
2.88	3.76	5.26	5.77	5.91	2.44	2.9	4.27	4.56	4.65
3.48	4.53	8.28	9.07	9.7	3.08	3.75	6.82	7.84	8.26
3.05	5.25	7.79	6.33	10.91	2.74	4.09	6.69	7.44	9.23
2.73	3.62	7.02	6.76	6.8	2.96	6.99	11.42	8.56	7.56
1.98	3.1	5.85	6.27	5.02	3.39	6.64	7.92	7.7	9.78
3.22	3.94	3.91	5.78	6.19	2.44	2.9	4.27	4.56	4.65
3.14	4.48	8.1	7.8	8.59	3.08	3.75	6.82	7.84	8.26
3.26	4.73	8.03	10.73	8.04	2.74	4.09	6.69	7.44	9.23

EMRA - MFI				
Medium	IL-7 (pg/ml)			
	0.01	0.1	1	10
2.87	4.62	5.77	4.94	4.03
3.46	3.31	3.52	3.87	4.57
2.65	2.99	3.68	3.82	3.87
2.71	2.89	3.65	4.08	4.39
2.95	3.83	5.61	6.23	7.42
2.43	2.96	4.3	4.24	4.43
1.85	2.25	3.66	4.35	4.77
2.88	2.82	3.1	3.51	4.09
2.78	3.48	5.85	5.3	6.07
2.76	3.29	4.11	4.6	4.19

Supplemental Table 5. Ex vivo Bcl-2 expression of CD8⁺ T-cells

Study ID	Status	Fibrosis	Genotype	Age	MFI		
					Unstained	Isotype	Baseline
I102	HCV	-	1B	40	2.02	3.32	21.37
I103	HCV	4	1	62	2.33	3.43	17.86
I105	HCV	0-1	1	62	2.53	5.92	23.05
I106	HCV	0-1	3A	63	2.40	3.77	18.34
I107	HCV	0-1	3	52	2.49	3.69	15.66
I100	HIV-HCV	1	2	54	2.27	4.43	22.34
I104	HIV-HCV	0-1	1	62	2.55	4.54	31.34
I109	HIV-HCV	0-1	1	52	2.53	2.9	14.99
I110	HIV-HCV	0-1	3A	59	2.32	3.16	20.62
I111	HIV-HCV	3	1A	46	2.95	2.99	12.35

Supplemental Table 6. Production of Bcl-2 in response to IL-7

Study ID	Status	Fibrosis	Genotype	Age	MFI					
					Medium	IL-7 (pn/ml)				
						0.01	0.1	1	5	10
I053	HCV	1	1	62	14.1	16.6	22	23.6	27.7	28.11
I055	HCV	0-1	2	56	17.07	16.23	18.23	19.53	22.59	25.17
I057	HCV	4	1B	58	22.33	13.87	14.6	17.93	24.42	21.82
I058	HCV	2	1	57	23.38	14.66	15.75	19.11	20.5	23.58
I060	HCV	1-2	1A	48	16.53	17.24	17.67	24.47	23.5	27.16
I061	HCV	0-1	1A	31	14.76	15.29	18.55	23.97	24.35	24.07
I062	HCV	2-3	1B	53	20.11	21.14	19.8	22.24	29.33	30.28
I072	HCV	0-1	1	26	15.28	13.9	18.17	23.84	27.73	25.71
I073	HCV	0-1	1A	33	17.48	18.42	25.4	38.65	36.24	40.11
I059	HIV-HCV	0-1	1A	55	24.92	24.33	26.4	33.15	34.02	41.05
I066	HIV-HCV	-	1B	53	17.53	16.62	18.86	23	27.42	25.63
I067	HIV-HCV	0-1	4	64	16.64	19.35	17.44	18.81	28.23	27.44
I075	HIV-HCV	-	1	56	20.39	18.82	19.64	28.44	27.36	26.5
I084	HIV-HCV	0-1	1A	45	15.38	17.95	24.54	31.38	30.44	30.67
I108	HIV-HCV	0-1	1	49	6.71	6.67	7.57	12.47	12.23	12.03
I109	HIV-HCV	0-1	1	52	8.55	8.61	10.56	14.16	14.3	14.15
I110	HIV-HCV	0-1	3A	59	15.2	8.97	15.82	22.56	24.31	26.19

Supplemental Table 7. Proliferation of CD8⁺ T-cells in response to IL-7

Study ID	Status	Fibrosis	Genotype	Age	Medium	IL-7 (10ng/ml)	PHA (0.2ug/ml)	IL-7 (10ng/ml) + PHA (0.2ug/ml)
I053	HCV	1	1	62	7.10	25.3	30.28	71.47
I054	HCV	0-1	1B	50	16.99	28.2	21.7	79.94
I055	HCV	0-1	2	56	9.04	42.26	8.62	50.89
I057	HCV	4	1B	58	6.08	26.78	19.93	55.87
I058	HCV	2	1	57	25.19	41.28	36.32	57.74
I060	HCV	1-2	1A	48	6.19	18.23	33.59	60.04
I061	HCV	0-1	1A	31	1.62	17.18	15.19	43.22
I062	HCV	2-3	1B	53	4.33	14.13	9.28	44.6
I059	HIV-HCV	0-1	1A	55	9.03	13.2	48.95	58.32
I066	HIV-HCV	-	1B	53	1.30	1.43	13.48	18.81
I067	HIV-HCV	0-1	4	64	3.47	4.09	4.61	16.92
I111	HIV-HCV	3	1A	46	2.15	14.78	18.04	42.42

Supplemental Table 8. Raw Cq and relative to RPS18 values for measurement of SOCS gene expression

Study ID	Status	Fibrosis	Genotype	Age	Baseline			
					SOCS1		SOCS3	
					Cq	Relative to RPS18	Cq	Relative to RPS18
I078	HCV	4	1A	59	30.72	9.64138	29.64	10.65611
I080	HCV	0-1	1B	59	31.22	16.49324	30.05	19.63909
I098	HCV	0-1	1A	55	30.4	30.35378	29.4	31.3761
I099	HCV	3	1A	58	31.21	5.42692	30.13	6.05485
I101	HCV	2	1 and 4	52	32.24	2.00072	31.21	2.24236
I051	HIV-HCV	-	1A	54	29.6	11.5808	29.49	6.44595
I052	HIV-HCV	4	3	35	30.53	10.22419	29.25	12.79741
I071	HIV-HCV	3	2B	59	30.56	8.76351	30.22	5.86048
I094	HIV-HCV	0-1	1A	45	30.13	5.76675	28.84	7.19818
I110	HIV-HCV	0-1	3A	59	31.04	14.00549	29.8	17.39154
Unstimulated				IL-7 (10ng/ml)				
SOCS1		SOCS3		SOCS1		SOCS3		
Cq	Relative to RPS18	Cq	Relative to RPS18	Cq	Relative to RPS18	Cq	Relative to RPS18	
32.19	2.93129	31.52	2.58496	30.19	24.56666	31.11	6.97211	
32.77	2.66127	31.47	3.61765	30.11	16.09973	31.15	4.21556	
31.36	6.9191	31.03	4.69346	28.36	29.41483	30.22	4.22005	
31.06	10.18827	30.83	6.41611	29.44	12.46336	30.31	3.59407	
31.75	4.97884	31.35	3.58818	29.87	13.65371	30.35	5.17188	
31.32	5.10475	31.1	2.7587	31.73	3.24143	31.08	3.22261	
31.43	1.86454	31.46	1	29.62	8.56615	30.33	2.74587	
31.19	5.75465	30.56	5.47096	29.66	15.7692	30.26	5.86048	
32.02	3.64221	31.77	2.39783	29.14	11.30115	30.87	1.80829	
33.51	1.24784	32.25	1.69155	30.15	10.87724	31.59	2.18489	

Supplemental Table 9. Subsets distribution of CD8⁺ T-cells from liver biopsied HCV⁺ individuals

Study ID	Location	Fibrosis	Genotype	Age	% of CD8 ⁺			
					Naïve	CM	EM	EMRA
I089	Blood	4	3	62	32.95	17.34	18.33	31.38
I089	Liver	4	3	62	11.64	38.93	39.62	9.81
I090	Blood	3	3	55	17.44	26.51	40.13	15.93
I090	Liver	3	3	55	10.71	35.71	50.12	3.46
I093	Blood	0	3	23	35.53	26.51	22.04	15.19
I093	Liver	0	3	23	18.82	35.0	36.95	9.24
I101	Liver	2	1 and 4	52	3.70	46.64	43.85	5.81
I100	Blood (HIV-HCV)	1	2	54	24.16	12.24	45.85	17.75
I100	Liver (HIV-HCV)	1	2	54	4.89	61.17	33.12	0.82

Supplemental Table 10. Expression of CD127 on CD8⁺ T-cells from liver biopsied HCV⁺ individuals

Study ID	Location	Fibrosis	Genotype	Age	mCD127 expression (%)			
					Naïve	CM	EM	EMRA
I089	Blood	4	3	62	81.54	56.09	32.44	10.63
I089	Liver	4	3	62	92.07	63.65	35.38	33.70
I090	Blood	3	3	55	51.15	36.58	35.82	11.89
I090	Liver	3	3	55	94.36	43.50	15.08	31.03
I093	Blood	0	3	23	65.16	22.06	14.45	9.46
I093	Liver	0	3	23	66.82	12.29	5.79	19.65
I101	Liver	2	1 and 4	52	72.07	51.08	33.11	9.71
I100	Blood (HIV-HCV)	1	2	54	79.78	61.27	12.76	9.18
I100	Liver (HIV-HCV)	1	2	54	66.77	28.71	15.31	36.36

Supplemental Table 11. Expression of pSTAT5 in CD8⁺ T-cells from liver biopsied HCV⁺ individuals

Study ID	Location	Fibrosis	Genotype	Age	Medium	IL-7 (0.1ng/ml)	IL-7 (1ng/ml)	IL-2 (100ng/ml)	IL-15 (10ng/ml)
I090	Blood	3	3	55	3.46	19.49	16.4	8.83	22.46
I093	Blood	0	3	23	3.42	9.48	9.05	9.13	14.68
I090	Liver	3	3	55	29.16	28.89	28.92	31.65	30.09
I093	Liver	0	3	23	12.21	13.71	13.66	13.89	14.64
I101	Liver	2	1 and 4	52	14.41	19.95	19.75	19.66	21.46
I100	Liver (HIV-HCV+)	1	2	54	31.51	35.67	36.69	41.36	44.18

Supplemental Table 12. Expression of basal Bcl-2 in CD8⁺ T-cells from liver biopsied HCV⁺ individuals

Study ID	Location	Fibrosis	Genotype	Age	MFI	
					Unstained	Baseline
I102	Blood	-	1B	40	2.02	21.37
I103	Blood	4	1	62	2.33	17.86
I105	Blood	0-1	1	62	2.53	23.05
I106	Blood	0-1	3A	63	2.40	18.34
I107	Blood	0-1	3	52	2.49	15.66
I100	Liver (HIV-HCV)	1	2	54	5.25	25.50
I101	Liver	2	1 and 4	52	3.47	14.04

Contribution of collaborators

Phenotypic analysis of CD8⁺ T cells in whole blood was done by Lorna Carrasco-Medina, laboratory technician, with assistance from Stephanie Burke.