Role of macrophage subsets in $CD8^+$ T cell dysfunction in chronic HCV infection

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Abstract

Chronic HCV infection causes generalized $CD8^+$T cell impairment, not limited to HCV-specific $CD8^+$ T cells. Infiltrating monocyte-derived macrophages contribute to a micro-environment that could impact $CD8^+$T cells trafficking through the liver. Macrophages can differentiate into pro-inflammatory (M1) and anti-inflammatory (M2a, M2b, and M2c) subsets. Whether macrophage subset generation in chronic HCV infection is altered and if that has a subsequent impact on $CD8^+$T cell functions was not known. I have shown phenotypic alterations in both M1 and M2 macrophages in chronic HCV infection. In particular, M1 from advanced fibrosis patients show increased CD86 expression, reduced spontaneous TNF-α and increased spontaneous IL-10 production. In uninfected controls, co-culturing $CD8^+$T cells with M1 macrophages significantly increased the percentage of $CD107a^+$ and IFN-γ^+ $CD8^+$T cells in a contact-dependent manner. Similar autologous co-cultures between M1 and $CD8^+$T cells from patients with chronic HCV infection showed that M1 significantly reduced the percentage of IFN-γ^+ $CD8^+$T cells, even though patients displayed elevated IFN-γ^+CD8^+ T cells at baseline prior to culture. Overall, I demonstrated the altered phenotype of macrophages generated from patients with chronic HCV infection. I also showed the ability of M1 macrophages to induce IFN-γ^+CD8^+T cells in normal donors and their opposite impact when the cells are derived from chronic HCV infected patients.

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List of Abbreviations

AAM	Alternatively activated macrophage
BCL-2	B cell lymphoma 2
CAM	Classically activated macrophage
CCR/CXCR	Chemokine receptor
CLDN-I	Claudin-I
CMV	Cytomegalo virus
CTLA-4	Cytotoxic lymphocyte antigen 4
DAMP	Danger associated molecular patterns
DC	Dendritic cells
DC-SIGN	Dendritic cell specific intracellular adhesion molecule 3 grabbing non-Integrin
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ER	Endoplasmic Reticulum
FCS/FBS	Fetal calf/bovine serum
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDL	High density lipoprotein
HIV	Human Immunodeficiency virus
HSC	Hepatic stellate cells
IFN	Interferon
IL	Interleukin
IRF-3	Interferon regulatory transcription factor
KC	Kupffer cell
LAG-3	Lymphocyte activation gene 3
LAMP-1	Lysosomal associated membrane protein 1
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
LSEC	liver sinusoidal epithelial cells
mAb	Monoclonal antibody
MAVS	Mitochondrial Antiviral signaling protein
M-CSF	Macrophage colony stimulating factor
MDM	Monocyte-derived macrophage
MF	Myofibroblasts
MIP-1α	Macrophage inflammatory protein 1 alpha
MMP	Matrix metalloproteases
MΦ	Macrophage
NF-κB	Nuclear factor- kappa B
NK	Natural Killer
NLR	Nod-like receptor
NS	Non-structural
NTR	Non-translated regions
OCLN-I	Occludin-I
PAMP	Pathogen associated molecular patterns

PBS	Phosphate buffered saline
PD-1	Programmed death 1
PD-L1	Programmed death ligand 1
PFA	Paraformaldehyde
pH	Power of hydrogen
PI	Propidium iodide
PKR	Phosphokinase receptor
PRR	Pattern recognition receptor
RIG-I	Retinoic acid inducible gene-I
RNA	Ribonucleic acid
SR-BI	Scavenger receptor class B type I
SVR	Sustained virologic response
TcR	T cell receptor
TGF	Tumor Growth Factor
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
TNX	Treatment
WHO	World Health Organization

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Chapter 1: Introduction

1.0 Hepatitis C Virus (HCV) infection

Since the discovery of Hepatitis C virus (previously known as Non-A, Non-B Hepatitis) (1), great strides have been made in understanding the immunopathogenesis of this disease. The World Health Organization (WHO) in their 2016 Fact Sheet estimated that over 71 million people worldwide are currently living with chronic Hepatitis C virus (HCV) infection, among which 399,000 individuals would die annually due to HCV-related liver cirrhosis or HCC (hepatocellular carcinoma). About 70-80% of individuals with the HCV infection go on to develop a chronic form of the disease, with only the remaining group being able to clear the infection in the acute phase; if left untreated, the disease progression can cause liver tissue scarring, or fibrosis, which can lead to cirrhosis and even HCC (2-6).

As a blood-borne pathogen, HCV is commonly transmitted through transfusion of infected blood and blood products, re-use of needles and syringes in clinics and hospital, shared use of syringe needles by injection drug users, dialysis patients, and vertical transmission from mother to child (7-10). Changes in health policy in most first world countries now require screening for HCV using antibody-based and/or RNA testing of blood samples before transfusion of blood and blood products and transplantations, which has greatly reduced transmission through these routes (11-14). Furthermore, the screening of at-risk populations and improving their linkage to care has allowed the identification, diagnosis, screening and treatment of large portions of the population previously living with HCV infection (12). For Canada in particular, the major transmission routes among the population in recent times has been through the sharing of syringes by injection drug users (14, 15).

Early treatment of HCV infection consisted of IFN-α (interferon alpha) with or without Ribavirin (antiviral) and while this treatment allowed 20-50% of patients to achieve viral clearance, depending on their viral genotype, gender, age, and other factors; it still had side effects in many individuals (16-18). Such side effects often included fatigue, psychological imbalance, and symptoms of blood disorders (19). Recently, great advances were made in designing drugs called direct acting antivirals (DAAs) which were able to target viral enzymes such as the RNA polymerase NS5B and the serine protease NS3/4A among others and inhibit them, thus preventing viral replication (20, 21). These orally administered drugs increased the sustained virologic response (SVR) rate to over 90% and they were also well tolerated (21-24).

However, the risk of developing HCC remains even after viral clearance from the body for up to ten years (25). Some patients who progress to advanced fibrosis or cirrhosis, do not experience full fibrosis regression after achieving SVR either (26). Furthermore, the expense of therapy and risk of re-infection among at-risk populations means DAA based treatment is either not accessible or not effective in the long-term as re-exposure may occur (25, 27, 28). Therefore, it remains essential to develop both a prophylactic vaccine to protect the uninfected population, and also a therapeutic or curative vaccine for an already infected population which may be at risk for re-infection (29, 30).

There are several immune impairments in individuals living with early and advanced liver disease caused by chronic HCV infection, some of which will be highlighted in the following sections. It is important to get a better understanding of such cellular immune impairments and their association with liver fibrosis, to develop best therapeutic strategies and to treat individuals who have already reached early and advanced levels of liver fibrosis.

1.1 The virology of HCV infection

HCV is a small, single-stranded RNA virus which infects hepatocytes in both humans and chimpanzees (31-33). It belongs to the *Hepacivirus* genus in the Flaviviridae family (33, 34). The virus displays great diversity in its genetic sequence, allowing it to evade the immune system and enabling it to frequently establish a persistent, chronic infection (31, 32).The genome translates to a polyprotein, which can then be cleaved to form the various viral proteins. It forms the non-structural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B, as well as the structural proteins: core and envelope proteins (E1 and E2) (35, 36). It also forms the hydrophobic peptide called p7 (35, 36). The 5' and 3' ends of the open reading frame have non-translated regions (NTRs), with the 5' end containing an internal ribosome entry site (IRES) that allows for the replication of the viral genome (35).

Circulating HCV comes into contact with the liver as it flows through the liver sinusoids, and it is possible that the cells lining the sinusoids such as Kupffer cells (KC) are able to capture them and thus bring them in contact with hepatocytes using a surface protein called DC-SIGN (dendritic cell-specific intercellular adhesion molecule three grabbing non integrin) (37, 38). The virus is then able to infect hepatocytes via a multi-step process as illustrated in Figure 1 (from review (39)).

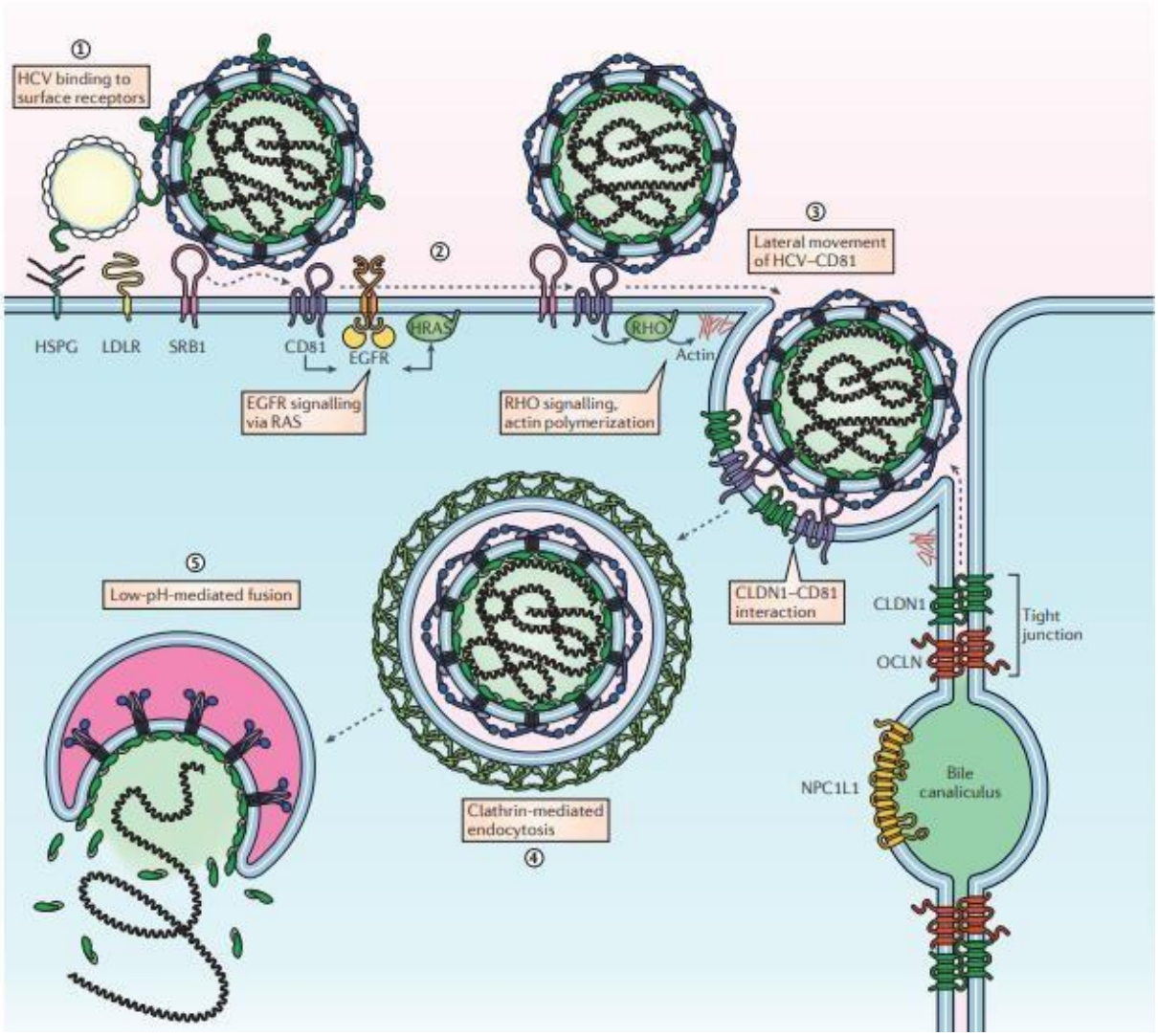

①
HCV binding to surface receptors
HSPG
LDLR
SRB1
CD81
EGFR
HRAS
②
EGFR signalling via RAS
RHO
Actin
RHO signalling, actin polymerization
③
Lateral movement of HCV–CD81
CLDN1–CD81 interaction
CLDN1
OCLN
Tight junction
NPC1L1
Bile canaliculus
Clathrin-mediated endocytosis
④
⑤
Low-pH-mediated fusion

Figure 1

Figure 1: Illustration of host factors involved in HCV entry into human hepatocytes from Review (39). HCV entry. HCV LVPs (lipoviroparticles) attach to the cell surface by interaction with HSPG, LDLR and SR-BI. SR-BI may delipidate HCV-associated lipoproteins and induces conformational changes in E2, exposing the CD81 binding site (step 1). TfR1 (transferrin receptor 1) plays an unknown, post-CD81 role in HCV entry (not shown). Interaction of E2 with CD81 then activates signal transduction through EGFR and Ras as well as Rho GTPases (step 2). These signaling events promote lateral movement of CD81-HCV complexes to sites of cell-cell contact (step 3), interaction of CD81 with CLDN1, and HCV internalization via clathrin-mediated endocytosis (step 4). The low pH of the endosomal compartment induces HCV fusion (step 5).

However, it must be noted that CD 81 alone is not enough for viral entry as the process requires several co-receptors (40, 41). Other host factors which play a role in this process include the scavenger receptor class B type I (SR–BI) responsible for internalizing high-density lipoproteins (HDL), LDL receptor, DC-SIGN and the tight junction proteins: claudin-1(CLDN1) and occludin-1 (OCLN). Furthermore, viral factors such as the envelope proteins E1 and E2 (38, 41-45) also play a crucial role in facilitating the entry of the virus into host cells.

The initial attachment of the virus to the hepatocyte happens through the Heparan Sulfate proteoglycans (HPLG) or LDL receptors(46). SR-BI is highly expressed on hepatocytes and is able to bind to lipoproteins such as LDL and HDL, as well as the E2 viral protein (41). After the initial attachment of the virus, the viral E2 protein binds to the extracellular loop of the CD81 receptor, while forming a complex with multiple proteins and co-factors at the cell surface(39, 46). This induces the signal transduction pathway involving EGFR (epidermal growth factor receptor), Ras GTPase and Rho GTPase which help the movement of the virus towards tight junctions where they enter the cell by clathrin-mediated endocytosis, eventually releasing the viral genome into the hepatic cytoplasm (39, 40, 46, 47) (43). The less extensively studied protein NPC1 (Niemann-Pick C1) is a cholesterol absorption receptor which also plays a role in the entry of the virus into hepatocytes, however, their exact role the entry pathway is poorly understood (39, 48). The virus then fuses with the endosomal compartment in a low-pH dependent manner (39, 49).

Once a cell is infected, the virus can also spread by direct cell-to-cell contact from one hepatocyte to the next, allowing it to evade exposure to neutralizing antibodies, as indicated by *in vitro* studies (50).

Once in the cytoplasm, the genome is first translated using the IRES which binds specifically to the 40S ribosome subunit, resulting in the large polyprotein formation at the endoplasmic reticulum (ER) (43, 51). As mentioned earlier, the viral genome forms a single polyprotein (made up of 3,000 amino acids) that is processed by host cell signalase and signal peptidase and two viral proteases (NS2/3 and NS3/4A) to form several structural and non-structural proteins with different functions (31, 32, 43, 51). The NS proteins which do not contribute to the structure of the virus are the ones that help in the viral replication process (51). Following the formation of these proteins, they associate with the ER membrane and form replication machinery to form the positive strand RNA using the RNA-dependent RNA polymerase NS5B enzyme (52). The ER membrane is altered by NS4B to form a membraneous web, which possibly provides support for the replication complex (51). Several host factors are also involved in the replication process such as FKBP8 (FK506-binding protein 8) and Hsp90 (heat shock protein) which have been shown to form a complex with NS5A (51). Newly formed RNA genome is again used as a template for further replication of the strand and these are eventually assembled to make virions (52). The newly formed strands are moved to assembly sites with the help of NS3/4A or NS5A, and NS2, while the p7 protein is responsible for combining core proteins, the replication complex and glycoproteins (52). The core protein and genomic RNA form the nucleocapsid, while the envelope is formed by the glycoprotein complex (52).

After being assembled in the ER, the virus eventually buds off with the help of the cellular machinery called endosomal-sorting complex required for transport (ESC-RT), and is released from the cell through a secretory pathway (51-53).

1.2 HCV Genotypes

Based on phylogenetic differences, the virus can be categorized into separate groups, meaning that any two HCV isolates will be placed in the same group if there is sequence homology in at least two regions of the individual virus genome (54-56). These groups that

vary in their sequences are called clades and several similar genotypes are grouped within each clade to simplify the nomenclature (54-56). For the purpose of this thesis report, I will refer to such clades as genotypes. So far, HCV isolates from infected patients across the world have helped identify a total of six different genotypes (referred to as genotype 1,2,3,4,5 and 6) (54-56). It is important to note that different geographic regions have a predominance of one genotype over others. For instance, genotype 1a and 1b are most frequently found in populations living in the United States and Europe, while genotype 2a and 2b are found throughout North America, Europe and Japan (54). On the other hand, genotype 3 is commonly observed in Southeast Asia, Indonesia, and the Indian sub-continent (54, 56).

Since the sample population in this project is from Ottawa, Canada, it is important to note that studies have previously shown genotype 1a to be the most common one in Canada, followed by 1b and 3a; small proportions of the population were positive for genotype 2a, 2b and 4a (57). It is important to note that with increasing immigration from countries which have a high occurrence of HCV infection, the frequency of infected population and common genotypes found are also expected to change with the change in demographic. This means, addressing only the health outcomes and therapeutic success of specific viral genotype infections are no longer enough in a high immigration population to eradicate this disease, and a more holistic approach needs to be taken.

1.3 Immunopathogenesis of HCV infection

The liver is an immunological organ with a dual vascular supply, where the hepatic artery and hepatic vein are joined by the hepatic portal vein; it is this portal circulation that exposes the liver to food antigens post-absorption (58). It is because of this unique design of the liver

that we find the organ to have developed strategies for immune tolerance upon exposure to portal contents (58). However, the liver can still produce an effective immune response to hepatotropic viral infections, but the mechanism requires dendritic cells to activate T cells in secondary lymphoid organs (58). This balanced system allows the activation and clearance of viral liver infections without causing severe liver inflammation. However, this system of balance often fails in the case of HCV infection (58). A subdued immune response to the infection results in viral persistence whereas a potent response may risk severe inflammation and fibrosis of the liver, leading to progressive organ damage and development of cirrhosis (58)

In the case of chronic HCV infection, the liver's histology indicates the common features of chronic hepatitis such as inflammation in the liver lobules and expansion of the hepatic portal tracts (59). In order to understand the immunopathogenesis of HCV infection, it is important to know how the first line of defense (from the innate immune system) and second line of defense (from the adaptive immune system) may fail because of viral factors, to give rise to the chronic state of the disease.

1.3.1 Innate immune response to HCV infection

Once the virus infects hepatocytes, the first line of response comes from the infected hepatocytes themselves (58, 60). The identification of the virus as non-self takes place through the pattern recognition receptors (PRR) in the cytosol of infected hepatocytes (60). Different parts of the virus and its intermediates are identified by different host receptors. For instance, the viral internal ribosomal entry site (IRES) can be identified by the host's RNA-dependent protein kinase R (PKR), whereas the HCV 3' poly U/U3 sequence is recognized by RIG-I

(retinoic acid inducible gene-I) (58, 60-63). Larger RNA intermediates of HCV replication are recognized by TLR (toll-like receptor) 3 (58, 64). The TLRs can sense them from within endosomal compartments, and Nod-like receptors (NLRs) sense cytosolic viral patterns, metabolites, and so on (65, 66). TLRs are a class of PRRs, that can be found on various cells of the immune system including macrophages, DC (dendritic cells), mast cells, and B-cells, where they help the cells to identify PAMPs (pathogen-associated molecular patterns) and DAMPs (danger associated molecular patterns) (67). TLR3 is expressed in endosomes of various cell types, including hepatocytes, KC (Kupffer cells) and scavenger cells (68, 69). These result in the production of pro-inflammatory chemokines and cytokines (69, 70). Apart from these, there are also non-traditional PRRs such as PKR (Protein Kinase R), that can bind to viral nuclei acid (dsRNA) and interacts with mitochondrial antiviral signal (MAVS) protein (71-73). Figure 2 illustrates some of the mechanisms by which viral factors interfere with the development of a potent interferon response.

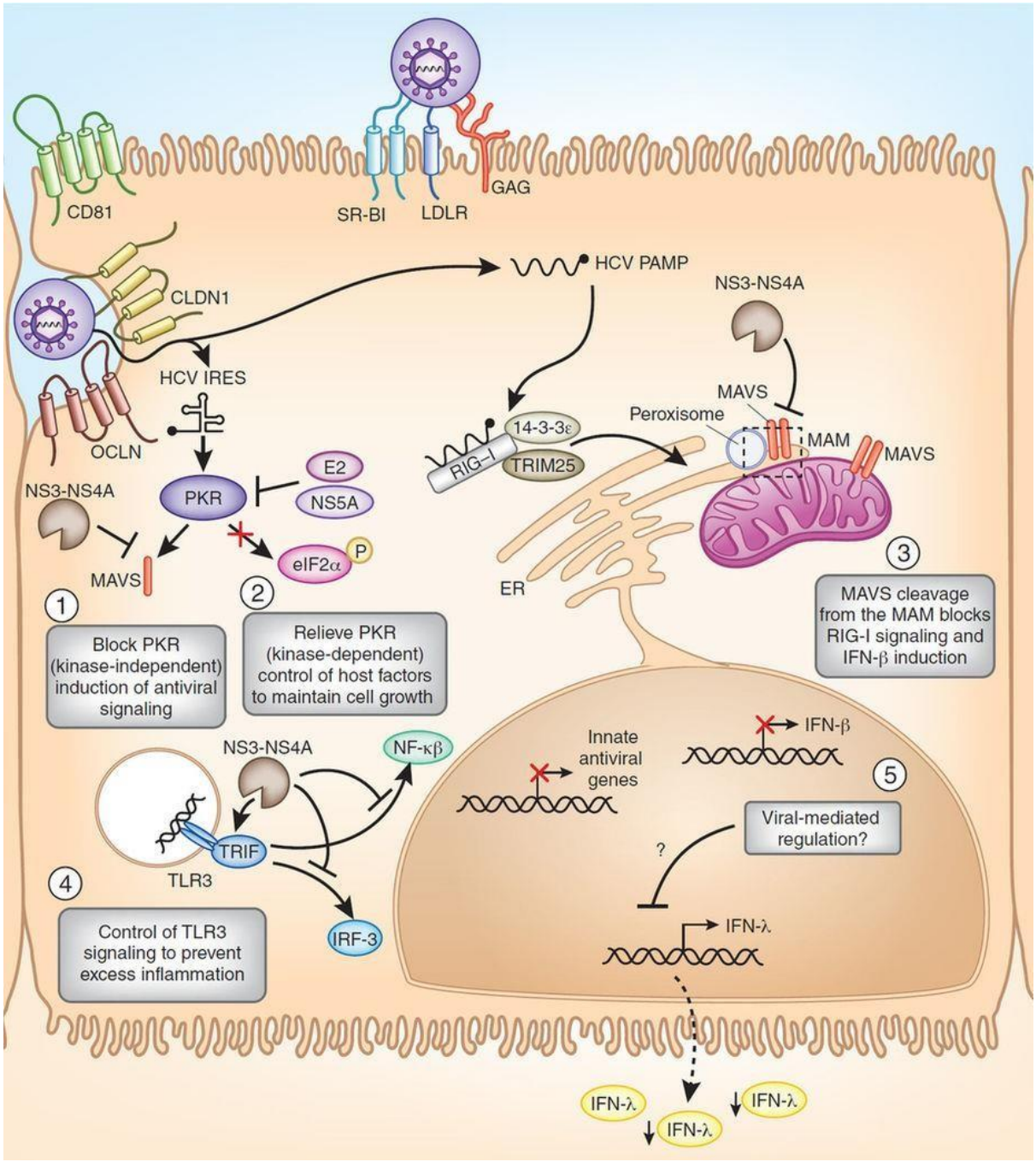

CD81
SR-BI
LDLR
GAG
CLDN1
OCLN
HCV IRES
HCV PAMP
NS3-NS4A
PKR
E2
NS5A
eIF2α
P
MAVS
1
Block PKR (kinase-independent) induction of antiviral signaling
2
Relieve PKR (kinase-dependent) control of host factors to maintain cell growth
RIG-I
14-3-3ε
TRIM25
Peroxisome
MAM
MAVS
ER
3
MAVS cleavage from the MAM blocks RIG-I signaling and IFN-β induction
NS3-NS4A
NF-κβ
TRIF
TLR3
4
Control of TLR3 signaling to prevent excess inflammation
IRF-3
Innate antiviral genes
IFN-β
5
Viral-mediated regulation?
?
IFN-λ
IFN-λ
IFN-λ
IFN-λ

Figure 2

Figure 2: HCV control of IFN induction and immune evasion from Review (60). Immune evasion by HCV in the hepatocyte occurs at several points during viral infection. The proposed regulation is shown here, where the HCV NS3/4A protease cleaves the signaling adaptors MAVS (on the mitochondrial-associated ER membrane (MAM; in the region indicated by the dashed box) and TRIF to inactivate (1) PKR, (3) RIG-I, and (4) TLR3 signaling pathways to prevent induction of immunomodulatory innate antiviral genes and IFN-β allowing for HCV replication. (5) HCV infection control of IFN-λ induction is not yet defined; (2) HCV E2 and NS5A proteins inactivate PKR kinase-dependent activation of the host translation factor eIF2α to reactivate protein translation during infection.

The binding of HCV to RIG-I leads to a conformational change, causing oligomerization and translocation of it from cytosol to intracellular membranes (60, 61, 64, 74). Following that, the activated PKR and RIG-I signals subsequently activate the signaling molecule MAVS (mitochondrial activating signal protein), leading to the activation of TBK1 (TANK binding kinase 1) (64). TBK1 results in the activation of interferon regulatory factor 3 (IRF3) which results in the production of the cytokine IFN-β (58, 75, 76). The activated TLR 3 receptors can also result in the production of IFN-β, however, they work through the Toll/IL-1 receptor domain -containing adapter inducing IFN- β (TRIF) (58, 64). Downstream of this signaling cascade, the TLR 3 works through TBK 1 and IRF 3 similar to RIG-I and produces IFN-β (58, 64).

Even after detection and activation of innate immunity, the virus is able to establish a chronic infection in about 85% of individuals, while the remaining group can achieve spontaneous clearance after the acute infection (77, 78). It is important to recognize, therefore, that the virus can evade a potent innate immune response in several ways. The NS5A protein is able to suppress the function of PKR resulting in viral resistance to the antiviral cytokine, IFN-γ (71, 72). The NS3/4A protease is able to block RIG-I signaling by cleaving MAVS from the mitochondrial membrane (79-81). It is essential for MAVS to remain anchored to the membrane in order for subsequent signal transduction to take place (79, 80, 82). This same viral factor also targets TRIF (Toll-like receptor 3 adapter protein) and cleaves part of it, to eventually reduce the abundance of this protein in infected state (81). Studies have shown that the virus can use PKR to promote infection by inhibiting translation of ISG (interferon stimulated genes) (73, 83). These are only some of the important innate responders to an HCV infection and I have summaried how the virus is able to dampen or evade the immune response and continue to establish a persistent infection.

Apart from this innate response from infected hepatocytes, the liver macrophage populations (including Kupffer cells) produce IL-1β and IFN-β in the infected liver (84, 85). The myeloid DC (mDC) subset of dendritic cells are able to produce IFN-λ whereas the plasmacytoid DC (pDC) subset produces IFN-α (86-89). Although infected hepatocytes themselves can have a defective response to HCV due to viral interference, there is still abundant type I (IFN-α/β) and type III (IFN- λ) interferon in the infected liver from non-parenchymal cells (64, 90).

PRR activation also results in the production of chemokines which help to increase recruitment of leukocytes to the infected liver (58). Furthermore, when the innate immune system fails to clear the infection, they begin to activate branches of the adaptive immune system.

1.3.2 Adaptive immune response to HCV infection

When it comes to adaptive immune responses to HCV infection, we see activation and response from both the cellular and humoral component of this branch of the immune system. Adaptive response can be observed about 2 to 3 months after HCV infection takes place (64). For the cellular response, a potent response from virus-specific $CD4^+$ and $CD8^+$ T cells is necessary to clear the infection (91-95). Among patients who can spontaneously resolve the infection from an acute phase, the effective T cell response is observed in combination with increased production of the cytokines IL-2, IFN-γ and TNF-α (64, 92, 93, 96).

The IFN-α produced bypDC are able to activate both $CD4^+$ T helper cells and $CD8^+$ T cells (also known as CTLs) (58, 97, 98). IL-12 is produced by mDC which then further activate $CD4^+$ T helper cells and $CD8^+$ T cells (97, 98). Apart from such cytokine-mediated activation,

immature DC take up and process HCV antigen which they then carry to lymph nodes (97, 98). They then mature and are able to activate naïve $CD4^+$ T helper cells as well as naïve $CD8^+$ T cells; the T helper cells produce TNF-α and in the presence of the IL-12 from mDCs, they differentiate to Th1 activated state (58, 97, 98). The Th1 drive maturation of naïve $CD8^+$ T cells by producing IFN-γ and IL-2, and these activate cells begin to proliferate and travel to the site of infection- at the liver (58, 97, 98). In individuals who are able to clear the HCV infection, these $CD8^+$ T cells that are specific for HCV antigens would then induce targeted cell lysis of the HCV-infected hepatocytes and eradicate the infection (97, 98).

Additionally, the virus can also manage to evade detection by substitution in their amino acid sequence, resulting in reduced processing of those antigens (T cell epitopes) and subsequently a diminished T cell response to cells presenting them (99). Even when the virus is detected by T cells, they are often able to induce dysfunction in the T cells, greatly reducing the impact of the immune response.

Most individuals who are able to clear the infection show a broad and early response from the virus-specific $CD4^+$ T cell population, while those who become chronic have either a very late response from these cells, or they have an early response followed by a transient drop in their response afterwards (95, 100, 101). Individuals with chronic infection often have a low number of $CD4^+$T (Th1) effector cells, with reduced production of IFN-γ, IL-2, poor proliferation and an overall state of cellular exhaustion indicated by upregulation of inhibitory molecules such as Tim-3 (T-cell immunoglobulin and mucin-domain containing-3), PD-1 (programmed death molecule -1) and CTLA-4 (cytotoxic T lymphocyte associated protein -4) (101-104). Other studies indicate that patients with chronic infection have increased numbers of $CD4^+$ regulatory T cells (known as Tregs) in the liver, which suppress HCV-specific $CD8^+$ T cells (105, 106).

Similarly, the $CD8^+$ T cells also lose their ability to launch a potent immune attack on the infected cells as they experience increased levels of apoptosis during both the acute and chronic phases of HCV infection (107). These cells also enter a state of cellular exhaustion, with upregulated inhibitory surface coreceptors such as PD-1, Tim-3 and LAG-3 (lymphocyte activation gene-3) among others (108, 109). Co-expression of inhibitory proteins (such as 2B4, CD160 and PD-1) on these virus-specific $CD8^+$ T cells also correlated with reduced T cell effector functions such as cell proliferation, delays in T cell differentiation and other anomalies (110). In chronic HCV infected individuals, these cells not only displayed reduced proliferation, but also lower antigen-specific cytotoxicity and lower production of the antiviral cytokine IFN- γ (111).

For clearance of HCV infection, an early and strong response is needed from the $CD8^+$ T cells, but it has been reported that such a response can take several months to appear; this happens due to a delay in their induction, resulting in poor outcomes (112). On the other hand, it is the role of the humoral branch of the adaptive immune system to form neutralizing and non-neutralizing antibodies (nAbs, nNAbs) against the virus (113). One of the mechanisms by which HCV evades the humoral immune system is by cell-to-cell transfer of the virus, which allows them to continue their infectious lifecycle without exiting the cell and being exposed to neutralizing antibodies (50, 114). Apart from protecting itself from exposure to nAbs, the virus is also able to protect itself by preventing the antibody from successfully binding to the viral epitope; for instance, the glycans on the envelope protein E2 are able to mask and thus protect important regions of this viral protein from binding to their specific antibodies (115, 116).

Although HCV does not cause a holistic and general suppression of the immune system, they are able to evade and counteract immune responses in various ways, some of which have been summarized in this introduction.

1.4 Liver inflammation and fibrosis in HCV infection

As the virus establishes a persistent infection within the host liver, a wide range of inflammatory cytokines and chemokines are produced that drive inflammation and eventually cause scarring, or fibrosis, of the liver tissue (117). One of the cells that stimulate the hepatocyte to produce cytokines such as IL-6, IL-8, MIP-1α (macrophage inflammatory protein) and MIP-1β are the HSCs (hepatic stellate cells) (117, 118). It is the activation of these HSCs, possibly activated by TGF-β1 from infected hepatocytes, that leads to its fibrogenic phenotype (119-122). Myofibroblasts (MF) either formed from HSCs or from activated fibroblasts also plays a role in fibrogenesis, and together they are responsible for collagen, MMPs (matrix metalloproteases), and other components in the extracellular matrix (ECM) deposition (122-124).

As the disease progresses, the extent of fibrosis also increases; this can be assessed and allocated a 'score' based on the degree of liver stiffness using several methods; these include liver biopsies or less invasive methods such as transient elastography (also referred to as a Fibroscan) among others (125). Essentially, a fibroscan device has a probe which is positioned to send a 50MHz wave to the patient's liver and the same device has a transducer which helps to measure the velocity of the wave as it passes through the liver (126). This velocity can then generate a value to assess liver stiffness (126). One such scoring system that is widely used to stage the degree of liver fibrosis is called the METAVIR scale which was created based on a

large population of reference liver samples, and this scale allocates a numerical value to the fibrosis stage (127, 128). These stages include F0 with no or minimal fibrosis, F1 where the tissue shows fibrous portal expansion, F2 fibrosis with periportal septa, F3 where it has numerous septa with structural distortion but no liver cirrhosis, and F4 where it displays cirrhosis of the liver (128, 129).

1.5 Impairment of $CD8^+$ T cells in HCV infection

1.5.1 Function of $CD8^+$ T cells in normal individuals

It is well documented that CTL (cytotoxic lymphocytes) or $CD8^+$ T cells are crucial in the adaptive control and clearance of intracellular pathogens and tumors (130). Once activated, these cells are able to launch an immune response by targeted lysis of infected cells and production of pro-inflammatory cytokines such as IFN-γ and TNF-α (130). These cells are able to kill their targets using the faster mechanism involving the cytolytic molecule perforin and the slower mechanism involving Fas ligand (131).

Cytolytic proteins such as perforin are housed within granules and secreted by $CD8^+$ T cells along with granzymes; where perforins 'perforate' the target, and lead to activation of certain caspases, eventually leading to their death (131-133). An excellent indicator of this degranulation are the markers LAMP-1 (lysosomal associated membrane protein-1), LAMP-2 and CD63 (134). LAMP-1 also known as CD107a, fuses with the surface of $CD8^+$ T cells upon release of the granular contents (134). Previous studies have established the correlation between CD107a expression and the cytotoxic functions of $CD8^+$ T cells, making it a great marker of their functionality (134).

In perforin-dependent method of killing by $CD8^+$ T cells, secretory granules carrying serine proteases (referred to as granzymes/granule-associated enzymes) are released onto target cells along with perforin (135, 136). Granzymes form a group of homologous enzymes which can trigger various pathways of cell death within the target (135). Among five different granzymes found in humans, type A and B are the most frequently available (137). Once the $CD8^+$ T cells are activated, the granules move towards the immunological synapse that forms between the T cell and the target cell expressing the antigenic peptide (135). The contents of the granules are released, and the granzymes enter the target cell via microscopic pores created by perforin in a complex chain of events (136). In the case of Granzyme B, death is induced via cleavage of caspase-3 and proteolysis of other caspase substrates (135). On the other hand, Granzyme A can induce apoptosis in a caspase-independent mechanism (135). Granzymes H, K, and M are often referred to as "orphans" since their exact role and function are yet to be elucidated (137). Interestingly, there is a lot of diversity in terms of the expression of the different granzymes, perforin and IFN-γ by $CD8^+$ T cells; meaning that not all T cells will express all these proteins at a given time (136).

The perforin-granzyme cell death mechanism is the main method by which $CD8^+$ T cells kill virus-infected cells (138). The Fas ligand method, where cell death receptor proteins on target cells (e.g. Fas) bind to their ligands on $CD8^+$ T cells, results in caspase-dependent cell death; however, this mechanism is primarily used by the body to kill off auto-reactive lymphoid cells, and not for infected or transformed targets (138).

However, as mentioned earlier, induction of apoptosis is not the only function of an activated $CD8^+$ T cell. Production of pro-inflammatory cytokines such as IFN-γ not only help to activate other immune cells and create an antiviral impact, but it can even help the $CD8^+$ T cell itself (139). A recent publication has illustrated how the autocrine and/or paracrine IFN-γ

was able to promote the speed and cytotoxicity of $CD8^+$ T cells (139). The production of IFN-γ by activated antigen-specific $CD8^+$ T cells even correlated positively with their ability to lyse target peptide-pulsed cells (140). However, $CD8^+$ T cells are also able to produce and secrete IFN-γ without activation through specific antigen presentation; this subset of memory $CD8^+$ T cells that can produce the cytokine in an antigen-independent manner possibly do so with the help of cytokines IL-12 and IL-18 (141).

In order for T cells to become activated in the typical antigen-dependent manner, an activation complex needs to form between its TcR (T cell receptor) and the corresponding cell's class I MHC (major histocompatibility complex) presenting the peptide (142). In $CD8^+$ T cells, this complex is further stabilized by the CD8 surface protein co-receptor (143). In $CD4^+$ T cells, the TcR engagement must be accompanied by co-stimulation of the two co-receptors: CD3 and CD28 (144). CD28 receptor has two different ligands, CD80 and CD86, both of which are potent stimulators for T cell activation (145).

1.5.2 Impairment of HCV-specific $CD8^+$ T cells in HCV infected patients

Since $CD8^+$ T cells are one of the major effectors of the adaptive immune system, failure of a robust immune response from HCV-specific $CD8^+$ T cells acts as a strong indicator for the development of a persistent, chronic infection (94). T cell exhaustion and dysfunction among the virus-specific T cell populations has been well described (146). In such scenarios, the HCV-specific T cells ($CD8^+$ T cells in particular) are found to have upregulated inhibitory markers such as PD1, CTLA4 (cytotoxic lymphocyte antigen 4) and LAG3 among others leading to a state of 'exhaustion' (147). However, studies have also shown that the impairment

in $CD8^+$ T cell function in individuals with chronic HCV infection are not limited to their HCV-specific cell populations, but also effect 'bulk' $CD8^+$ T cells (148).

1.5.3 Generalized $CD8^+$ T cell impairment in HCV infected patients

CMV-specific $CD8^+$Tcells derived from chronic HCV infected individuals display a significantly lower expression of perforin, Fas and markers associated with T cell maturation (compared to healthy controls) (148). Another study reported that 'bulk' $CD8^+$ T cells from chronic HCV infected patients showed increased apoptosis after a T cell receptor stimulation (149). Our research group has previously shown that upregulation of the survival marker Bcl-2 in response to IL-7 was reduced in bulk $CD8^+$ T cells derived from chronic HCV infected patients compared to healthy controls (150). Furthermore, this reduction in Bcl-2 upregulation was significantly higher in patients with increasing levels of liver fibrosis (150). Evidently, the dysfunction is not limited to virus-specific $CD8^+$ T cells and extends to all $CD8^+$ T cells in such chronically infected individuals. However, the cause for such a generalized impairment is not well understood.

In order to understand the cause of such generalized impairment among $CD8^+$ T cells from individuals chronically infected with HCV, we attempted to investigate the role of innate cells that contribute to the cytokine microenvironment of the infected tissue.

Blood from the portal vein and hepatic artery passes through the vascular tubes called sinusoids, which are lined by cells known as LSEC (liver sinusoidal epithelial cells) (151, 152). The porous nature of these cells lining the sinusoids facilitate interaction of T lymphocytes with the hepatocytes as well as liver macrophages (Figure 3) (151, 152). Studies using electron micrography have shown T lymphocytes extend through fenestrations on the

LSEC membrane and have direct contact with hepatocytes (151-154). The sinusoidal endothelium have unique structural features such as the absence of typical basement membrane, which allows it to exchange microparticles between the liver tissue and the blood (153, 154). However, the LSECs are not the only cells which help T lymphocyte interaction with the liver (155). The resident liver macrophages called Kupffer cells allow circulating $CD8^+$ T cells to adhere and interact with infected liver tissue (155). It is evident that all circulating T lymphocytes come into contacted with the liver and its cytokine milieu. In the next section, I have discussed how, in normal, healthy individuals, the liver and its innate cells are able to maintain a state of immune tolerance and why they may be expected to be altered in chronic HCV infection.

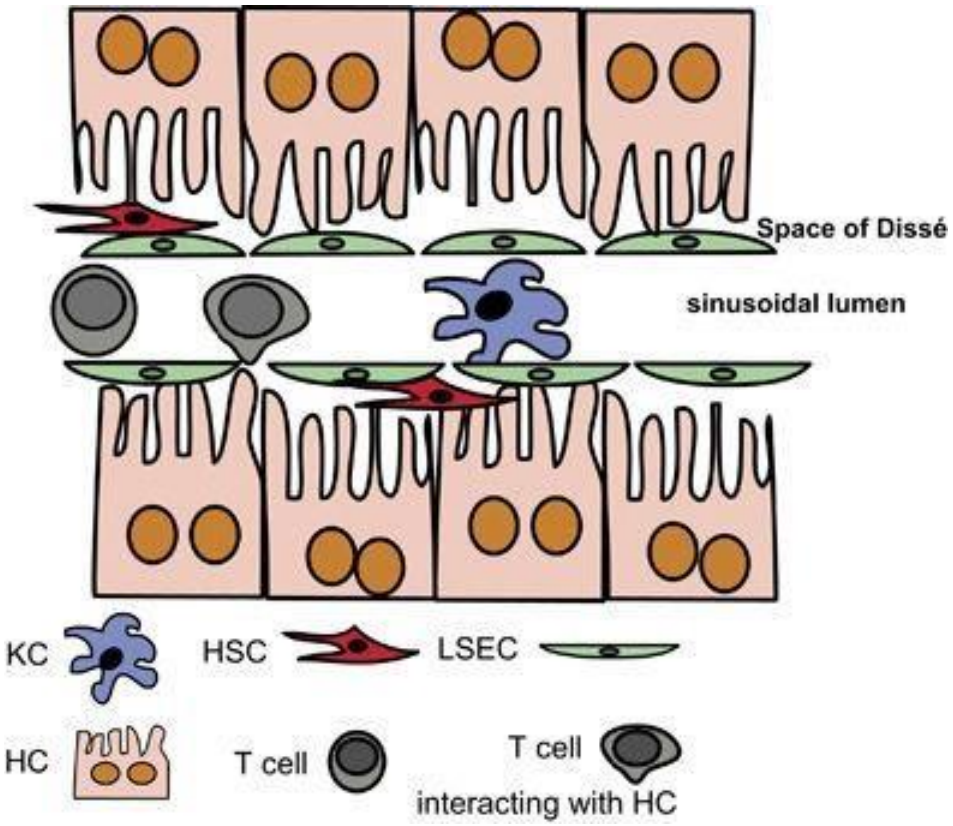

Space of Dissé
sinusoidal lumen
KC
HSC
LSEC
HC
T cell
T cell
interacting with HC

Figure 3

Figure 3: Liver sinusoidal structure & physiology allows liver macrophages to come into close contact with T lymphocytes in the blood circulation from Review (152). Schematic representation of the microanatomy of the liver sinusoids and their cellular composition. The hepatocytes are separated from the sinusoidal blood flow by the liver sinusoidal LSECs that create the Space of Dissé and shield the hepatocytes from sinusoidal blood flow. Between the LSECs and the hepatocytes, hepatic HSCs are interspersed. In the sinusoidal lumen, KCs and passenger leukocytes are located. Note that T cells can form intimate contacts with microvilli from hepatocytes, but also LSECs or KCs, which enables priming of T cells in the liver. HSCs, hepatic stellate cells; KCs, Kupffer cells; LSECs, liver sinusoidal endothelial cells.

1.6 Role of macrophages in immune activation & tolerance

1.6.1 Liver macrophage populations

Although the liver is assumed to be made up of cellular units of hepatocytes alone, a third of the organ's cells are actually non-hepatocytes, which include KC, LSEC, HSC (hepatic stellate cells), and lymphocytes among others (156). KC comprise the largest population of fixed macrophages in the body and make up almost 20% of the non-hepatocyte cell populations in the liver (156). There is some controversy regarding the origin of KC population being regenerated solely from yolk-sac derived pre-existing cells within the liver or from a continuous supply of infiltrating, bone-marrow derived monocytes (154, 157). Regardless, during liver inflammation, the resident population of innate cells are joined by large numbers of infiltrating monocytes, which differentiate into macrophages (M Φ) or dendritic cells (DC) (156, 158). Hepatic macrophages play a crucial role in liver homeostasis and immunity by responding to DAMPs and PAMPs and promoting the formation of inflammasomes (159).

These liver-resident macrophages also play an important role in induction of the immune tolerance attribute of the liver. For instance, the KC are able to patrol sinusoids and take up antigens without inducing an inflammatory response (152). They do so by producing suppressors such as IL-10, nitric oxide, TGF-β among others (152). However, the same KC are also able to produce immune activating factors such as TNF-α, IL-6, IL-1β and more (152). Both KC and infiltrating macrophage populations are able to play a dual role in pro- and anti-inflammatory immune response and modulate activation and tolerance. To understand the diverse functions of macrophages in the liver (or any other such organ), it is important to understand the reason behind inducing and controlling inflammation. Inflammation from

infection often results from an immune response to the foreign pathogen which has altered homeostasis. However, the inflammatory response generated to restore homeostasis is itself able to damage tissue for which it needs to be tightly regulated (157).

Monocytes originate in the bone-marrow and circulate in the blood for about 2 days, during which time they are recruited to infected or damaged tissue. If such a recruitment signal is not present, the monocytes die (157). Stromal cells within the blood and tissue are able to produce a growth factor called M-CSF (macrophage colony stimulating factor) that can induce the development of monocytes to macrophages (157). Once they become macrophages, they are able to play numerous roles in both defense and homeostasis of the tissue. However, which function they play is dependent on the subset that they can polarize to. The plasticity of macrophages allowed them to respond to the signal in their immediate environment and form diverse subsets that may be pro- or anti-inflammatory in their response (160).

As I mentioned earlier, the unique portal blood supply of the liver brings it into constant contact with bacterial endotoxins and other such antigens (161). As a result, the liver needs to carefully maintain a balance between immune activation and immune tolerance to avoid a constant state of immune activation and inflammation (161). Previously (in section 1.4) I discussed one such mechanism of immune suppression involving T cell activation at secondary lymphoid sites. In fact, when T cells are activated in the liver, it results in an exhaustive state where effector functions are severely limited and the T cells die prematurely (152). However, other cell populations such as resident liver macrophages also play an important role to maintain this state of tolerance. For instance, at least three cell populations including DC, KC and Tregs (regulatory T cells) produce IL-10 known to be an anti-inflammatory cytokine (152). It is well documented that HSCs can prevent the activation of naïve $CD8^+$ T cells (152, 162). They can even induce early apoptosis of activated $CD8^+$ T cells they encounter by increasing

the expression of the PD-L1 (163). Although the function of DC, LSEC and HSC in the suppression of $CD8^+$ T cell functions is well described, the possible role of KC and MDM (monocyte derived macrophage_ is poorly understood.

While immune tolerogenic nature of the liver although meant to control excessive inflammation, can also make room for pathogens to escape complete eradication; this often results in the development of a chronic infection (164).

1.7 Macrophage subsets

As mentioned earlier, the heterogenous cell population of macrophages are able to polarize into pro- and anti- inflammatory states (or subsets) depending on the type of stimuli they encounter (165). For instance, when they encounter pro-inflammatory cytokines such as IFN-γ or TNF-α, they adopt an M1-like or classically activated macrophage (CAM) phenotype (165-167). On the other hand, when they receive stimuli from anti-inflammatory cytokines such as IL-4, IL-10 or IL-13, they form an M2-like or alternatively activated macrophage (AAM) phenotype, which are immune-tolerant in nature (165, 167, 168). The M2 subset can be identified on the basis of a range of markers such as glycoproteins, scavenger receptors, enzymes, cytokines, and more (169). Even within the M2 population, there are differences in the function and phenotype of the macrophages which allows them to be subdivided into three sub-categories: M2a, M2b and M2c (168). M2a are the ones mainly responsible for tissue repair after injury and are formed in response to IL-4, IL-13 and parasitic or fungal infection (31, 169). The M2b is a regulatory subset, formed in response to immune complexes and bacterial lipopolysaccharide (LPS) stimulation; they help to dampen the effects of an immune

response to control excessive inflammation (31, 170). M2c subset formed in response to IL-10, TGF-β, and glucocorticoids and are known as the "deactivated" subset (169, 170).

1.7.1 M1 Macrophages

Once macrophages have an M1 phenotype, they have a Th1 type of immune response where they produce TNF-α, IL-1, ROS (reactive oxygen species), iNOS (inducible nitric oxide synthase), but have low expression of arginase. This subset, therefore, has a strong anti-microbial and anti-tumoricidal activity (171). This subset is mostly induced by intracellular pathogens, pathogenic by-products of microbes, and pro-inflammatory cytokines such as IFN-γ and TNF-α. Such subsets when generated *in vitro* display a high expression of CD80 and IL-12 in comparison with M0 (unpolarized) and M2 subsets (172). CD206, on the other hand, is expressed by both M1 and M2 macrophage subsets but has a higher expression on M2 macrophages (172-175). Another study showed high expression of CD80 and CD86 on M1 when compared to M0 (31). Once stimulated with LPS, M1 were able to secrete significant amounts of IFN- γ, TNF-α, IL-12, and IL-23 (31). Studies using different in vitro models to generate the subsets have reported slight variations in their resulting phenotype based on surface protein expression and cytokine profiles.

1.7.2 M2 Macrophage subsets

At basal levels, all the M2 subsets (M2a, M2b and M2c) are able to secrete IL-10 (31). This anti-inflammatory cytokine binds to its receptor (IL-10R1-R2 dimer) and inhibits expression of pro-inflammatory cytokines (167). In terms of surface receptors, the M2a subset shows a high expression of CD86 while M2c subset showed very low expression of this marker

(31). M2a and M2c produced low levels of IL-6 and IL-1β. Furthermore, M2c display a significantly high expression of CD163. The in vitro generated M2b subset showed high expression of CD80 and CD14 surface receptors and produced high amounts of IL-6 and TNF-α; although this subset produced comparatively low amounts of most cytokines (except IL-5 and IL-10) after being challenged with LPS and other TLR stimulants (31). The M2b subset also promotes Th2 responses like the rest of the M2 family members, however, is unique in its ability to cross-talk with B-cells, exhibit impairment in their phagocytotic ability, are able to switch from IL-12 production to IL-10 production, while increasing antigen presentation (167). In short, the combination of LPS and IL-1β, the two cytokines used to generate this subset *in vitro* give it a mixed phenotype and function with features of both M1 and M2 family (167).

1.7.3 Macrophage subsets in chronic HCV infection

Not much is known about the state of MDM subsets in the context of chronic HCV infection. There is some controversy regarding the possibility of monocytes and macrophages also becoming infected by HCV (176, 177). It is possible that macrophages are more infective by certain genotypes of HCV over others, and that they allow a protective area of the virus to replicate and multiply without undergoing cell death (177). Not only can they help to promote the progression of HCV infection, but they have been implicated in playing a role in extra-hepatic manifestations of HCV infection (177, 178). These diseases include B-cell non-hodgkins lymphomas, mixed cryoglobulinemia and autoimmune thyroiditis and are associated with a state of chronic inflammation and deposition of immune complex (177, 178).

Some studies have shown that the serum of HCV-infected patients was able to successfully infect monocytes, macrophages and DC (177, 179-181). It is believed that macrophages infected with HCV are able to live without cytotoxic effects, allowing the infection to grow and spread to areas to which the cell travels (177). Whether or not the monocytes and macrophages in patients with chronic HCV infection harbor a productive infection by the virus, they are still exposed to the virus and its proteins, which should result in a response or anomalous alteration in their functions and phenotype.

As HCV infection spreads rapidly through the liver tissue, KCs and MDMs come in contact with free viral nucleic acids and proteins. The double-stranded RNA (dsRNA) are able to trigger TLR3, while the single-stranded RNA (ssRNA) are able to trigger TLR8 possibly resulting in activation and subsequent production of type I interferons (176). KC have been shown to display the activated M1-like phenotype by increasing their MHC II expression in response to HCV infection (176).

In a typical viral infection, KC produces CCL2 (chemokine c-c motif ligand 2), which signals circulating monocytes to infiltrate the infected liver (176). Here, these monocytes produce the chemokine ligand CCL3 which in turn would recruits innate immune cells, driving a cascade of immune activation steps to eliminate the viral infection (176). In animal models of HCV infection, such a cascade resulting in chemokine and IFN-γ induced response was only observed in those who spontaneously resolved the infection at an acute stage (176). Therefore, it is important to consider if macrophages are able to form activated subsets when they are in the HCV-infected liver and whether they can function normally.

Animals with chronic HCV infection were observed to have a large macrophage population in their livers (176). In HCV transgenic mouse models, the number of M2 macrophages were higher in number within the liver tissue; surprisingly, this increase

corresponded with an increase both serum pro-inflammatory cytokines (such as TNF-α and IL-6) as well as anti-inflammatory cytokines (such as IL-4 and IL-10) in the serum (182). Another study has shown in both a humanized mouse model of the disease as well as in liver tissue from individuals with chronic HCV infection, that a higher number of macrophages had an M2 surface phenotype (compared to M1) and these macrophages were found in regions of the liver with the most fibrotic scars (183). It is evident that the M2 macrophages dominate the liver during infection, although their resulting cytokines do not necessarily adhere to the expected M2 cytokine profile as described in literature. It is also not known why most macrophages are displaying an M2 phenotype in an environment that is known to have chronic inflammation, and whether these populations identified as M1/M2 based on surface receptors are indeed functionally producing the traditional M1/M2 cytokines in the diseased condition.

One study has reported a direct role of macrophages in the activation of HSCs (pro-fibrotic cells); when the macrophages were exposed to HCV, they were able to activate HSC through production of CCL5 *in vitro*, leading to an increase in inflammatory and pro-fibrotic markers within HSCs (184). Furthermore, the viral core protein (HCV core) which is often found in the serum of infected patients was implicated in causing a dysfunction at the monocyte-level, which prevented them from fully being able to polarize into M1 and M2 macrophages (185, 186). The study found that the viral protein could inhibit the proper differentiation and polarization of monocytes via TLR2 by manipulating the STATs signaling pathway (186). Some of the major observations identified the ability of HCV core protein to inhibit expression of CD80 and CD86 and production of TNF-α by M1 macrophages (185, 186). For M2 subset, the HCV core protein was able to inhibit the expression of CD163, CD206, and the production of IL-10 (186). Overall, there appears to be dysfunction in

macrophages in HCV infection with respect to their polarization and functions, which could result in an altered effect of macrophages on $CD8^+$ T cells that requires further investigation.

1.8 Monocyte/Macrophage dysfunction in chronic HCV infection

An increase in cytokines and chemokines are not only limited to the hepatic environment but are also found in the circulation. Individuals with chronic HCV infection have elevated serum levels of IL-1α, IL-2, IL-10, IL-33, TNF-α, IL-6, IFN- γ and TGF-β among others (187-191). Along with cytokines, there is also an increased level of endotoxin and the viral protein HCV core in the serum of these individuals (191). This concoction of chemokines, cytokines and viral factors changes the environment to which the circulating blood cells including monocytes and T lymphocytes are exposed. Studies have found alterations in the phenotype of circulating monocytes in patients with chronic HCV infection. Monocytes displayed an increase in expression of the chemokine receptor CXCR3, CXCR4 and a decreased expression of CCR2 (192). Since several ligands of the CXCR3 receptor are products of the ISGs (interferon stimulated genes), its increase in expression could indicate a response to the infection (192). Interestingly, the ligand for CCR2 (CCL2) is increased in serum and liver of HCV infected patients; this reduction in the receptor's expression on monocytes may indicate a negative feedback to control monocyte recruitment to the liver (192).

Typically, when monocytes are exposed to various TLR stimulations, they are expected to develop tolerance (191). However, monocytes from patients with chronic HCV infection showed loss of tolerance and rather a hyperresponsiveness to TLR stimulation, resulting in

increased production of TNF-α (191). Since individuals with chronic HCV infection had increased levels of IFN-γ, endotoxins and HCV core protein in their serum, an *in vitro* experiment was conducted to see if exposure to such factors was causing a similar hyperresponsiveness of these cells (191). It was then discovered that IFN-γ pre-treated monocytes from uninfected controls were resulting in a loss of tolerance to a subsequent LPS or HCV core stimulation (191). Interestingly, after these 'hyper-responsive' monocytes were differentiated into macrophages in culture, they re-gain their ability to become tolerant to TLR stimulations (191). Although HCV is a hepatotropic disease, the change in cytokine and chemokine factors in the circulation as well as the presence of some viral factors results in anomalies in circulating cells such as monocytes. When these cells are eventually recruited to the liver, the resulting phenotype and function expected of them may be severely altered as well.

1.9 Impact of macrophage subsets on $CD8^+$ T cells activation & function

1.9.1 Contact dependent impacts

In extracellular infections involving $CD4^+$ T helper cells and APCs, the presentation of antigen by MHC class II molecules needs to be accompanied by a co-stimulatory stimulus from the CD80/CD86 ligands expressed on the APC, which activates the CD28 receptor on the T cell. However, in intracellular infections and tumors which activates $CD8^+$ T cells, the co-stimulation from CD80/86 ligands (anti-CD28) may not be required for activation and proliferation (193). This theory is based on the understanding that a potent stimulation by TcR engagement via antigen presentation alone is enough to activate naïve $CD8^+$ T cells to form

antigen-specific effector cells (193). A study using an artificial APC system showed that even a brief 2 hour contact-dependent stimulation by the antigen presenting cell was able to activate $CD8^+$ T cell to then form effector cells (194). A mouse model has shown that a TcR signal solely from antigen presentation to a $CD8^+$ T cell can lead to cellular anergy in the absence of CD28 co-stimulation, unless the antigenic stimulation is prolonged (195).

Macrophages, B cells and Dendritic cells are APCs, although in contrast to DCs, the ability of macrophages and B cells to activate naïve T cells is still controversial (196). It is possible that only certain subsets of activated macrophages have a high expression of activating molecules (such as MHC class I, co-stimulators, etc.) to stimulate the activation of naïve T cells (197). M1 macrophages are known to express high levels of the ligand CD80, while M1 and M2a both express high levels of CD86, but it can vary slightly depending on the model of macrophage generation (198-201). The ligands CD80 and CD86 as well as anti-CD28 mAb (monoclonal antibodies) were tested to observe CD28 stimulation via tyrosine phosphorylation of the receptor, and it was found that CD86 had a lower ability to induce such stimulation compared to CD80 and anti-CD28 mAb (202). M2a and M2c macrophage subsets generated using IL-4, IL-10 and TGF-β form an immunosuppressive subset with low CD86 and MHC class II expression and was able to reduce T cell expansion as well (203). They reduced proliferation by increasing the expression of PD-L2 (programmed death inhibitory ligand) an inhibitory molecule (204). Overall, these are some of the possible mechanisms by which macrophages can activate T cells via cell-to-cell contact. Alternatively, since these macrophage subsets are also able to produce signature cytokines, it is possible that they can impact T cell function through soluble factors.

1.9.2 Contact independent impacts

Macrophages produce cytokines upon activation and depending on the type of activation to M1 or M2 subsets, the cytokines produced can vary greatly. For instance, when classically activated to M1, macrophages produce large amounts of TNF-α, IL-1β, IL-6, IL-12, IL-23 and other pro-inflammatory cytokines (205). The M2b subset, interestingly, produce anti-inflammatory IL-10 but also pro-inflammatory cytokines such as TNF-α and IL-6 (205). M2c subset produces IL-10 and TGF-β, both of which are considered to be immune-suppressive; overall indicating that these cytokines can impact $CD8^+$ T cell function at certain concentrations in different ways (205). IL-6 has both pro-inflammatory and anti-inflammatory effects, depending on the conditions and cells upon which it acts. Its effects on $CD4^+$ T cells are well studied, where it promotes Th2 and Th17 functions as opposed to Th1, but its effects on $CD8^+$ T cell is not well investigated (206). IL-12 on the other hand promotes a more Th1 response in $CD4^+$ T cells, and in $CD8^+$ T cells it is able to induce cytotoxic functions (207). IL-10 is known to promote formation of regulatory T cells (Tregs) which are known to be strongly immune-suppressive (208). The study also showed that IL-10 can induce naïve T cells to produce more IL-10, IFN-γ and reduce production of the cytokines IL-4 and IL-13, which is a mixed cytokine profile (208).

1.10 Rationale and hypothesis

The generalized impairment of $CD8^+$ T cells in individuals with chronic HCV infection has been previously described, however, the possible role of by-stander cells such as

macrophages in mediating such dysfunction remains unknown (148-150). Similar exhaustion in $CD4^+$ T cells in such individuals have shown a decreased immune response to other viral vaccines such as those for HBV (Hepatitis B virus) (209). This highlights the importance of investigating whether macrophages induce dysfunction in T cells and the subsets of macrophages that may be involved.

Macrophages in pathological conditions can influence their tissue cytokine environment in a manner that aids disease progression and influence secondary cell populations, such as tumor-associated macrophages whose altered cytokine milieu in breast cancer potentially leads to increased tumor metastasis (210). *In vitro* studies have shown that monocyte-derived DC exposed to HCV resulted in an increased expression of IFN-β as well as reduced expression DC maturation markers (211). Another *in vitro* investigation showed HCV-induced differentiation of monocytes to macrophages result in a mixed M1/M2 phenotype, which display M2 surface receptors and a mixture of both M1 and M2 characteristic cytokines (212). It is therefore important to assess whether monocyte-derived macrophages generated from individuals with chronic HCV infection have an altered phenotype or cytokine profile compared to those who are uninfected; and if it does, how that altered phenotype may influence $CD8^+$ T cell function.

1.1.1 Hypothesis

I hypothesize that macrophage subsets activate $CD8^+$ T cells in healthy individuals. In chronic HCV infection, such macrophage subsets are impaired, resulting in altered $CD8^+$ T cell function.

Statement of objectives

To evaluate whether an altered phenotype exists in monocyte-derived macrophage subsets of chronic HCV infected individuals and its possible involvement in altering $CD8^+$ T cell function, my research project has the three following objectives:

- Assess if phenotypic differences exist between MDM subsets in healthy and chronic HCV infected individuals.
- Establish the role of each MDM subset in the induction of $CD8^+$ T cell functions in healthy and chronic HCV infected individuals.

Chapter 2: Materials and Methods

2.1 Study Subjects

Individuals recruited for the study were either healthy volunteers who were HCV uninfected (HCV^-), and those who were HCV mono-infected (HCV^+). For the HCV^+ group, a number of mandatory inclusion criteria were considered before collecting samples; these include:

- Individuals were chronic HCV patients (6 months or more HCV RNA^+ status)
- Individuals were untreated for HCV at time of blood draw (TNX^- treatment naïve)
- Individuals were not co-infected with Human Immunodeficiency Virus (HIV-) or Hepatitis B Virus (HBV-)
- Individuals had liver fibroscans done within last 12 months of blood draw

Peripheral blood was collected from human subjects by clinic nurses at the Viral Hepatitis Clinic and the Clinical Investigation Unit at The Ottawa Hospital. Sample collection and consenting method was approved by The Ottawa Health Science Network Research Ethics Board. Fibroscans of the liver were conducted by nurses at the Viral Hepatitis Clinic and The Ottawa Hospital Medical Imaging Department, and scores were then used to stage patients on the basis of their fibrosis using the METAVIR system. Patients were grouped into early fibrosis (F0-2) and late fibrosis (F3-4) stages. Characteristics of the patients have been summarised in table 1 below.

A

HCV+ Subject No.	Fibrosis Group	FibroScan Value (kPA)	Age	Sex	Ethnicity	Viral Load (IU/mL)	Genotype	AST (U/L)	ALT (U/L)
1	F0-2	4.3	57	F	Caucasian	2.08E+06	1a	13	28
2	F0-2	5.4	57	M	Caucasian	2.65E+06	1a	19	30
3	F0-2	5.2	58	M	Caucasian	2.59E+06	1	38	63
4	F0-2	7.6	20	F	East Indian	1.43E+03	1b	23	65
5	F0-2	4.6	37	F	Caucasian	6.20E+06	1	15	15
6	F0-2	9.9	67	F	Caucasian	3.75E+06	1a	16	23
7	F0-2	5.5	63	M	African	5.99E+06	2b	17	83
8	F0-2	7.6	50	M	Caucasian	4.23E+06	1a	41	58
9	F3-4	11	52	M	Native	3.46E+05	1a	24	26
10	F3-4	23.1	60	M	Caucasian	4.74E+06	3	78	111
11	F3-4	16	54	M	Caucasian	6.61E+04	1a	35	54
12	F0-2	4.8	41	M	Caucasian	4.43E+05	1a	47	61
13	F3-4	27	60	M	Caucasian	1.09E+06	1a	104	109

B

Uninfected Donor No.	Age Group
1	<40 Years
2	<40 Years
3	<40 Years
4	<40 Years
5	<40 Years
6	<40 Years
7	<40 Years
8	<40 Years
9	<40 Years
10	Years
11	**>40 years**
12	**>40 years**
13	**>40 years**

Table 1: Characteristics of Patients with Chronic HCV Infection and Healthy Donors who participated in the study. (A) All HCV-infected patients who agreed to donate their blood samples to the study provided information regarding their age, sex, ethnicity, and diagnostic markers including their viral load, liver fibroscan values, ALT and AST levels. The Viral Hepatitis Clinic at The Ottawa General Hospital allocated a Fibrosis group score using the METAVIR scale based on the FibroScan values. **(B)** Uninfected donors only provided information verbally regarding their HCV^- (negative) status and age-group with a cut-off value of 40 years that was used to separate two age groups.

2.2 Isolation of blood mononuclear cells

Human PBMC was isolated from whole blood as previously described (31, 150, 213). Peripheral blood was collected from patients and donors in heparinized tubes, following which the blood was pooled together and processed to isolate peripheral blood mononuclear cells (PBMC) on the same day. PBMC were isolated using a method called density gradient centrifugation, where 30mL of blood is layered over 15mL of lymphoprep (Stemcell technologies Inc., British Columbia, Canada) and centrifuged at 470xg for 30 minutes with no break on the centrifuge. The resulting layers formed include the plasma layer at the top (which is discarded), the 'buffy' coat containing isolated mononuclear cells (which is collected and transferred to a separate tube), and the bottom layers containing lymphoprep and red blood cells which are discarded with the tube. Cells are then washed with PBS (phosphate buffered saline) and centrifuged at 470xg for 10 minutes 2 consecutive times before counting and culture.

2.3 Isolation of monocytes and generation of macrophage subsets

Monocytes were isolated from PBMC and differentiated into macrophage subsets as shown in Figure 4 and as previously demonstrated (214, 215). Isolated PBMCs were first resuspended at $3X10^6$ cells/mL in serum-free Iscove's modified DMEM 1X media (Thermo Fischer Scientific, New York, USA) and 20ml was plated in a T75 tissue-culture treated flasks (Thermo Fischer Scientific, New York, USA). Cells were incubated at 37°C, 5% CO_2, for 3 hours to separate the monocytes from the PBMC by adherence method. Non-adherent cells were then washed off using 15ml of PBS for each of the 3 consecutive washes. Following this, 20ml of 1XDMEM was added to each flask, containing 10%FCS (fetal calf serum) (Corning,

Virginia, USA), 10units/ml of penicillin/streptomycin (Thermo Fischer Scientific, New York, USA) and 20ng/ml of M-CSF (Monocyte colony stimulating factor) (R&D Systems, Minnesota, USA). The monocytes were then cultured at 37°C, 5% CO_2 for 6 days to allow them to differentiate into macrophages. The media was discarded and replenished with fresh media on the fourth day.

Once macrophages have been generated on day 7, the cells are washed and treated with 12ml Accutase cell detachment solution (Innovative Cell Technologies, California, USA) in each T75 flask and incubated for 30 minutes at 37°C, 5% CO_2. Following this, the cells were gently scraped using a cell scraper (Sarstedt Ag & Co, Germany) and transferred to a 50ml Falcon tube for washing with PBS. The cells were then counted and resuspended at 250,000/ml in 1XDMEM containing 10% FCS and Pen/Strep. They were seeded at this concentration in 12-well polystyrene plates (Thermo Fischer Scientific, New York, USA), with 1ml in each well. To each well, polarizing cytokines were added to generate each of the four macrophage subsets (M1, M2a, M2b and M2c) and the untreated cells were designated as M0. The cytokines used were IFNγ (20 ng/ml) (Thermo Fischer Scientific, New York, USA) to generate M1 macrophages, IL-4 at 20 ng/ml (R&D Systems, Minnesota, USA) for M2a macrophages, LPS (lipopolysaccharide) 1 µg/ml (Sigma-Aldrich) and IL-1β at 10 ng/ml (R&D Systems, Minnesota, USA) for M2b macrophages and IL-10 (10 ng/ml) (R&D Systems, Minneapolis, Minnesota) to generate the M2c macrophages. They were incubated with these cytokines for polarization into these subsets at 37°C, 5% CO_2 for 48 hours. The polarized macrophages were then washed twice with PBS and cultured with fresh media for 72 hours before analysis.

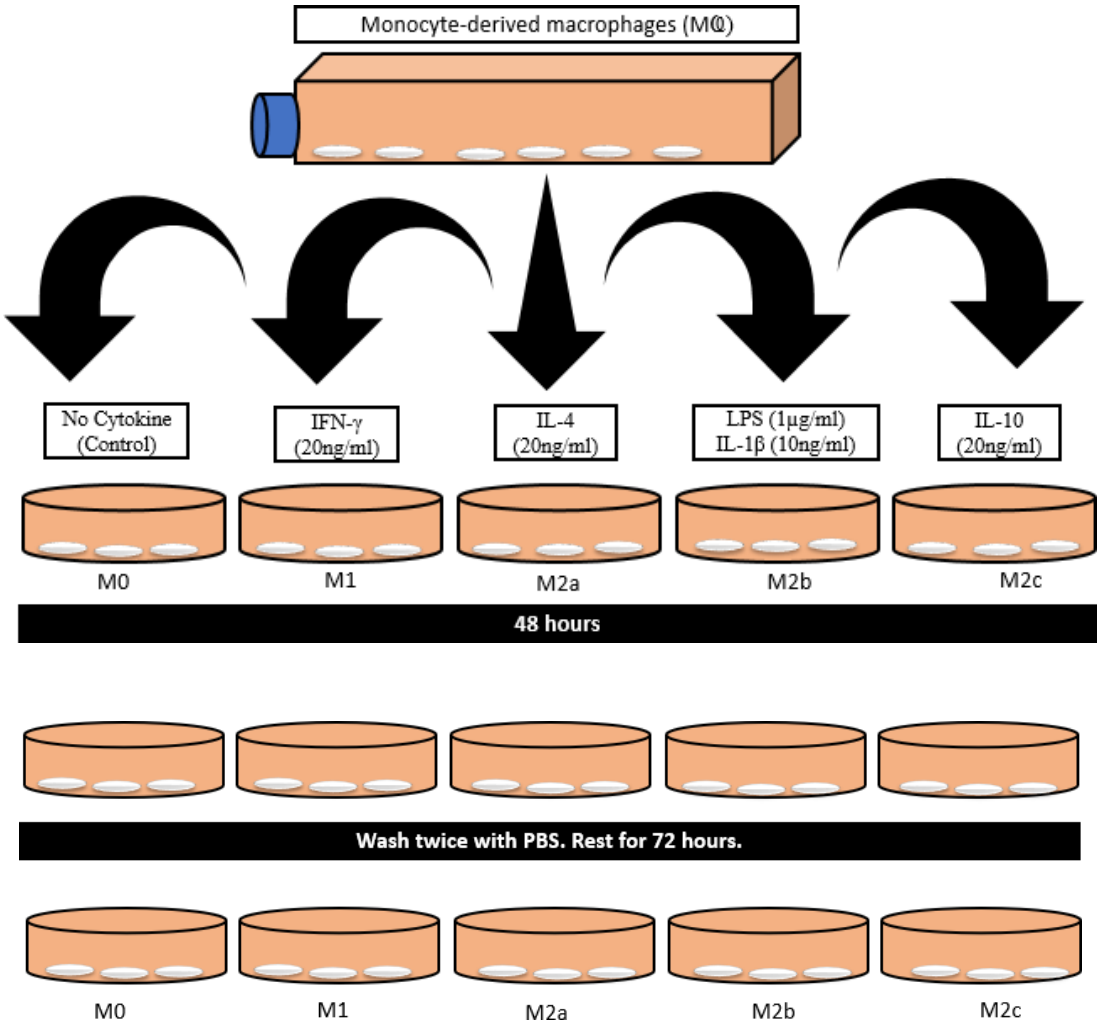

Monocyte-derived macrophages (MΦ)
No Cytokine (Control)
IFN-γ (20ng/ml)
IL-4 (20ng/ml)
LPS (1µg/ml) IL-1β (10ng/ml)
IL-10 (20ng/ml)
M0
M1
M2a
M2b
M2c
48 hours
Wash twice with PBS. Rest for 72 hours.
M0
M1
M2a
M2b
M2c

Figure 4

Figure 4: Generation of Macrophage Subsets from human PBMC. PBMC were isolated and plated in serum-free media in T-75 flasks at 3million PBMC/ml for 3 hours. Non-adherent cells were then washed off by washing twice with PBS and M-CSF (20ng/mL) was added to complete media (DMEM containing 10% FBS and Pennicillin/Streptamycin) for 6 days. Media was changed once on day 4. Monocyte-derived macrophages were then washed and fresh DMEM media containing 10% FBS, Pennicillin/Streptamycin, and cytokines were added to polarize them into the four subsets: M1, M2a, M2b and M2c. One well received only media as an unpolarized control (M0). These cells were cultured for 48 hours, after which they were washed twice with PBS and cultured with fresh media for another 72 hours before phenotype analysis.

2.4 Isolation of $CD8^+$ T cells from frozen PBMC

PBMC are resuspended in 90% FCS and 10% DMSO (Di-methyl sulfoxide) (Sigma-Aldrich, Ontario, Canada) for cryopreservation at $10X10^6$ cells/ml. They are transferred to cryogenic tubes (Thermo Fischer Scientific, New York) with 1ml of the mixture per tube and stored at -80°C until use. Three days before the $CD8^+$ T cells are required for experiments, the tubes are thawed on a bead bath at 32°C for 4 minutes, then mixed with 1ml warm complete media 1XRPMI-1640 (Roswell Park Memorial Institute medium) from Thermo Fischer Scientific, New York, containing 20% FCS, Pen/Strep. Thawed cells were then washed with PBS and centrifuged at 470xg two consecutive times before counting and culturing overnight in complete media (20% FCS and Pen/strep).

$CD8^+$ T cells were then isolated as previously described (150, 213). To isolate the $CD8^+$ T cells, PBMC were resuspend at $100X10^6$ cells/ml in MACS buffer (0.5% BSA, 2mM EDTA, PBS). A CD8 immunomagnetic positive selection kit II (Stemcell Technologies, British Columbia, Canada) was first titrated out to select for $CD8^{high}$ cells only. Selection cocktail from the kit was added to at 5µl/100µl of PBMC and incubated for 3 minutes at room temperature. Following this, the magnetic nanobeads were added at 5µl/100µl for 3 minutes at room temperature. The mixture was then made up to 2.5ml volume using MACS buffer, mixed well and placed in a magnet for 3 minutes. Cells bound to the tube were $CD8^+$ and remaining liquid was discarded. The tube was removed from the magnet and made up to 2.5ml using MACS buffer, and the processed was repeated a total of three times. Finally, $CD8^+$ T cells were collected and cultured overnight in complete media (20% FCS and Pen/strep) at 37°C, 5% CO_2. Purity of the isolated $CD8^+$ T cells was assessed using CD3 and CD8 surface staining on flow cytometry and on average a purity of 92% ± 2 was observed.

2.5 Cell surface phenotyping of MDM subsets

Polarized macrophages were washed with PBS stained with fluorescence-conjugated antibodies for CD163, CD206, and CD86 (BioLegend, California, USA) for analysis using flow cytometry. Staining was done for 15 minutes, in the dark, at 4°C in PBS containing 1% BSA and 10% FCS. Cells were washed with PBS, fixed using 4% PFA (para-formaldehyde) and fluorescence was measured using FC500 Navios^{TM} flow cytometer (Beckman Coulter, Ontario, Canada). 5,000 events were collected in the lymphocyte gate during initial data acquisition. Markers were set based on unstained cell control and FL-1 controls (which contained all fluorescent markers except one, for each of the fluorochromes used).

2.6 Secreted cytokine quantification in cell supernatants

Supernatants from cell cultures were collected, centrifuged to remove cells and debris, and frozen in 1.5ml low-retention Eppendorf tubes (Sigma-Aldrich, Germany) at -20°C until use. On day of experimental analysis, supernatant samples were thawed on a bead bath at 32°C for 15 minutes; HCV inactivation was carried out using 1% Triton-x and 0.3% Tributyl phosphate added to the samples so that 10% inactivating solution and 90% supernatant sample were mixed together and incubated at 37°C on a bead bath for 3 hours before use.

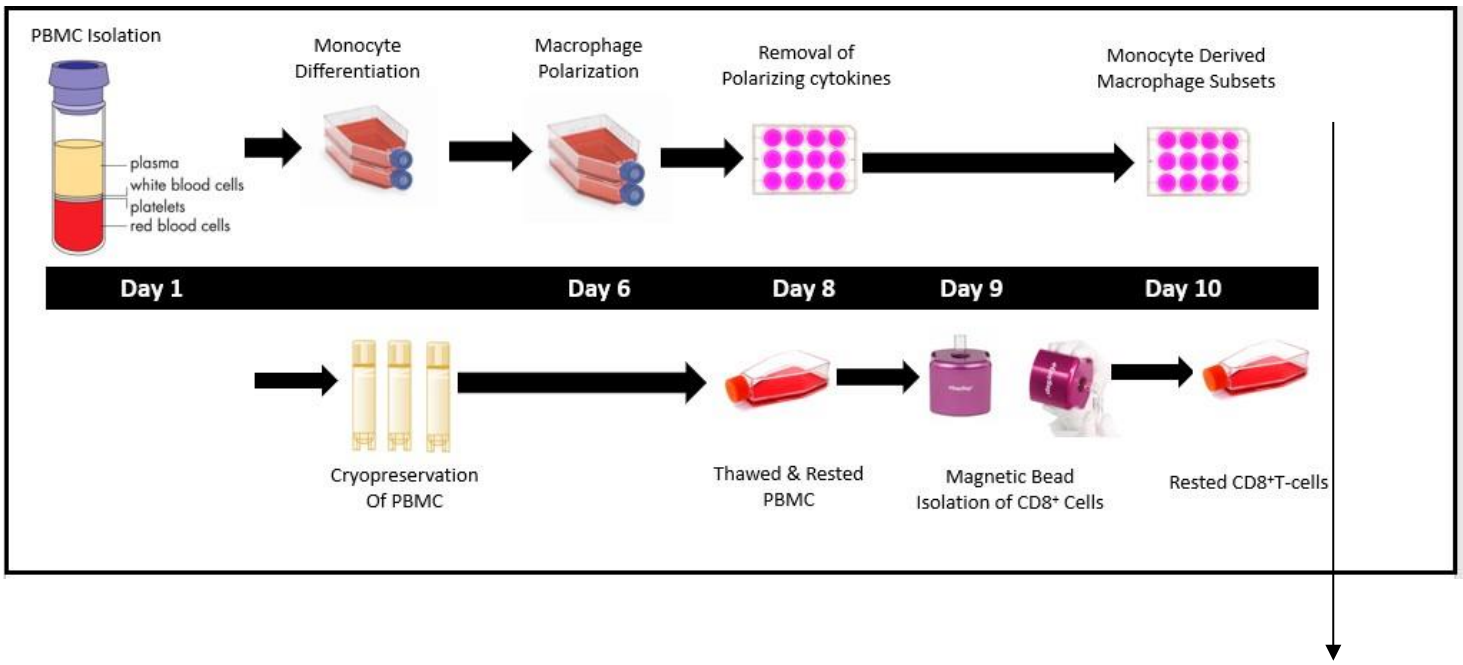
Cytokines in supernatant were quantified using a custom-designed panel of fluorescence bead-based quantification kit and analyzed using a Magpix system (EMD Millipore, Etobicoke, Ontario, Canada). Kits were used to quantify cytokines IL-6, TNF-α in a screening assay and IL-10, IL-12, IFN-γ in a high sensitivity assay (R&D Systems,

Minnesota, USA). Cytokines IFN-γ and perforin were selected from a custom T cell panel (EMD Millipore, Massachusettes, USA) for separate experiments.

2.7 Establishment of macrophage subset and $CD8^+$ T cell co-culture

Co cultured were established between autologous macrophages and $CD8^+$ T cells as described in Figure 5. 500µl of macrophages (at 100,000 cells/ml) were seeded into wells in a 24-well polystyrene plate (Thermo Fischer Scientific, New York, USA) with and without transmembrane inserts (Corning, New York, USA). Membranes of the transwell inserts were made of polyethylene terephthalate (PET) with 0.4µm pores to prevent movement of cells between the compartments, while allowing soluble factors to travel. They were then polarized into subsets as described previously, washed and cultured for 72 hours. To this, $CD8^+$ T cells isolated from the same individuals were added to establish an autologous co-culture at a ratio of 1:4 (MΦ:Tcell). The $CD8^+$ T cells were resuspended at $2X10^6$ cells/ml, and 100µl was added to each well either directly (in direct culture) or in a secondary well containing a transmembrane base (culture across transwell). They are mixed well and incubated at 37°C, 5% CO_2 for 24 or 48 hours, depending on which functional assay would be conducted on the $CD8^+$ T cells.

A


PBMC Isolation
plasma
white blood cells
platelets
red blood cells
Monocyte Differentiation
Macrophage Polarization
Removal of Polarizing cytokines
Monocyte Derived Macrophage Subsets
Day 1
Day 6
Day 8
Day 9
Day 10
Cryopreservation Of PBMC
Thawed & Rested PBMC
Magnetic Bead Isolation of CD8+ Cells
Rested CD8+T-cells

B

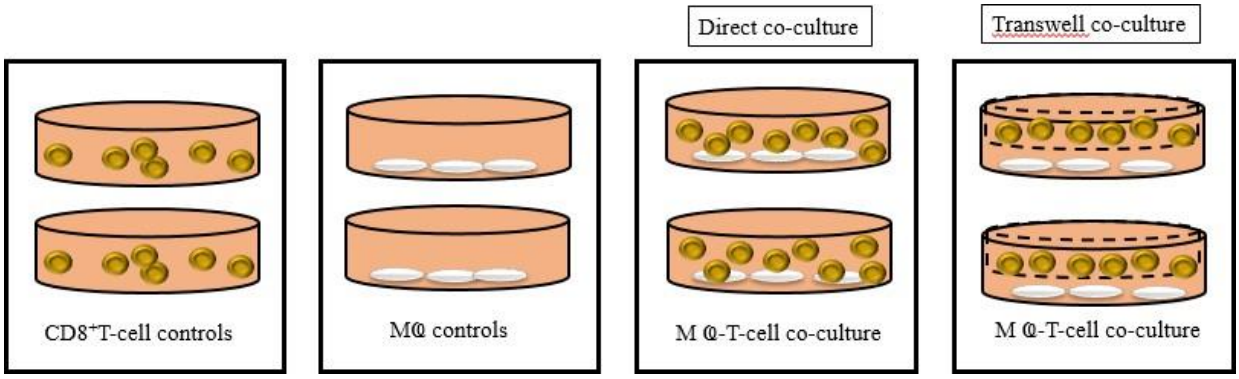

Direct co-culture
Transwell co-culture
CD8+T-cell controls
MΦ controls
M Φ-T-cell co-culture
M Φ-T-cell co-culture

Figure 5

Figure 5: Macrophage-T cell Co-Culture Methodology. Macrophage subsets and $CD8^+$ T cells were isolated from the same donor and a co-culture was established under different culture plates. (A) While monocytes isolated from PBMC and differentiated into macrophage subsets, part of the donor's PBMC is cryopreserved for later use. On day 8, the frozen PBMC is thawed, cultured overnight and then sorted to isolate the $CD8^+$ T cells. On day 10, the macrophage subsets and the $CD8^+$ T cells are co-cultured together. (B) The culture was established either in direct co-culture where both cell types have cell-to-cell contact, or alternatively they were cultured across a transwell membrane. As controls, macrophage subsets alone and T cells alone were also cultured in parallel.

2.8 Measurement of intracellular perforin in $CD8^+$ T cells

Intracellular perforin was analyzed at 48 hours following co culture as described previously (213). At 48 hours, non-adherent cells were collected, transferred to polypropylene tubes (BD Biosciences, Massachusetts, USA) and washed with PBS. The cells were surface stained for CD8 (conjugated with Allophycocyanin) for 15 minutes in the dark at 4°C. After surface staining was complete, cells were washed and fixed using 4% PFA in the dark for 15 minutes at room temperature. Cells were permeabilized using a permeabilization buffer (saponin) with about 200,000cells/100µl. 10µl of perforin antibody (conjugated with Fluorescein isothiocyanate) was added to each sample for intracellular staining for about 20 minutes at 4°C in the dark. Cells were then washed and resuspended in 300 µl 1% BSA PBS and analyzed using flow cytometry within an hour.

2.9 Measurement of surface CD107a in $CD8^+$ T cells

CD107a, the degranulation marker, was analyzed at 48 hours following co culture as previously described (213). At 42 hours following culture, non-adherent cells were transferred to polypropylene tubes (BD Biosciencs, Massachusetts, USA) and washed with PBS. They were then resuspended in 100µl of complete 1XDMEM media (10% FCS, Pen/Strep.) containing 10 µl of dilute golgi transport inhibitor containing monensin (2µM) (BD Biosciences, Ontario, Canada) and 5µl of CD107a antibody (conjugated with Fluorescein isothiocyanate) for 6 hours at 37°C, 5% CO_2 in the dark. Cells were then washed with PBS and stained with CD8 (conjugated with Allophycocyanin) for 15 minutes at 4°C in the dark.

Cells were washed again and resuspended in 300 µl 1% BSA PBS and analyzed using flow cytometry within an hour.

2.10 Measurement of intracellular IFN-γ in $CD8^+$ T cells

Intracellular IFN-γ was analyzed at 24 hours following co-culture as described previously (213). For the last 6 hours of those 24 hours, non-adherent cells from the co-culture were transferred to polypropylene tubes, washed, and resuspended in 100µl of media containing 10µl of dilute golgi transport inhibitor containing monensin (2µM) and incubated at 37°C, 5% CO_2. Cells were then washed with PBS and surface stained using CD8 antibody (conjugated with Allophycocyanin) for 15 minutes at 4°C in the dark. Cells were washed and fixed using 4% PFA in the dark for 15 minutes at room temperature. Following that, the cells were washed again permeabilized using 100µl of permeabilization buffer (saponin) containing 5 µl of IFN-γ antibody (conjugated with Fluorescein isothiocyanate) and incubated for 30 minutes in the dark 37°C, 5% CO_2. Cells were washed and resuspended in 300 µl 1% BSA PBS and analyzed using flow cytometry within an hour.

2.11 Analysis and Statistics

All flow cytometry data was analyzed using FCS Express Research Edition 4.0 (De Novo Software, Los Angeles, California, USA). Standard curves and protein quantification was calculated through an automatic report generation using the xPONENT® on the Magpix™ system. Graphs were designed and statistical tests were carried out on GraphPad Prism 5.0 software (San Diego, California, USA). For all statistical tests, a paired or unpaired student's

t-test was used (with a 95% confidence interval) unless otherwise specified. Where necessary, Multivariate Data Analysis and a one-way ANOVA Dunnett post-test was carried out. Data is presented as mean ± standard deviation.

Chapter 3: Results

Objective#1 Phenotype of monocyte-derived macrophage subsets derived from normal & HCV infected individuals

Human blood monocytes are able to terminally differentiate into macrophages or dendritic cells depending on the type of growth factor they are exposed to (216). Upon exposure to monocyte-colony stimulating factor (M-CSF), these precursor cells form a heterogenous population of macrophages, that are able to respond to various immune stimuli by reprogramming itself into different subsets (216). It has been reported that monocytes from patients with chronic HCV infection display various dysfunctions, such as reduced IL-6, TNF-α, IL-1β production in response to TLR stimulation, among others (217, 218). Hence, it was of interest to investigate whether such dysfunctional monocytes from HCV infected patients would have corrected or whether differentiated macrophages and their subsets maintained the dysfunctional phenotype and functional status.

I isolated blood monocytes from each group of patients and healthy donors and differentiated them *in vitro,* followed by cytokine-induced polarization into M1, M2a, M2b and M2c subsets. M0 macrophages were the control which was differentiated but not polarized into any subset This allowed us to then analyze the surface receptors and secreted cytokines from each macrophage subset following polarization.

Objective#1A. Surface phenotype of macrophage subsets from normal and chronic HCV infected individuals

Surface receptors CD86, CD206 and CD163 were chosen to help distinguish between the subsets in healthy individuals, due to their differential expression on macrophage subsets

relative to each other. These were also chosen to observe differences in activation of the subsets between healthy controls and HCV infected patients. Once I generated polarized macrophages subsets, I cultured them for 48 hours; following this, I observed their viability for 30 hours in culture using a live cell imagine system and staining with propidium iodide (PI). We then stained for the three surface receptors and analyzed using flow cytometry. The gating is shown in Figure 6A.

When assessing the proportion of cells expressing the CD86 marker, I observed a significant increase in their percentage in M0 ($p=0.04$) and M1 ($p=0.01$) macrophage subsets in advanced fibrosis patients compared to healthy controls (Figure 6B-C). All other subsets showed no significant difference among each other and HCV infected stages (Figure 6D-F). I also observed a significant increase in the expression of CD86 per cell (MFI) in M1 macrophages ($p=0.01$) derived from individuals with advanced fibrosis compared to healthy controls (Figure 7B). All the remaining subsets showed no significant differences in the expression of this co-stimulatory molecule (Figure 7A,C-E). The histogram shows three representative donors, one from each group indicating healthy, early fibrosis and late fibrosis, and shows their respective CD86 expression (Figure 7F).

CD163 receptor is found in elevated levels on M2c macrophages and have often been linked to chronic inflammatory diseases including HCV where the protein is cleaved and found in the serum in its soluble form (sCD163) (219). This biomarker of chronic HCV is found in increased amounts in the soluble form with increasing levels of liver fibrosis in patients (219). However, my studies revealed that the surface expression on macrophages from patients with chronic HCV patients did not differ from healthy controls, neither in proportion of cells expressing the marker nor in their expression per cell of this receptor (Figure 8, Figure 9).

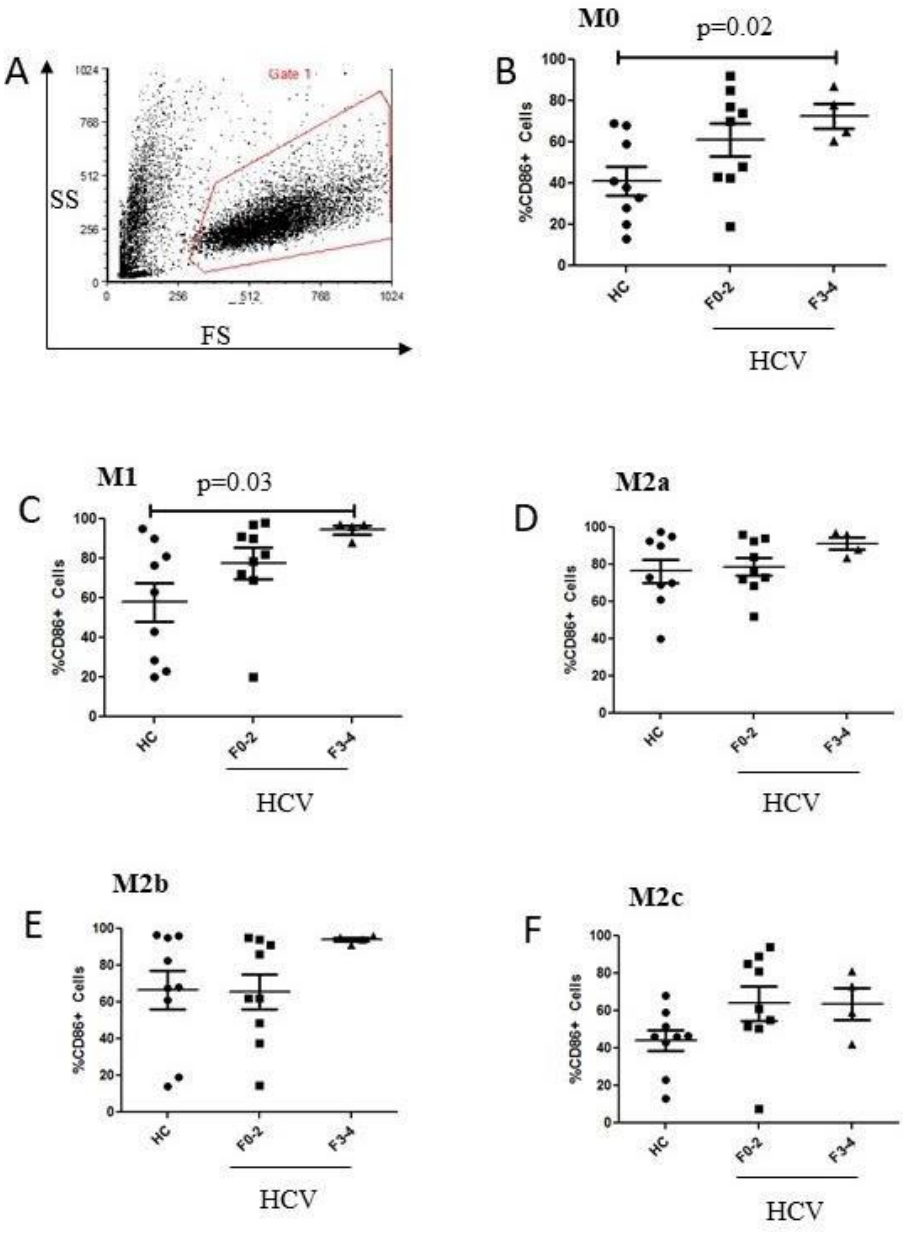

A
Gate 1
SS
FS
1024
768
512
256
0
256
512
768
1024
B
M0
p=0.02
%CD86+ Cells
HC
F0-2
F3-4
HCV
C
M1
p=0.03
D
M2a
E
M2b
F
M2c

Figure 6

Figure 6: M0 and M1 macrophage subset from HCV-infected advanced fibrosis patients have an increased percentage of CD86$^+$ cells. Surface staining of macrophage subsets from healthy controls (HC, n=9), early fibrosis (F0-2, n=9) and advanced fibrosis (F3-4, n=4) were performed and analyzed using flow cytometry. **(A)** Representative macrophage gating strategy. The percentage of CD86$^+$ cells is shown in figures **(B)** M0 **(C)** M1 **(D)** M2a **(E)** M2b **(F)** M2c Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

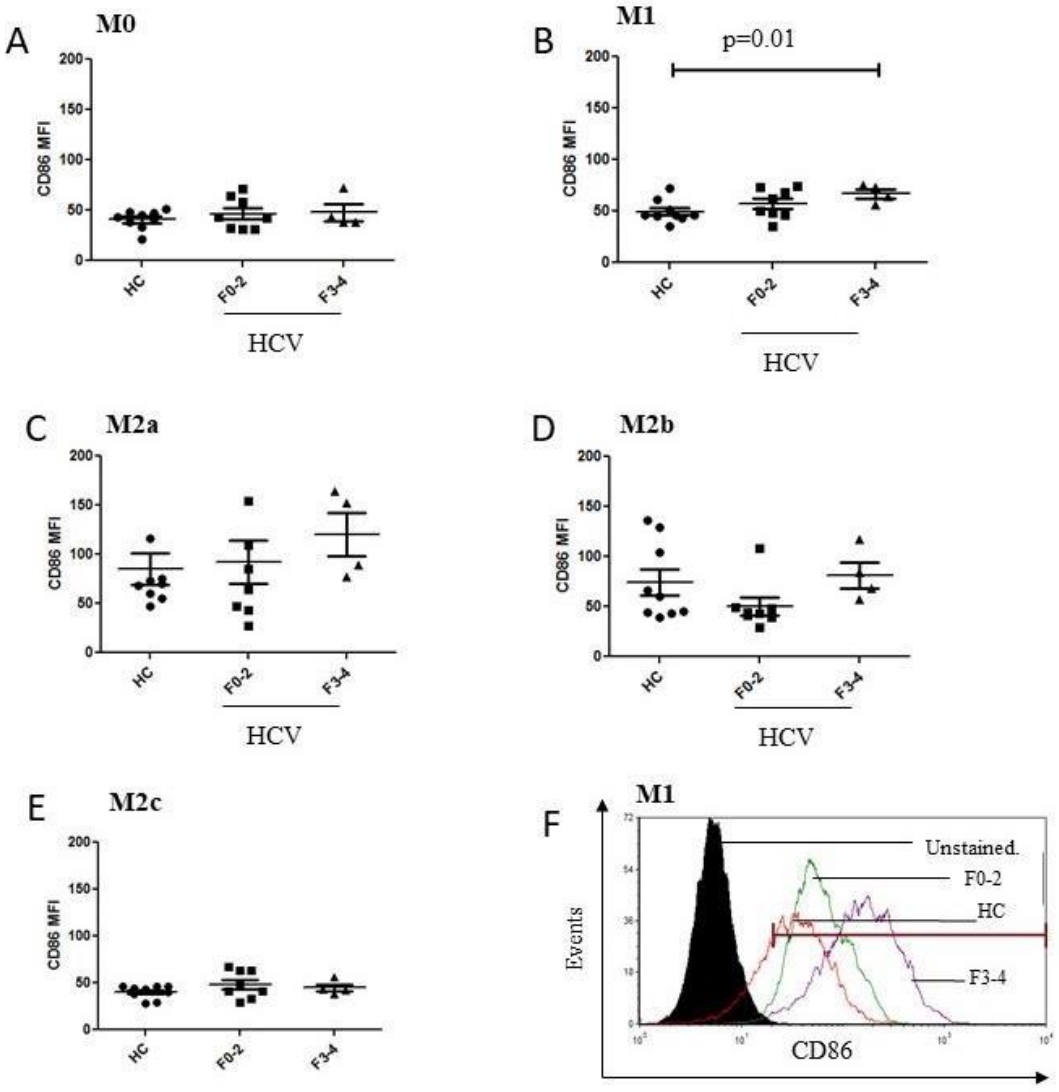

A
M0
CD86 MFI
200
150
100
50
0
HC
F0-2
F3-4
HCV
B
M1
p=0.01
CD86 MFI
HC
F0-2
F3-4
HCV
C
M2a
CD86 MFI
HC
F0-2
F3-4
HCV
D
M2b
CD86 MFI
HC
F0-2
F3-4
HCV
E
M2c
CD86 MFI
HC
F0-2
F3-4
F
M1
Events
Unstained.
F0-2
HC
F3-4
CD86

Figure 7

Figure 7: M1 macrophage subsets from HCV-infected advanced fibrosis patients express increased levels of CD86^{+} cells. Surface staining of macrophage subsets from healthy controls (HC), early fibrosis (F0-2) and advanced fibrosis (F3-4) were performed and analyzed using flow cytometry. The expression of CD86 per cell is shown in figures **(A)** M0 (HC n=9, F0-2 n=8, F3-4 n=4) **(B)** M1 (HC n=9, F0-2 n=8, F3-4 n=4) **(C)** M2a (HC n=8, F0-2 n=8, F3-4 n=4) **(D)** M2b (HC n=9, F0-2 n=8, F3-4 n=4) **(E)** M2c (HC n=9, F0-2 n=8, F3-4 n=4). Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant. **(F)** A representative histogram from an uninfected donor, an early fibrosis and a late fibrosis donor with chronic HCV is shown.

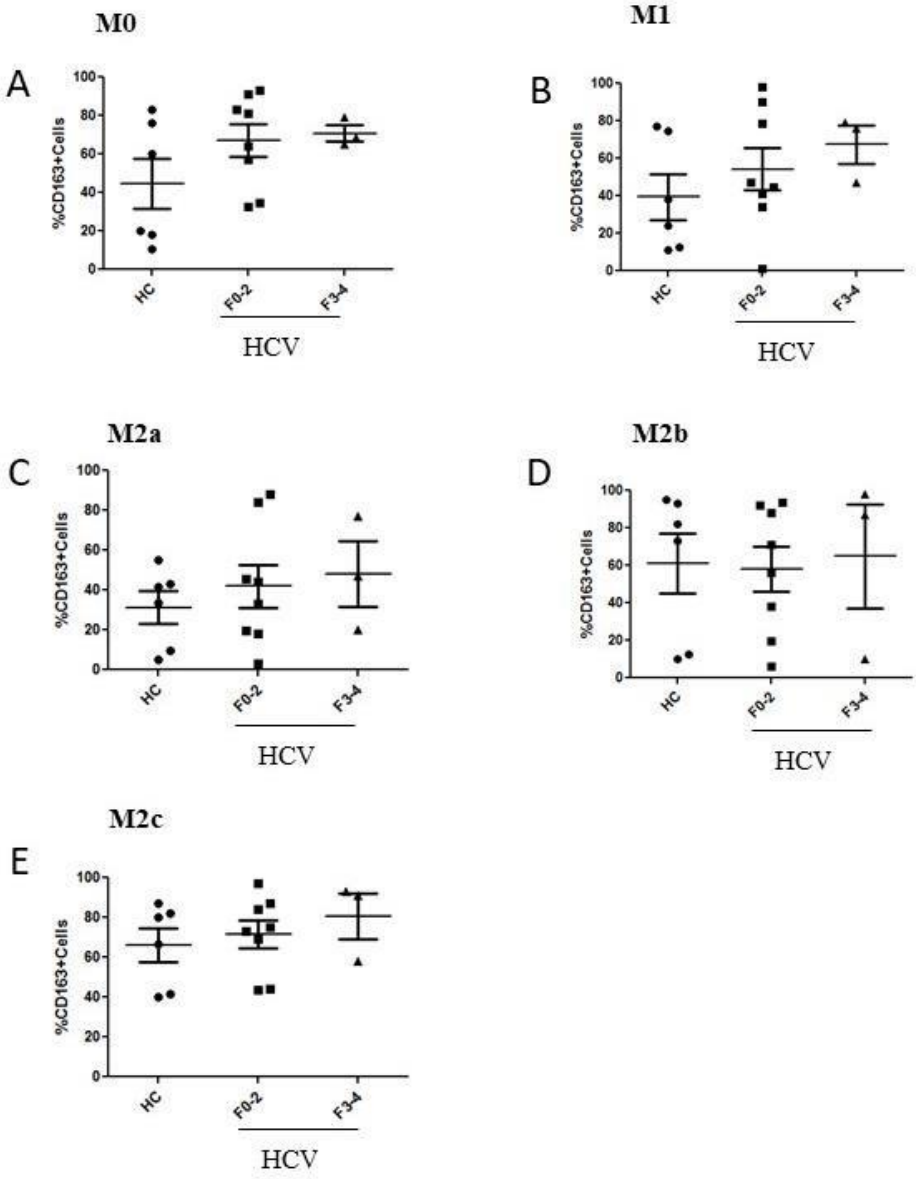

M0
A
%CD163+Cells
HC
F0-2
F3-4
HCV
M1
B
%CD163+Cells
HC
F0-2
F3-4
HCV
M2a
C
%CD163+Cells
HC
F0-2
F3-4
HCV
M2b
D
%CD163+Cells
HC
F0-2
F3-4
HCV
M2c
E
%CD163+Cells
HC
F0-2
F3-4
HCV

Figure 8

Figure 8: Macrophage subsets derived from early and advanced fibrosis HCV-infected patients and healthy controls have similar percentage of CD163$^+$ cells. Surface staining of macrophage subsets from healthy controls (HC, n=6), early fibrosis (F0-2, n=8) and advanced fibrosis (F3-4, n=3) were performed and analyzed using flow cytometry. The percentage of CD163$^+$ cells are shown in figures **(A)** M0 **(B)** M1 **(C)** M2a **(D)** M2b **(E)** M2c. Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

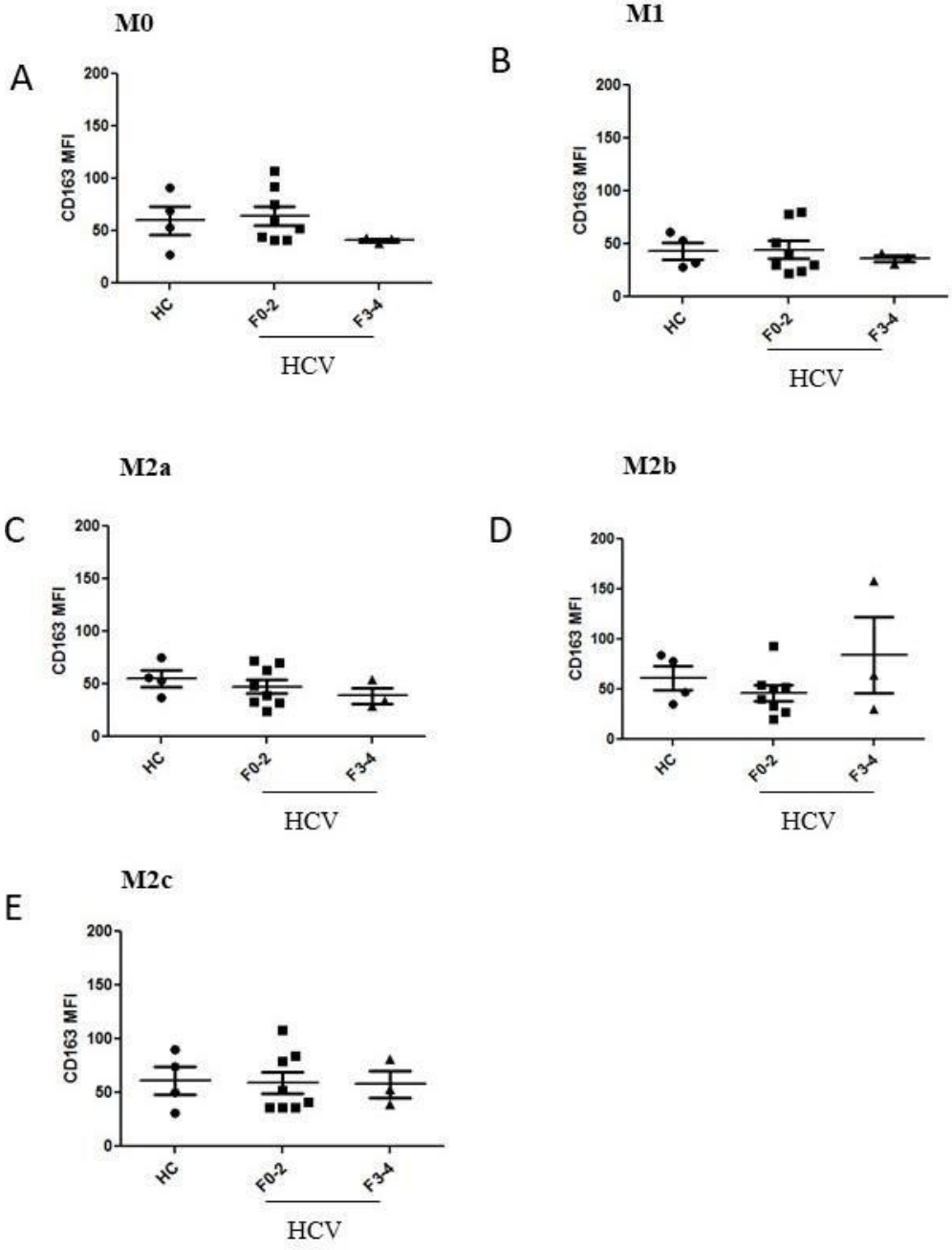

M0
A
CD163 MFI
200
150
100
50
0
HC
F0-2
F3-4
HCV
M1
B
CD163 MFI
200
150
100
50
0
HC
F0-2
F3-4
HCV
M2a
C
CD163 MFI
200
150
100
50
0
HC
F0-2
F3-4
HCV
M2b
D
CD163 MFI
200
150
100
50
0
HC
F0-2
F3-4
HCV
M2c
E
CD163 MFI
200
150
100
50
0
HC
F0-2
F3-4
HCV

Figure 9

Figure 9: Macrophage subsets derived from HCV-infected patients with early fibrosis, advanced fibrosis and healthy controls express similar levels of CD163. Surface staining of macrophage subsets from healthy controls (HC, n=4), early fibrosis (F0-2, n=8) and advanced fibrosis (F3-4, n=3) were performed and analyzed using flow cytometry. The percentage of $CD163^+$ cells are shown in figures **(A)** M0 **(B)** M1 **(C)** M2a **(D)** M2b **(E)** M2c. Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

CD206, the mannose receptor, is expressed at higher levels on M2c macrophages in healthy donors and plays a role in promoting fibrosis through its secretion of CCL18 and TGF-β (169, 175). Due to its association with fibrosis, it was of interest to observe any differences in their expression with advancement of liver fibrosis in chronic HCV infection. Surprisingly, the proportion of cells expressing CD206 was significantly reduced in M2c macrophages from HCV-infected patients with advanced liver fibrosis (Figure 10E), other subsets showed no significant differences (Figure 10 A-D). While the percentage of cells expressing CD206 was reduced in HCV infection, there was no actual change in the expression of the receptor per macrophage (Figure 11).

Objective#1B. Secreted cytokine profile of macrophage subsets derived from normal and chronic HCV infected individuals

To assess differences in secreted cytokine profiles of macrophage subsets between normal, healthy controls and HCV-infected patients, a combination of pro- and anti-inflammatory cytokines were selected that include IL-6, TNF-α, IL-10, IL-12 and IFN-γ. These cytokines were quantified from the supernatants of the macrophage subsets that were cultured for 72 hours.

TNF-α As described in literature, M1 macrophages from healthy donors, spontaneously produced high levels of TNF-α (Figure 12B) (205, 220-223). All the other subsets produced very low amounts of this cytokine, usually below 50pg/mL (Figure 12A, C-E).

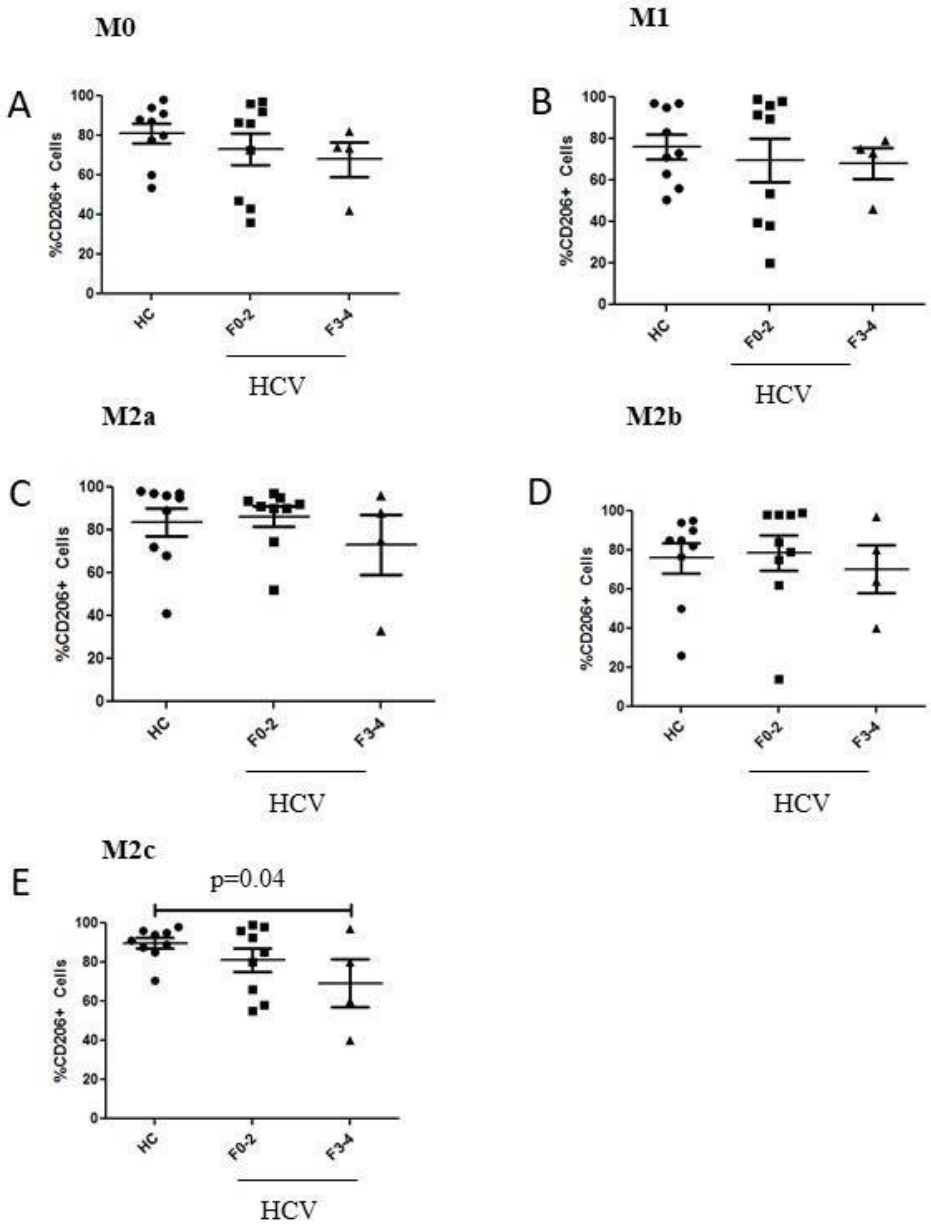

M0
A
%CD206+ Cells
HC
F0-2
F3-4
HCV
M1
B
%CD206+ Cells
HC
F0-2
F3-4
HCV
M2a
C
%CD206+ Cells
HC
F0-2
F3-4
HCV
M2b
D
%CD206+ Cells
HC
F0-2
F3-4
HCV
M2c
E
p=0.04
%CD206+ Cells
HC
F0-2
F3-4
HCV

Figure 10

Figure 10: M2c macrophage subsets in HCV-infected patients with advanced fibrosis have a lower percentage of CD206$^+$ cells. Surface staining of macrophage subsets from healthy controls (HC, n=9), early fibrosis (F0-2, n=9) and advanced fibrosis (F3-4, n=4) were performed and analyzed using flow cytometry. The percentage of CD206$^+$ cells are shown in figures **(A)** M0 **(B)** M1 **(C)** M2a **(D)** M2b **(E)** M2c. Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

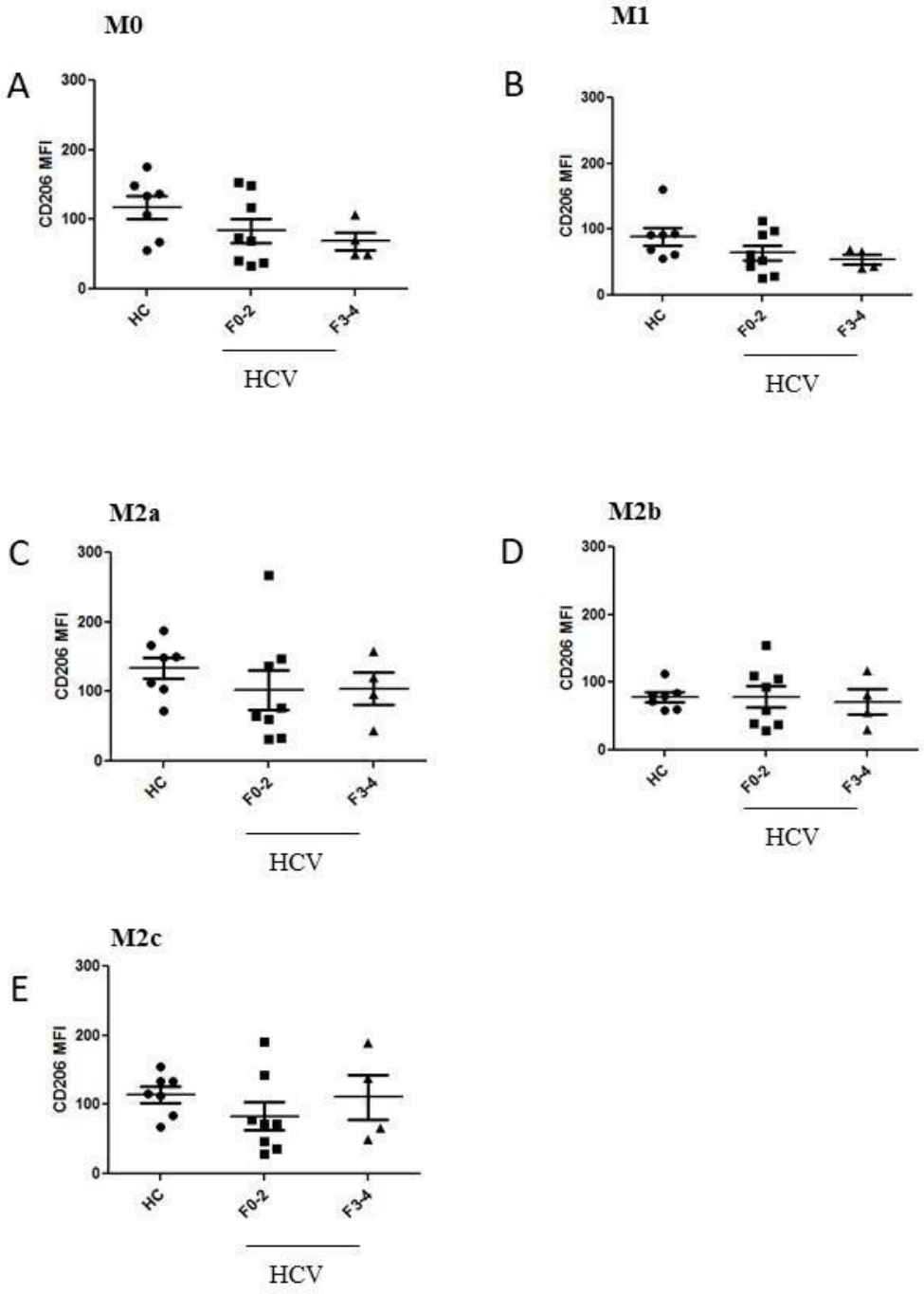

M0
A
CD206 MFI
300
200
100
0
HC
F0-2
F3-4
HCV
M1
B
CD206 MFI
300
200
100
0
HC
F0-2
F3-4
HCV
M2a
C
CD206 MFI
300
200
100
0
HC
F0-2
F3-4
HCV
M2b
D
CD206 MFI
300
200
100
0
HC
F0-2
F3-4
HCV
M2c
E
CD206 MFI
300
200
100
0
HC
F0-2
F3-4
HCV

Figure 11

Figure 11: Macrophage subsets derived from HCV-infected patients with early fibrosis, advanced fibrosis and healthy controls have similar expression of CD206. Surface staining of macrophage subsets from healthy controls (HC, n=7), early fibrosis (F0-2, n=8) and advanced fibrosis (F3-4, n=4) were performed and analyzed using flow cytometry. The percentage of CD206 cells are shown in figures **(A)** M0 **(B)** M1 **(C)** M2a **(D)** M2b **(E)** M2c. Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

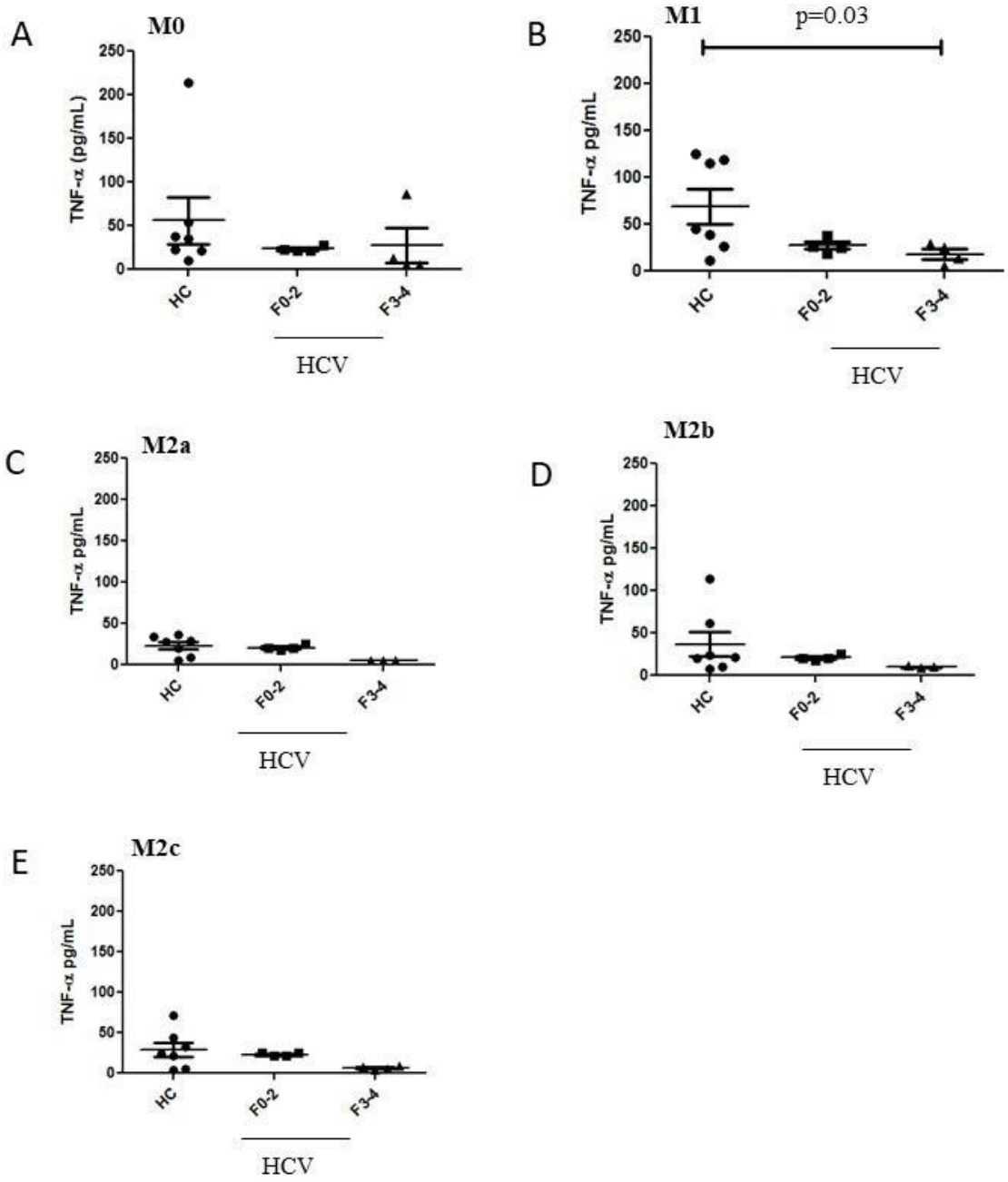

A
M0
TNF-α (pg/mL)
250
200
150
100
50
0
HC
F0-2
F3-4
HCV
B
M1
p=0.03
TNF-α pg/mL
250
200
150
100
50
0
HC
F0-2
F3-4
HCV
C
M2a
TNF-α pg/mL
250
200
150
100
50
0
HC
F0-2
F3-4
HCV
D
M2b
TNF-α pg/mL
250
200
150
100
50
0
HC
F0-2
F3-4
HCV
E
M2c
TNF-α pg/mL
250
200
150
100
50
0
HC
F0-2
F3-4
HCV

Figure 12

Figure 12: M1 macrophages in HCV-infected advanced fibrosis patients produce lower levels of spontaneous TNF-α. Macrophage subsets from healthy controls (HC) and chronic HCV-infected individuals with early fibrosis (F0-2) and advanced fibrosis (F3-4) were cultured for 72 hours. Following that, their supernatants were collected and quantified for TNF-α using a Luminex-based multiplex assay for each subset as shown by **(A)** M0 (HC n=7, F0-2 n=4, F3-4 n=4) **(B)** M1 (HC n=7, F0-2 n=4, F3-4 n=4) **(C)** M2a (HC n=7, F0-2 n=4, F3-4 n=3) **(D)** M2b (HC n=7, F0-2 n=4, F3-4 n=3) **(E)** M2c (HC n=7, F0-2 n=4, F3-4 n=4) . Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

In patients with advanced fibrosis (F3-4), there was a significant reduction in the level of spontaneous TNF-α production by M1 macrophages compared to HC (Figure 12B). The M1 macrophages derived from F0-2 group showed no significant difference in TNF-α production when compared to F3-4 and HC groups. All other subsets derived from early and late fibrosis stages of HCV infection showed low levels of TNF-α production comparable to their respective HC groups.

IL-6: Spontaneous production of IL-6 has been observed as a subset-defining feature of M2b macrophages among normal, healthy donors (31, 224, 225). Similarly, my results showed that M2b was the main subset producing significant amounts of this cytokine spontaneously (up to 2350 pg/mL) (Figure 13D). The other subsets of macrophages produced very low amounts of the cytokine in healthy controls, up 60pg/mL (Figure 13A-C, E).

I did not observe any statistical differences in the spontaneous production of IL-6 between HC, F0-2 and F3-4 groups (Figure13A-E). Due to sample limitations, some of the samples which were too concentrated and reached the upper limit of detection, could not be repeated after sample dilution (Figure 13D).

IFN-γ: In the case of IFN-γ, the M1 macrophages produced significant amounts of the cytokine spontaneously in healthy individuals, which is consistent with previous studies (31, 226) (Figure 14B). This ranged from 0.5pg/mL to 2800pg/mL. The other subsets derived from normal, healthy donors produced very low amounts of the cytokine mostly between 0.5pg/mL to 50pg/mL (Figure 14A, C-E).

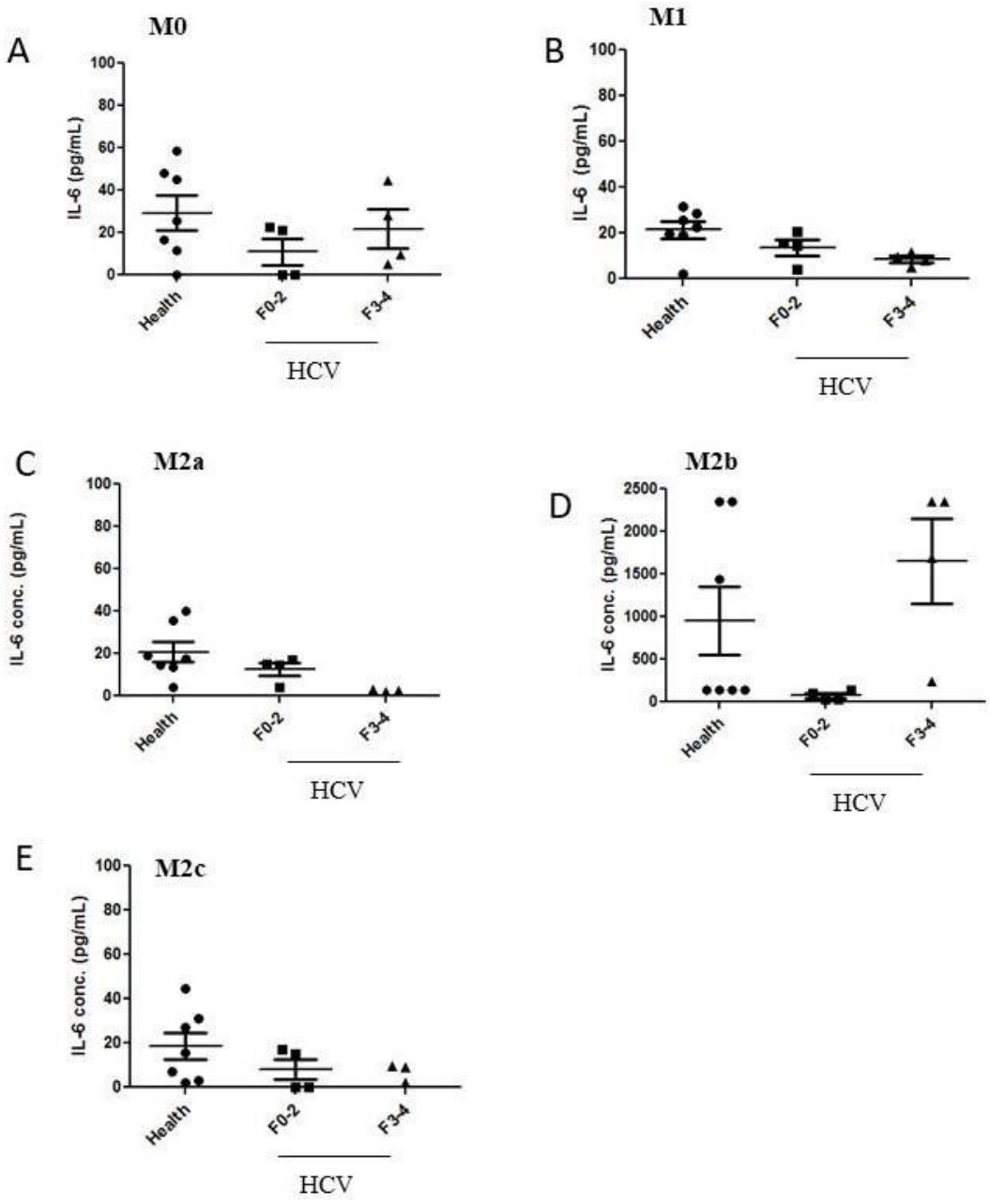

A
M0
IL-6 (pg/mL)
100
80
60
40
20
0
Health
F0-2
F3-4
HCV
B
M1
IL-6 (pg/mL)
100
80
60
40
20
0
Health
F0-2
F3-4
HCV
C
M2a
IL-6 conc. (pg/mL)
100
80
60
40
20
0
Health
F0-2
F3-4
HCV
D
M2b
IL-6 conc. (pg/mL)
2500
2000
1500
1000
500
0
Health
F0-2
F3-4
HCV
E
M2c
IL-6 conc. (pg/mL)
100
80
60
40
20
0
Health
F0-2
F3-4
HCV

Figure 13

Figure 13: Macrophage subsets derived from HCV-infected patients in early fibrosis, late fibrosis and healthy controls produce similar levels of IL-6. . Macrophage subsets from healthy controls and chronic HCV-infected individuals with early fibrosis (F0-2) and advanced fibrosis (F3-4) were cultured for 72 hours. Following that, their supernatants were collected and quantified for IL-6 using a Luminex-based multiplex assay for each subset as shown by **(A)** M0 (HC n=7, F0-2 n=4, F3-4 n=4) **(B)** M1 (HC n=7, F0-2 n=4, F3-4 n=4) **(C)** M2a (HC n=7, F0-2 n=4, F3-4 n=3) **(D)** M2b (HC n=7, F0-2 n=4, F3-4 n=4) **(E)** M2c (HC n=7, F0-2 n=4, F3-4 n=3). Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated on the graph. A value of $p \leq 0.05$ was considered to be statistically significant.

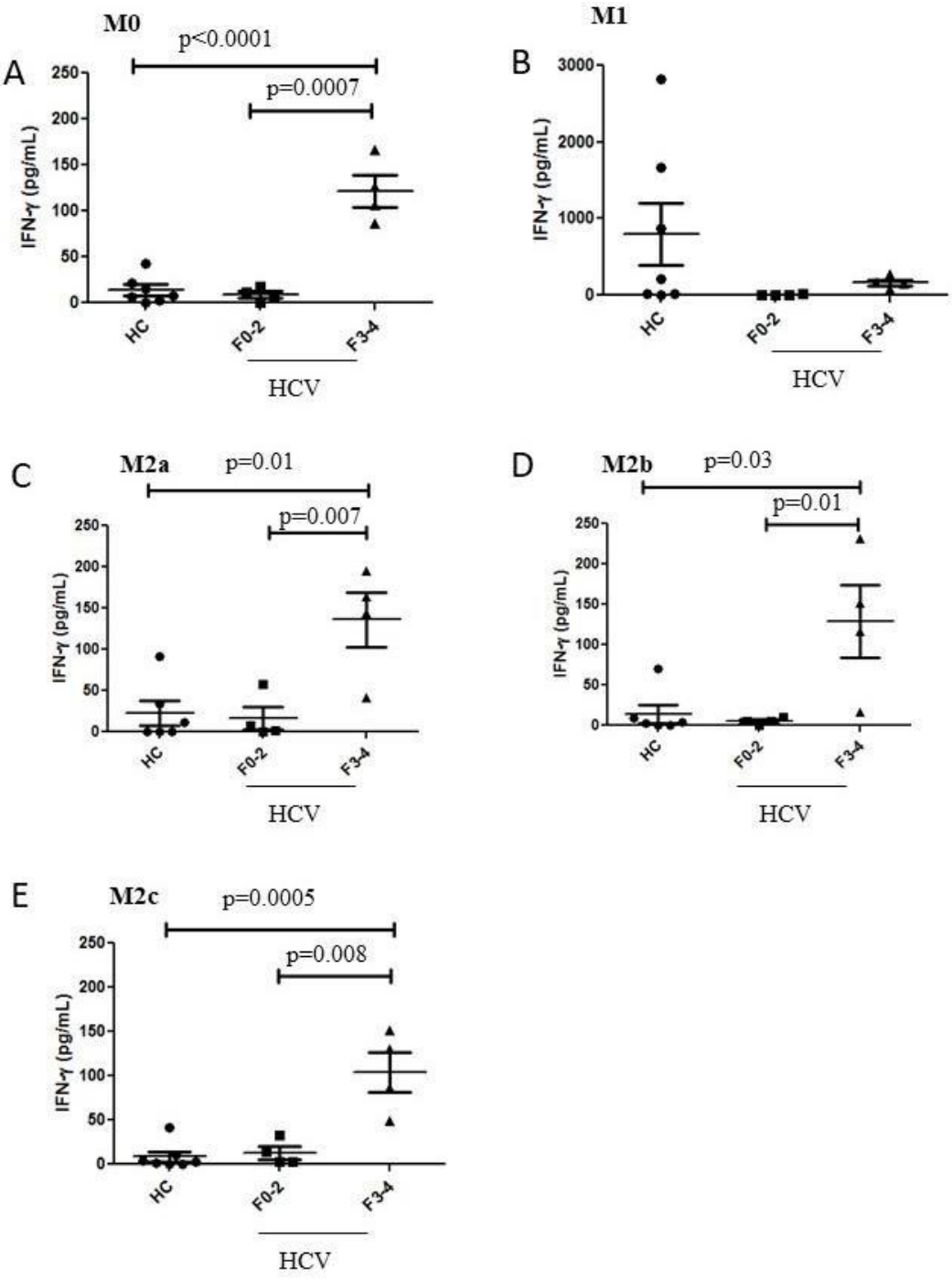

A
M0
p<0.0001
p=0.0007
IFN-γ (pg/mL)
250
200
150
100
50
0
HC
F0-2
F3-4
HCV
B
M1
3000
2000
1000
0
HC
F0-2
F3-4
HCV
C
M2a
p=0.01
p=0.007
IFN-γ (pg/mL)
HC
F0-2
F3-4
HCV
D
M2b
p=0.03
p=0.01
IFN-γ (pg/mL)
HC
F0-2
F3-4
HCV
E
M2c
p=0.0005
p=0.008
IFN-γ (pg/mL)
HC
F0-2
F3-4
HCV

Figure 14

Figure 14: M,0, M2a, M2b and M2c macrophage subsets from HCV-infected patients at advanced fibrosis produce high levels of spontaneous IFN-γ. Macrophage subsets from healthy controls (HC) and chronic HCV-infected individuals with early fibrosis (F0-2) and advanced fibrosis (F3-4) were cultured for 72 hours. Following that, their supernatants were collected and quantified for IFN-γ using a Luminex-based multiplex assay for each subset as shown in **(A)** M0 (HC n=7, F0-2 n=4, F3-4 n=4) **(B)** M1 (HC n=7, F0-2 n=4, F3-4 n=4) **(C)** M2a (HC n=6, F0-2 n=4, F3-4 n=4) **(D)** M2b (HC n=6, F0-2 n=4, F3-4 n=4) **(E)** M2c (HC n=7, F0-2 n=4, F3-4 n=4) . Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

However, I made an interesting observation at F3-4 stage, where all subsets other than M1 showed increased spontaneous production of IFN-γ (up to 230 pg/mL) compared to HC as well as F0-2 (Figure 14A,C-E). However, this increase was not to the same levels as M1 which produced up to 2800pg/mL (Figure 14B). The production of this pro-inflammatory cytokine by untreated M2 macrophages sheds light on the altered functionality of macrophages in chronic HCV infection at advanced stages of fibrosis. The macrophage subsets derived from F0-2 groups of HCV-infected patients, however, did not produce IFN-γ (Figure 14A-E).

IL-10: I then quantified the anti-inflammatory cytokine IL-10 with our high-sensitivity assay. Among normal, healthy donors, the spontaneous production of the cytokine was extremely limited. M1, M2a, and M2b produced an average of 10pg/mL or less while M0 and M2c barely produced detectable levels of the cytokine (Figure 15A-E).

Interestingly, patients with chronic HCV infection at F3-4 liver fibrosis displayed a significant increase in the spontaneous production of IL-10 among all macrophage subsets compared to F0-2 group (Figure 15A-E). The IL-10 production increased to about 20pg/mL for all the subsets at advanced fibrosis. With the exception of M1 and M2b subsets, this increase in spontaneous IL-10 production was also significant in comparison to healthy controls (Figure 15B, 15D). IL-10 produced by macrophage subsets derived from the F0-2 showed no significant difference when compared to the HC group.

IL-12p70: When quantifying the pro-inflammatory cytokine IL-12, I observed that macrophage subsets from healthy controls produce very low levels of the cytokine mostly below detectable levels (Figure 16A-E).

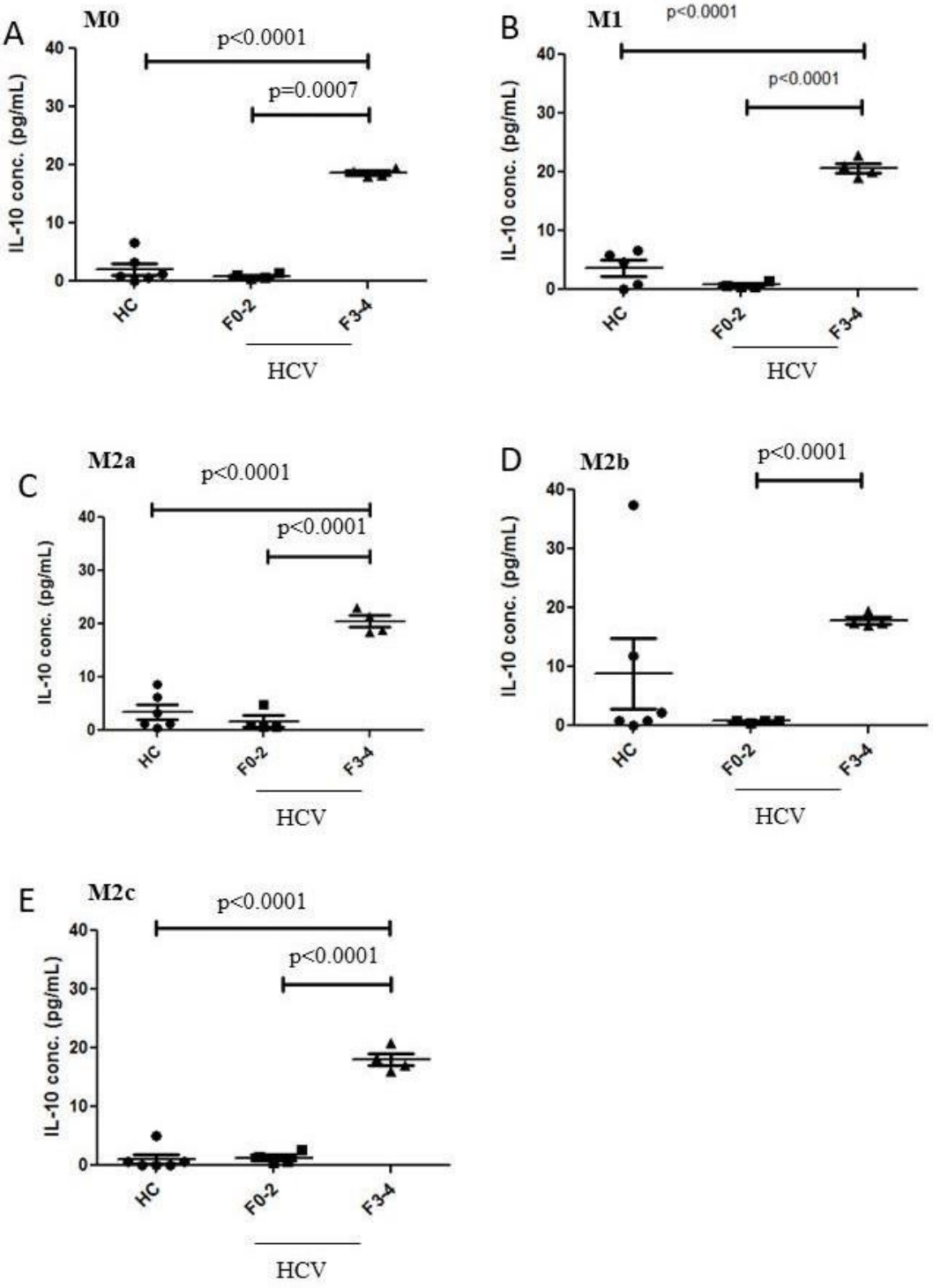

A
M0
p<0.0001
p=0.0007
IL-10 conc. (pg/mL)
HC
F0-2
F3-4
HCV
B
M1
p<0.0001
p<0.0001
C
M2a
p<0.0001
p<0.0001
D
M2b
p<0.0001
E
M2c
p<0.0001
p<0.0001

Figure 15

Figure 15: M0, M1, M2a and M2c macrophages from HCV-infected patients at advanced fibrosis spontaneously produce high levels of IL-10. Macrophage subsets from healthy controls and chronic HCV-infected individuals with early fibrosis (F0-2) and advanced fibrosis (F3-4) were cultured for 72 hours. Following that, their supernatants were collected and quantified for IL-10 using a Luminex-based multiplex assay. The cytokine was quantified in supernatants from each subset as shown in **(A)** M0 (HC n=6, F0-2 n=4, F3-4 n=4) **(B)** M1 (HC, n=5 F0-2 n=4, F3-4 n=4) **(C)** M2a (HC, n=6 F0-2 n=4, F3-4 n=4) **(D)** M2b (HC, n=6 F0-2 n=4, F3-4 n=4) **(E)** M2c (HC, n=6 F0-2 n=4, F3-4 n=4). Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

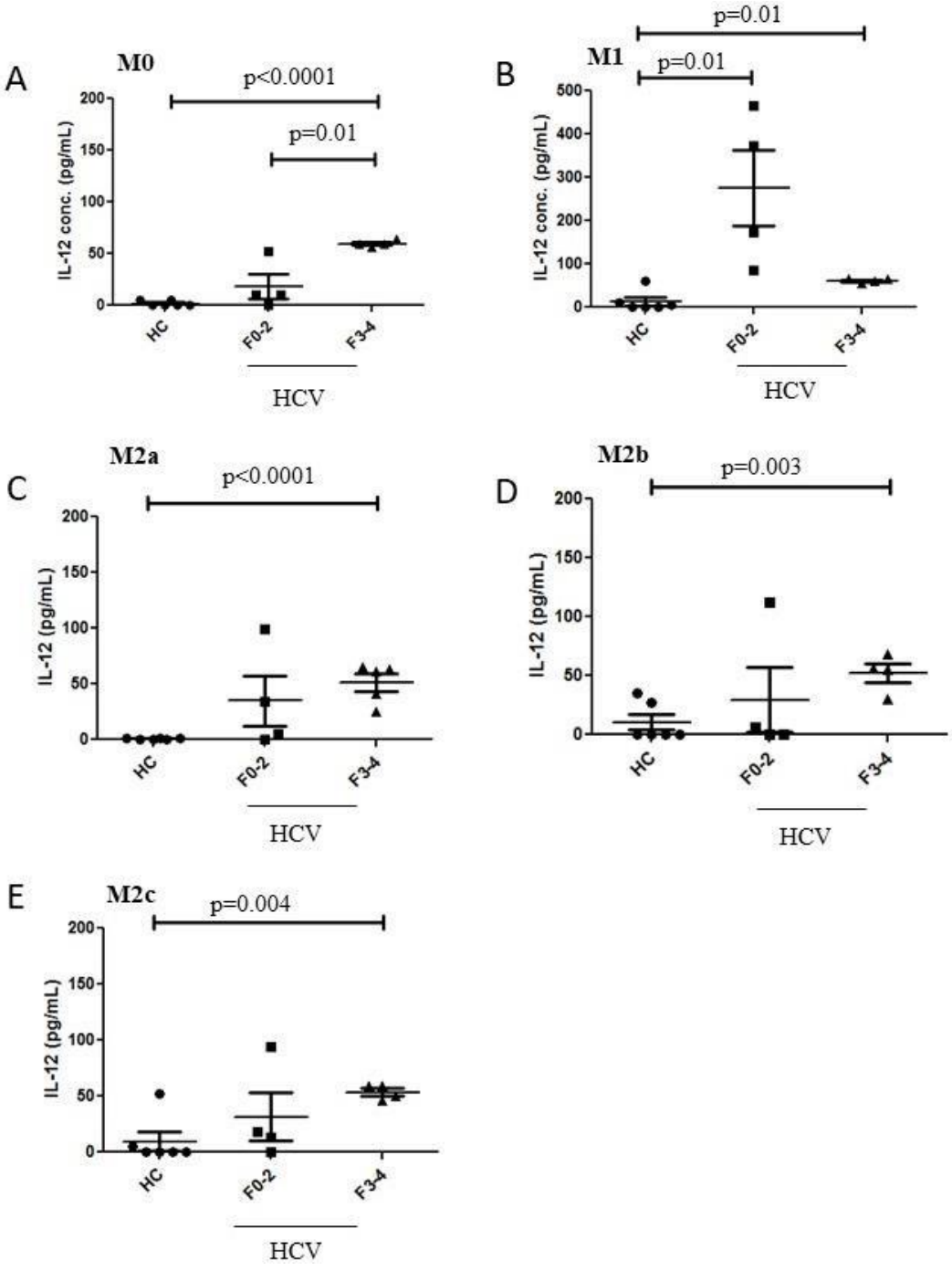

A
M0
p<0.0001
p=0.01
IL-12 conc. (pg/mL)
HC
F0-2
F3-4
HCV
B
M1
p=0.01
p=0.01
IL-12 conc. (pg/mL)
HC
F0-2
F3-4
HCV
C
M2a
p<0.0001
IL-12 (pg/mL)
HC
F0-2
F3-4
HCV
D
M2b
p=0.003
IL-12 (pg/mL)
HC
F0-2
F3-4
HCV
E
M2c
p=0.004
IL-12 (pg/mL)
HC
F0-2
F3-4
HCV

Figure 16

Figure 16: M0, M1, M2a M2b and M2c macrophage subsets from HCV-infected patients with advanced fibrosis spontaneously produce high levels of IL-12. Macrophage subsets from healthy controls (HC, n=6) and chronic HCV-infected individuals with early fibrosis (F0-2, n=4) and advanced fibrosis (F3-4, n=4) were cultured for 72 hours. . Following that, their supernatants were collected and quantified for IL-12 using a Luminex-based multiplex assay. The cytokine was quantified in supernatants from each subset as shown in **(A)** M0 **(B)** M1 **(C)** M2a **(D)** M2b **(E)** M2c. Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

In M1 subset, I saw significant increases in the spontaneous production of IL-12 in both early (up to 480pg/mL) and advanced fibrosis groups (up to 60pg/mL) (Figure 13B). For the M0 subset, the increase in production at advanced fibrosis was significantly higher (up to 70pg/mL) compared to HC and F0-2 groups (Figure 16A). For M2a, M2b and M2c subsets, I saw this increase in spontaneous IL-12 production at advanced fibrosis levels (F3-4) compared to HC only (Figure 16A, C-E).

Overall, I was able to observe multiple changes to the phenotype and cytokine profiles of macrophage subsets between normal, healthy donors and HCV-infected patients. Most of these changes were observed at advanced levels of liver fibrosis of HCV-infected individuals. It is imperative to investigate if the alterations in macrophage subsets I found in objective 1 can induce a dysfunction in $CD8^+$ T cells. However, before evaluating the impact on $CD8^+$ T cells in chronic HCV infection, I first established the relationship between each macrophage subsets and $CD8^+$ T cells in normal, healthy controls.

Objective#2 Role of macrophage subsets in $CD8^+$ T cell function induction among normal, healthy and chronic HCV infected individuals

In order to assess if the change in macrophage subset phenotype plays a role in modulating $CD8^+$ T cell dysfunction, I first investigated the relationship of these two cell types in uninfected, healthy controls. For this, I isolated macrophages and T cells from the same healthy donors and established autologous co-cultures between them at a ratio of 1:4 (macrophage: T cells). Following that, I analyzed the resulting impact of the co-culture on $CD8^+$ T cell functional molecules. Among the functions of cytotoxic T cells that I studied were the production of the immunomodulatory cytokine IFN-γ, the cytolytic perforating agent called perforin and the marker for degranulation, CD107a.

Objective#2A. M1 macrophages induce IFN-γ production and degranulation in $CD8^+$ T cells derived from normal uninfected controls

IFN-γ: IFN-γ is an important modulator of anti-viral response which is released by T cells soon after activation, but before the onset of their true cytolytic functions (140). To observe the IFN-γ secretory function of T cells, I cultured them with macrophage subsets for a total of 24 hours, after which the supernatant from the culture was collected and their IFN-γ production quantified. I observed that while T cells alone did not spontaneously produce any IFN-γ, once cultured with M1 subsets, the two cell types together produced significantly higher levels of IFN-γ (Figure 17 A). Such production was not seen in the supernatants containing T cells in combinations with any other macrophage subset (Figure 17A). However, our previous results from Aim 1 indicated that M1 macrophages from healthy controls do produce high levels of IFN-γ on their own (Figure 14B, and shown here as Figure 17B), it is not possible to attribute the released IFN-γ to the T cells in culture as a marker of their function.

To determine if M1 macrophages were able to induce IFN-γ within $CD8^+$ T cells, I repeated the culture and carried out intracellular staining of IFN-γ within cells positive for the CD8 marker. In this method, after culturing the T cells again for 24 hours, I treated the culture with golgi transport inhibitor containing monensin for the last 6 hours of culture, to prevent the release of cytokines from the cell populations. I then collected all non-adherent cells (which would primarily be only the $CD8^+$ T cells), surface stained them to isolate the $CD8^+$ populations and performed intracellular staining for IFN-γ. The schematic diagram showing intracellular IFN-γ production following co-culture with macrophages is shown in Figure 18. I confirmed that compared to the T cells alone, those that had been cultured with M1 and M2b subsets had a significantly increased percentage of cells that were positive for IFN-γ (Figure

19A). A representative donor's histogram indicates that T cells alone have no IFN-γ^+ population, however, those co-cultured with M1 subset show a smaller second population, indicating a subset of T cells that become IFN-γ^+ upon interacting with the macrophages (Figure 19C, 19E). For the same donor, we see that co-cultures with other subsets did not form IFN-γ^+ $CD8^+$ T cells (Figure 19B, D-F).

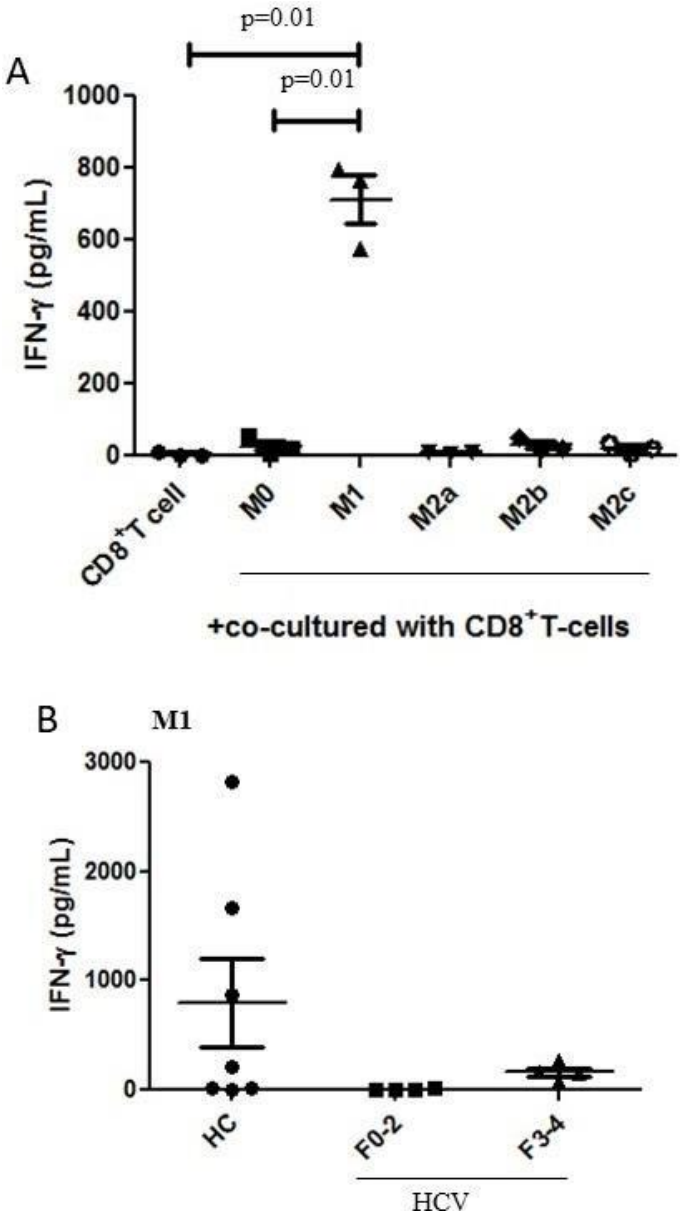

A
p=0.01
p=0.01
IFN-γ (pg/mL)
1000
800
600
400
200
0
CD8+T cell
M0
M1
M2a
M2b
M2c
+co-cultured with CD8+T-cells
B
M1
IFN-γ (pg/mL)
3000
2000
1000
0
HC
F0-2
F3-4
HCV

Figure 17

Figure 17: M1 macrophages following culture with $CD8^+$T cells induce IFN-γ production in healthy controls. (A) Macrophage subsets ($50X10^3$) and $CD8^+$ T cells ($200X10^3$) isolated form the same donors were cultured in a ratio of 1:4 (macrophage:T cell) for 24 hours. Supernatants were quantified for IFN-γ production using a Luminex-based multiplex assay (n=3). (B) IFN-γ production bymacrophage subsets alone is the same figure from Figure 14B and is shown here for clarity. Each symbol (dot/square/triangle) on the graph represents a unique donor (HC n=7, F0-4 n=4, F3-4 n=4). P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

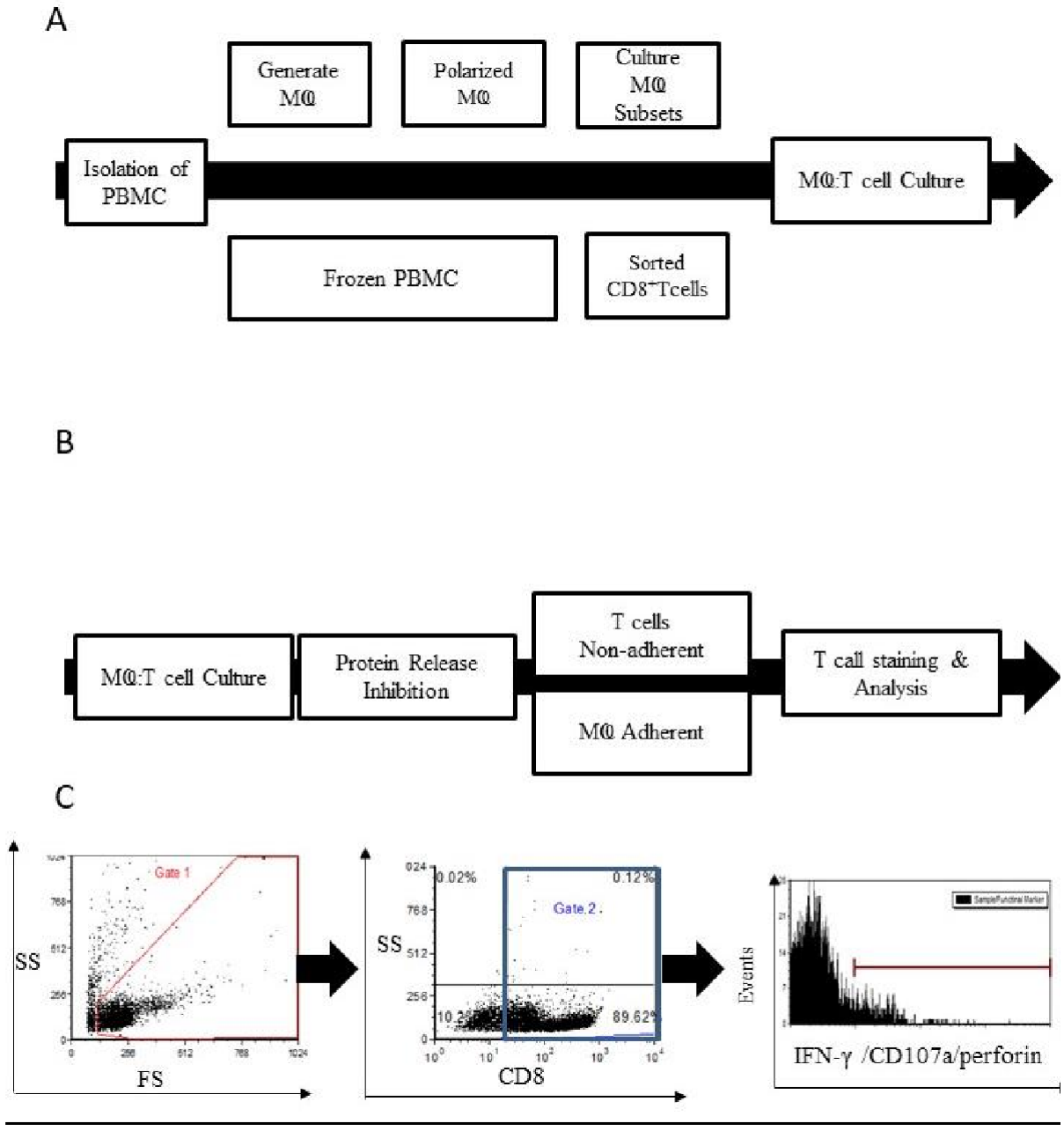

A
Generate MΦ
Polarized MΦ
Culture MΦ Subsets
Isolation of PBMC
MΦ:T cell Culture
Frozen PBMC
Sorted CD8⁻Tcells
B
MΦ:T cell Culture
Protein Release Inhibition
T cells Non-adherent
MΦ Adherent
T call staining & Analysis
C
Gate 1
SS
FS
0.02%
0.12%
Gate 2
SS
CD8
10.2
89.62
Events
IFN-γ /CD107a/perforin

Figure 18

Figure 18: Method outline and flow cytometry gating strategy for co-cultured $CD8^+$ T cell functional assays. (A) Method outline indicates the isolation of PBMC from whole blood, following by the freezing of half the PBMC sample while the remaining half is used to generate macrophages and polarize them into subsets. While macrophage subsets are cultured, the frozen PBMCs in parallel are thawed and sorted for $CD8^+$ cells, following which these two are cultured together. **(B)** Once culture period is complete, depending on assay requirements they are treated with protein export inhibitor and incubated. Non-adherent T cells are later removed and staining for desired protein and cell identification is carried out. **(C)** Gating strategy for flow cytometry is shown, indicating live cell gating, $CD8^+$ marker gating and functional protein marker gating.

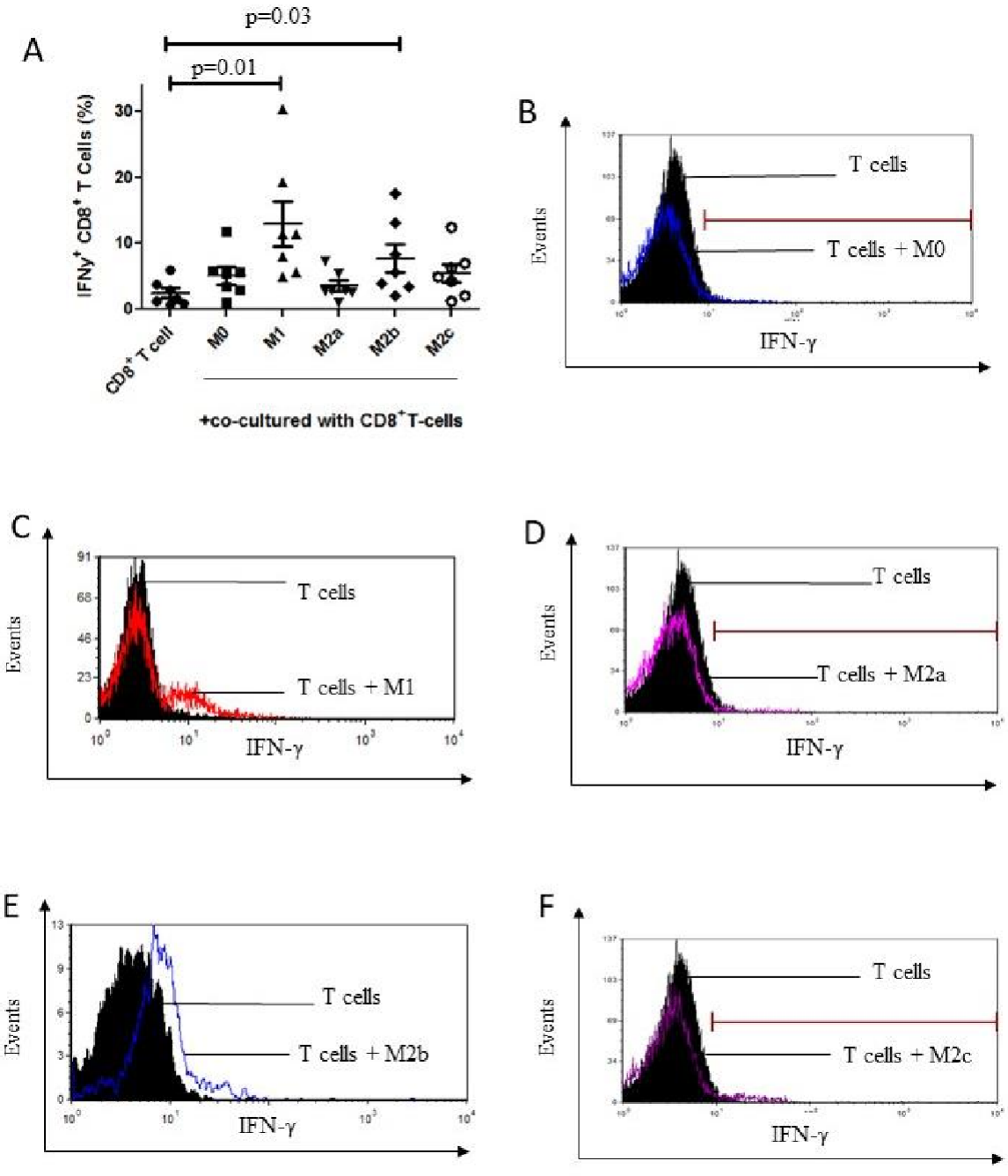

A
p=0.03
p=0.01
IFNγ+ CD8+ T Cells (%)
30
20
10
0
CD8+ T cell
M0
M1
M2a
M2b
M2c
+co-cultured with CD8+T-cells
B
T cells
T cells + M0
Events
IFN-γ
C
T cells
T cells + M1
Events
IFN-γ
D
T cells
T cells + M2a
Events
IFN-γ
E
T cells
T cells + M2b
Events
IFN-γ
F
T cells
T cells + M2c
Events
IFN-γ

Figure 19

Figure 19: M1 and M2b macrophages cultured with $CD8^+$ T cells have higher percentage of IFN-γ^+ $CD8^+$ T cells. $50X10^3$ macrophages were cultured with $200X10^3$ $CD8^+$ T cells isolated form the same donors, in a ratio of 1:4 (macrophage: T cell) for 24 hours. Non-adherent cells were harvested and analyzed for intracellular IFN-γ. **(A)** Percentage of $CD8^+$T cells that were IFN-γ^+ in each control and culture combination (n=7). Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant. Representative donor histogram indicates IFN-γ expression on $CD8^+$T cells alone and that co-cultured with **(B)** M0 **(C)** M1 **(D)** M2a **(E)** M2b and **(F)** M2c.

Perforin: I then quantified levels of perforin released into the supernatants of T cells cultured with each macrophage subset but this time we cultured the cells for 48 hours, allowing enough time for the cytolytic molecules to be produced and released. Interestingly, there was no difference in the level of perforin released by T cells alone and that released by T cells and macrophage subsets cultured together (Figure 20). To reconfirm that culturing with macrophage subsets does not impact T cell perforin production, we cultured the cells for 48 hours and collected the non-adherent cells, strained them to isolate the $CD8^+$ population, and finally performed intracellular staining for perforin. Once again, we observed no difference between the portion of $perforin^+$ cells among T cells alone and T cells cultured with various macrophage subsets (Figure 21).

CD107a: Finally, I assessed whether macrophage subsets induced degranulation of $CD8^+$ T cells by examining the expression of CD107a. For this, I cultured T cells with macrophage subsets for 48 hours, but for the last 6 hours of culture, we added golgi transport inhibitor containing monensin and CD107a antibody conjugated with a fluorescent marker. This allowed the CD107a to accumulate at the cell's surface after exocytosing granules and prevented their re-internalization into the cells. I found a striking increase in the percentage of $CD107a^+$ $CD8^+$ T cells among those that had been cultured with M1 macrophages compared to T cells alone (Figure 22A). However, none of the other macrophage subsets caused degranulation in $CD8^+$ T cells. Again, a representative donor's histogram is showing how T cells alone have no $CD107a^+$ population (in black) and those co-cultured with M1 subset show a smaller second population (blue), indicating a subset of T cells that become $CD107a^+$ upon interacting with the M1 macrophages (Figure 22C). For the same donor, we see that co-cultures with other subsets did not form $CD107a^+$ $CD8^+$ T cells (Figure 22B, D-F).

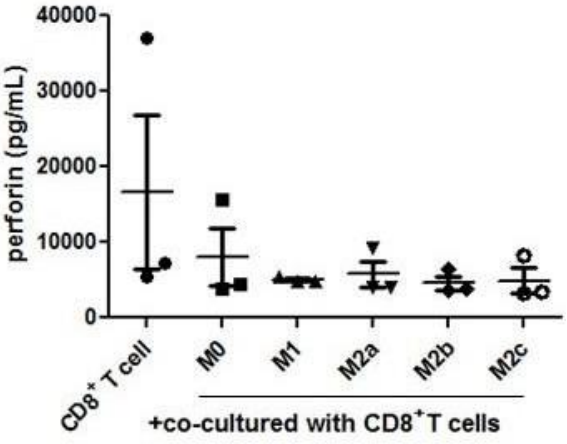

40000
30000
20000
10000
0
perforin (pg/mL)
CD8+ T cell
M0
M1
M2a
M2b
M2c
+co-cultured with CD8+T cells

Figure 20

Figure 20: Macrophage subsets cultured with $CD8^+$ T cells produce similar levels of perforin. $50X10^3$ macrophages were cultured with $200X10^3$ $CD8^+$T cells for 48 hours. Supernatants were quantified for perforin using a Luminex-based multiplex assay. Spontaneous production of perforin from $CD8^+$T cells alone and $CD8^+$T cells cultured with each respective macrophage subset (n=3) is shown. Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

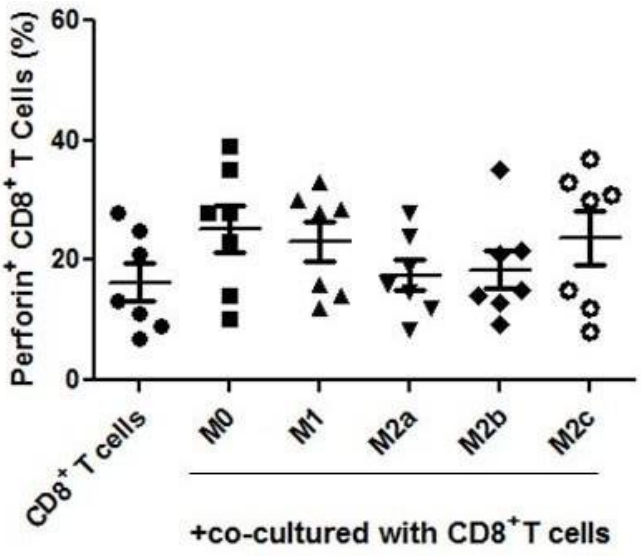

Perforin+ CD8+ T Cells (%)
60
40
20
0
CD8+ T cells
M0
M1
M2a
M2b
M2c
+co-cultured with CD8+ T cells

Figure 21

Figure 21: Macrophage subsets cultured with $CD8^+$ T cells show similar percentage of perforin$^+$ $CD8^+$T cells. $50X10^3$ macrophage subsets were cultured with $200X10^3$ $CD8^+$ T cells isolated form the same donors in a ratio of 1:4 (macrophage: T cell) for 48 hours. Non-adherent cells were harvested and analyzed for intracellular perforin. Percentage of perforin$^+$ $CD8^+$ T cells alone and those cultured with each macrophage subset are shown (n=7). Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

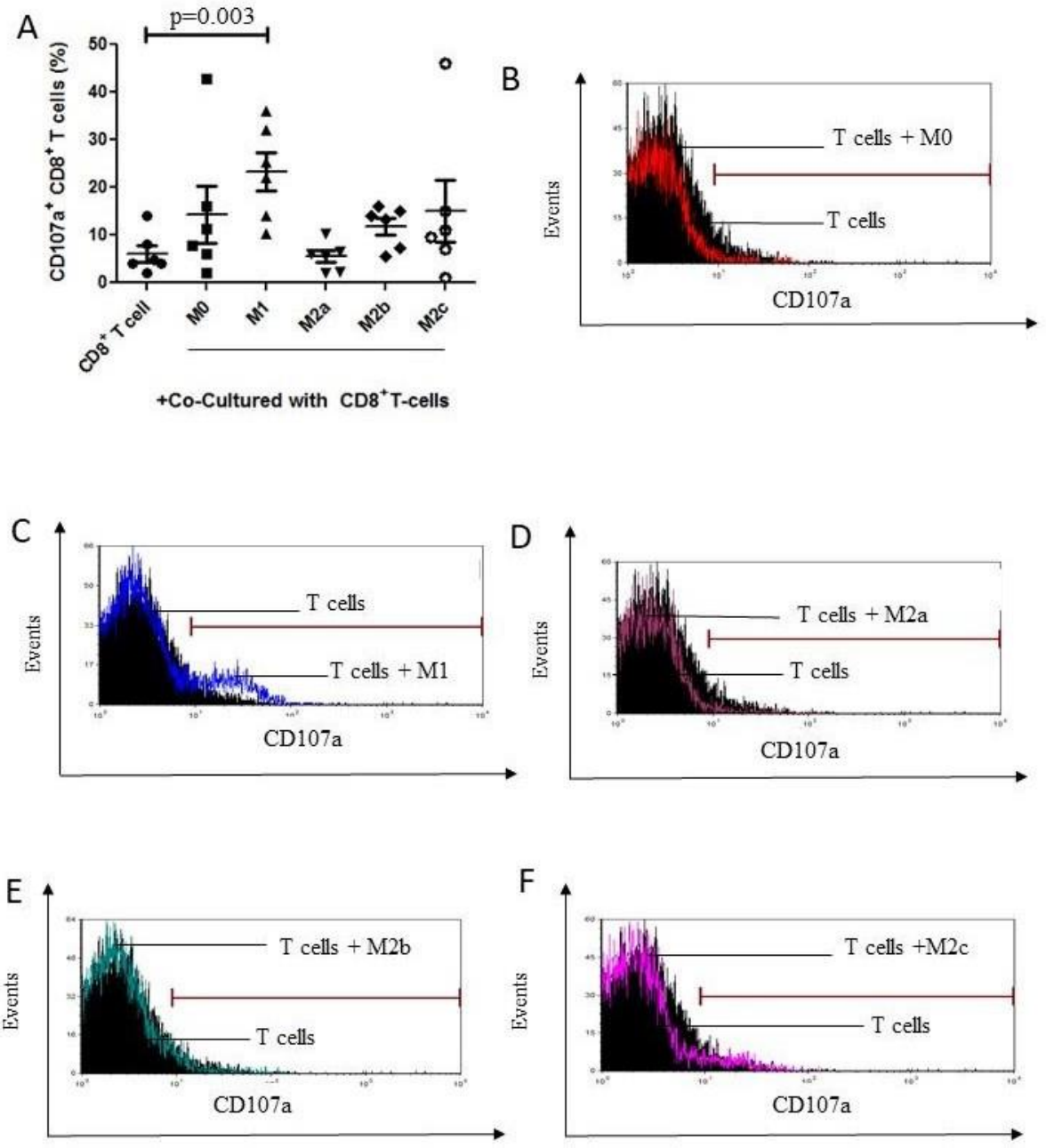

A
p=0.003
CD107a+ CD8+ T cells (%)
50
40
30
20
10
0
CD8+ T cell
M0
M1
M2a
M2b
M2c
+Co-Cultured with CD8+T-cells
B
T cells + M0
T cells
Events
CD107a
C
T cells
T cells + M1
Events
CD107a
D
T cells + M2a
T cells
Events
CD107a
E
T cells + M2b
T cells
Events
CD107a
F
T cells +M2c
T cells
Events
CD107a

Figure 22

Figure 22: M1 macrophages cultured with $CD8^+$T cells have increased percentage of $CD107a^+$ $CD8^+$ T cells. $50X10^3$ macrophages were cultured with $200X10^3$ T-cells for 48 hours. Non-adherent cells were harvested and analyzed for surface CD107a. **(A)** Percentage of $CD107a^+$ $CD8^+$ T cells in each condition (n=6). Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant. Representative donor histogram indicates CD107a expression in $CD8^+$ T cells alone and $CD8^+$T cells cultured with **(B)** M0 **(C)** M1 **(D)** M2a **(E)** M2b **(F)** M2c.

Overall, culturing T cells autologously with M1 macrophages is able to induce the production of the immunomodulator IFN-γ and degranulation of cytolytic protein as indicated by CD107a. However, there was no increase in the ability of M1 macrophages to induce the production of perforin.

Objective#2B. M1 macrophage induced IFN-γ production and degranulation of $CD8^+$ T cells is contact dependent

It was essential for us to understand if the M1 macrophages were modulating T cell function (IFN-γ and CD107a induction) by means of secreted molecules (such as cytokines and other soluble effectors) or through cell to cell contact. For this, I repeated the direct co-culture in parallel with a co-culture across a transmembrane. These membranes were selected to have pore sizes of 0.4μm in order to prevent macrophages and T cells from passing through to the other compartment. For the IFN-γ and CD107a assays, we then repeated the functional analysis for donors with both types of cell cultures in parallel.

We used cells from three new normal, healthy donors and observed that the significant increase in IFN-γ^+ $CD8^+$ T cells after culturing with M1 macrophages was still consistent with previous findings. Furthermore, this increase in IFN-γ^+ $CD8^+$ T cell populations was absent when the cells were cultured across a transwell membrane and this reduction was also statistically significant (Figure 23A). A representative histogram shows T cells cultured with M1 in direct contact (in black) where a second IFN-γ^+ positive peak can clearly be observed. In contrast, the histogram with the same donor's T cells cultured across a transmembrane with M1 (in red) shows the IFN-γ^+ population is absent (Figure 23B).

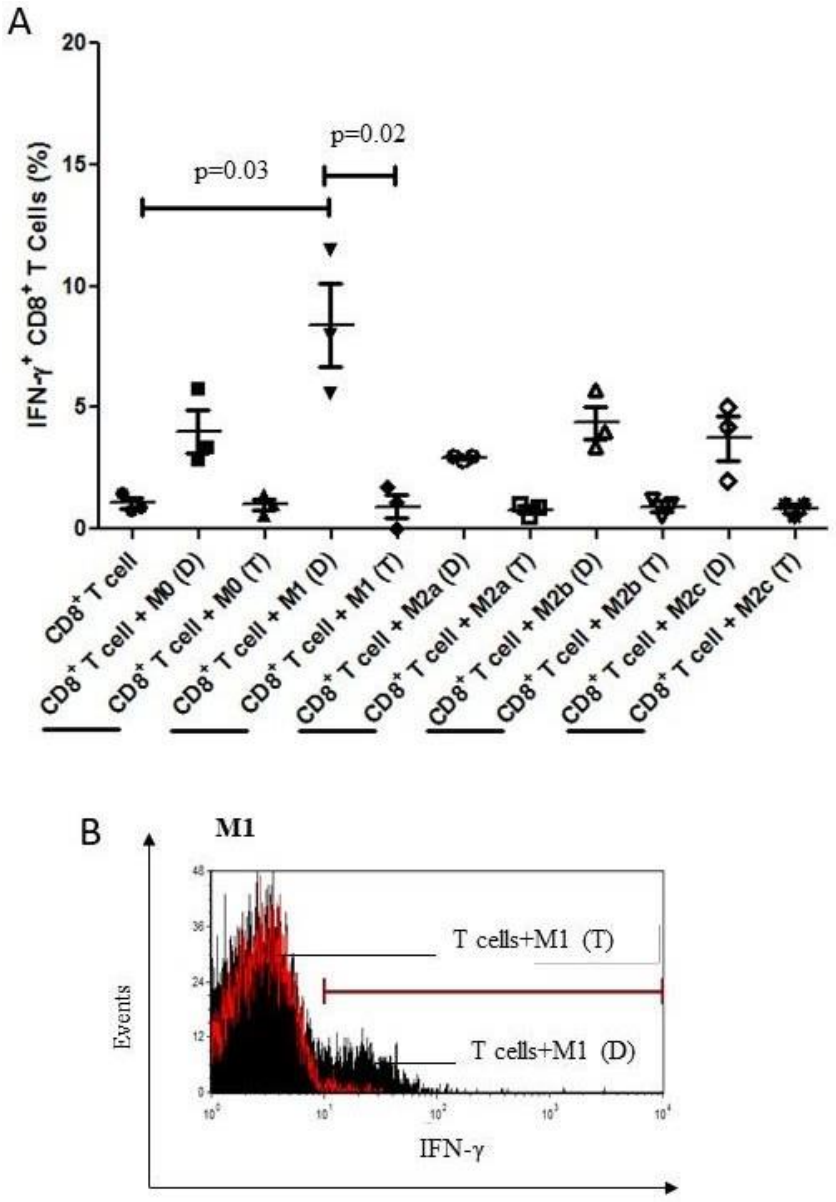

A
IFN-γ+ CD8+ T Cells (%)
20
15
10
5
0
p=0.03
p=0.02
CD8+ T cell
CD8+ T cell + M0 (D)
CD8+ T cell + M0 (T)
CD8+ T cell + M1 (D)
CD8+ T cell + M1 (T)
CD8+ T cell + M2a (D)
CD8+ T cell + M2a (T)
CD8+ T cell + M2b (D)
CD8+ T cell + M2b (T)
CD8+ T cell + M2c (D)
CD8+ T cell + M2c (T)
B
M1
Events
T cells+M1 (T)
T cells+M1 (D)
IFN-γ

Figure 23

Figure 23: IFN-γ induction in $CD8^+$ T-cells following co-culture is contact dependent. $50X10^3$ macrophages were cultured with $200X10^3$ T-cells for 24 hours. These cells were cultured either in direct contact or across a transmembrane. Non-adherent cells were harvested and analyzed for intracellular IFN-γ. **(A)** Percentage of IFN-γ^+ $CD8^+$T cells following either direct culture (as indicated by D) or across a transmembrane (as indicated by T) (n=3). Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant. **(B)** Representative donor histogram indicates $CD8^+$ T cells in direct and transwell co culture with M1 macrophages.

To analyze the mechanism of induction of degranulation in T cells by M1 macrophages, I performed a similar experiment with direct and transwell co cultures of macrophages and T cells and this time we assessed the proportion of $CD8^+$ T cells that were positive for CD107a. Similar to the IFN-γ assay findings, I observed that the induction of $CD107a^+$ T cells by M1 macrophages was contact dependent, and this impact was reversed when cultured across a transmembrane (Figure 24A). None of the other macrophage subsets induced such degranulation in T cells when in direct culture. A representative donor histogram depicts the formation of a small subset of T cells that were positive for CD107a (black) when T cells and M1 macrophages are in direct culture. The figure also shows the absence of this $CD107a^+$ subset of population when the same donor's T cells and M1 macrophages are cultured across a transmembrane (red) (Figure 24B).

Objective#2C. Role of M1 macrophages in modulating T cell function in chronic HCV infection

(i) $CD8^+$ T cells from HCV F02 secrete higher levels of IFN-γ

Before we began to investigate the macrophage and T cell relationship among HCV infected patients, it was important to identify the relative baseline levels of IFN-γ production by $CD8^+$ T cells from normal, healthy donors and chronically HCV-infected patients. We treated the PBMC from HCV-infected patients in an identical manner as for normal healthy donors (Figure 25A). My results show that a significantly higher proportion of $CD8^+$ IFN-γ^+ T cells in HCV infected patients compared to normal, healthy controls (Figure 25B). The representative histogram of one healthy donor (black) and one HCV-infected patient (blue) indicates a rightward shift in the baseline levels of IFN-γ^+ T cells in HCV patients compared

to the healthy controls (Figure 25C). It is important to note, however, that all patients in this experiment were in early stages of liver fibrosis, as we could not successfully recruit more patients in advanced fibrosis stage to complete this part of the study

(ii) M1 macrophages downregulate IFN-γ production in $CD8^+$ T cells following co culture in patients with chronic HCV infection

I then determined whether M1 macrophage co culture with $CD8^+$ T cells from HCV patients induce IFN-γ expression in T cells similar to the normal, healthy controls. My previous results have shown that M1 macrophages induce a IFN-γ^+ T cells population when cultured with autologous T cells from healthy controls (Figure 26C). I then repeated the M1 macrophage and T cell co-culture for the patient population (F0-2), and observed a striking difference compared to the results in normal, healthy donors. Not only did the culture of M1 macrophages and T cells in patients fail to induce the IFN-γ^+ population, but we actually observed a significant reduction of IFN-γ^+ T cells compared to increased expression of IFN-γ observed following the co culture with M1 macrophages in healthy, normal donors (Figure 26B and 26C, left panels). Representative donor histograms from healthy controls (Figure 26C, right panel) clearly indicate increased expression of IFN-γ^+ T cells in the co-cultured T cells in normal, healthy donors, whereas the histogram showing data for HCV-infected patients (Figure 26B, right panel) shows a decreased level of IFN-γ^+ T cells.

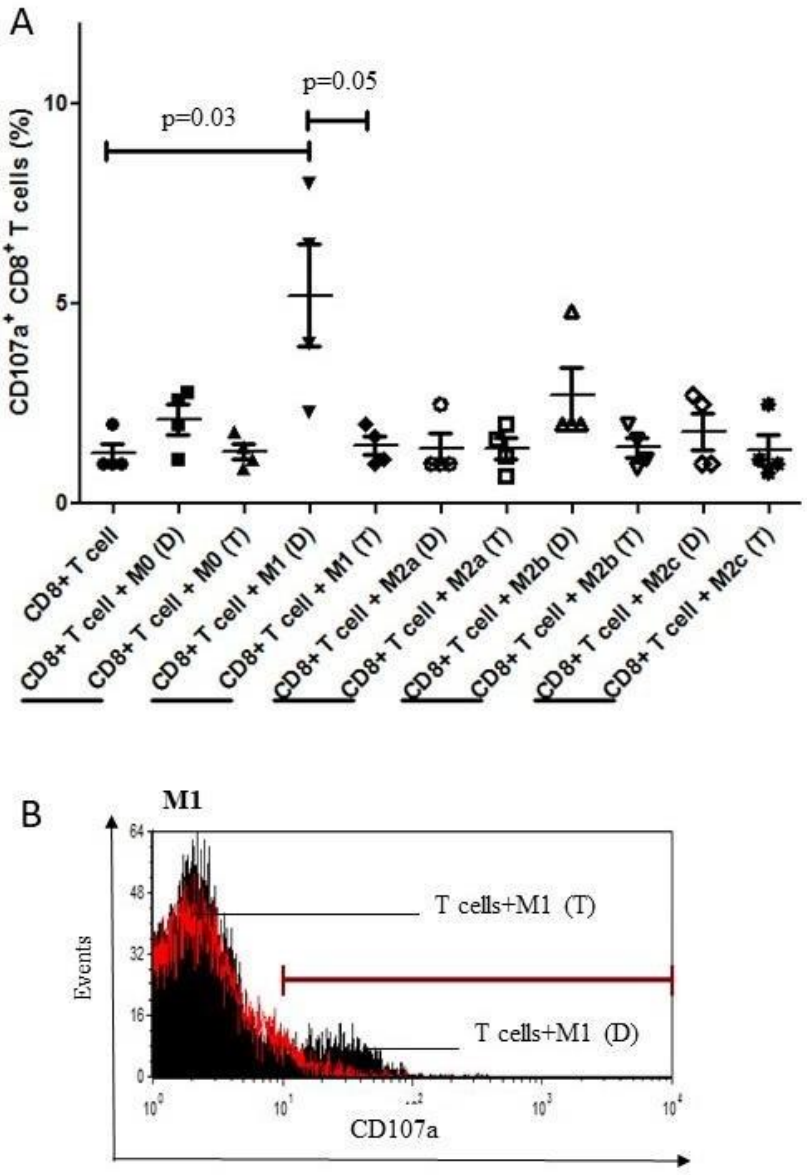

A
p=0.03
p=0.05
CD107a+ CD8+ T cells (%)
10
5
0
CD8+ T cell
CD8+ T cell + M0 (D)
CD8+ T cell + M0 (T)
CD8+ T cell + M1 (D)
CD8+ T cell + M1 (T)
CD8+ T cell + M2a (D)
CD8+ T cell + M2a (T)
CD8+ T cell + M2b (D)
CD8+ T cell + M2b (T)
CD8+ T cell + M2c (D)
CD8+ T cell + M2c (T)
B
M1
Events
64
48
32
16
0
T cells+M1 (T)
T cells+M1 (D)
CD107a

Figure 24

Figure 24: CD107a induction in $CD8^+$ T-cells following co-culture is contact dependent. $50X10^3$ macrophages were cultured with $200X10^3$ T-cells for 48 hours. These cells were cultured either in direct contact or across a transmembrane. Non-adherent cells were harvested and analyzed for intracellular CD107a. **(A)** Percentage of $CD107a^+$ $CD8^+$ T cells following either direct culture (as indicated by D) or across a transmembrane (as indicated by T) (n=4). Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant. **(B)** Representative donor histogram indicates $CD8^+$ T cells in direct and transwell co culture with M1 macrophages.

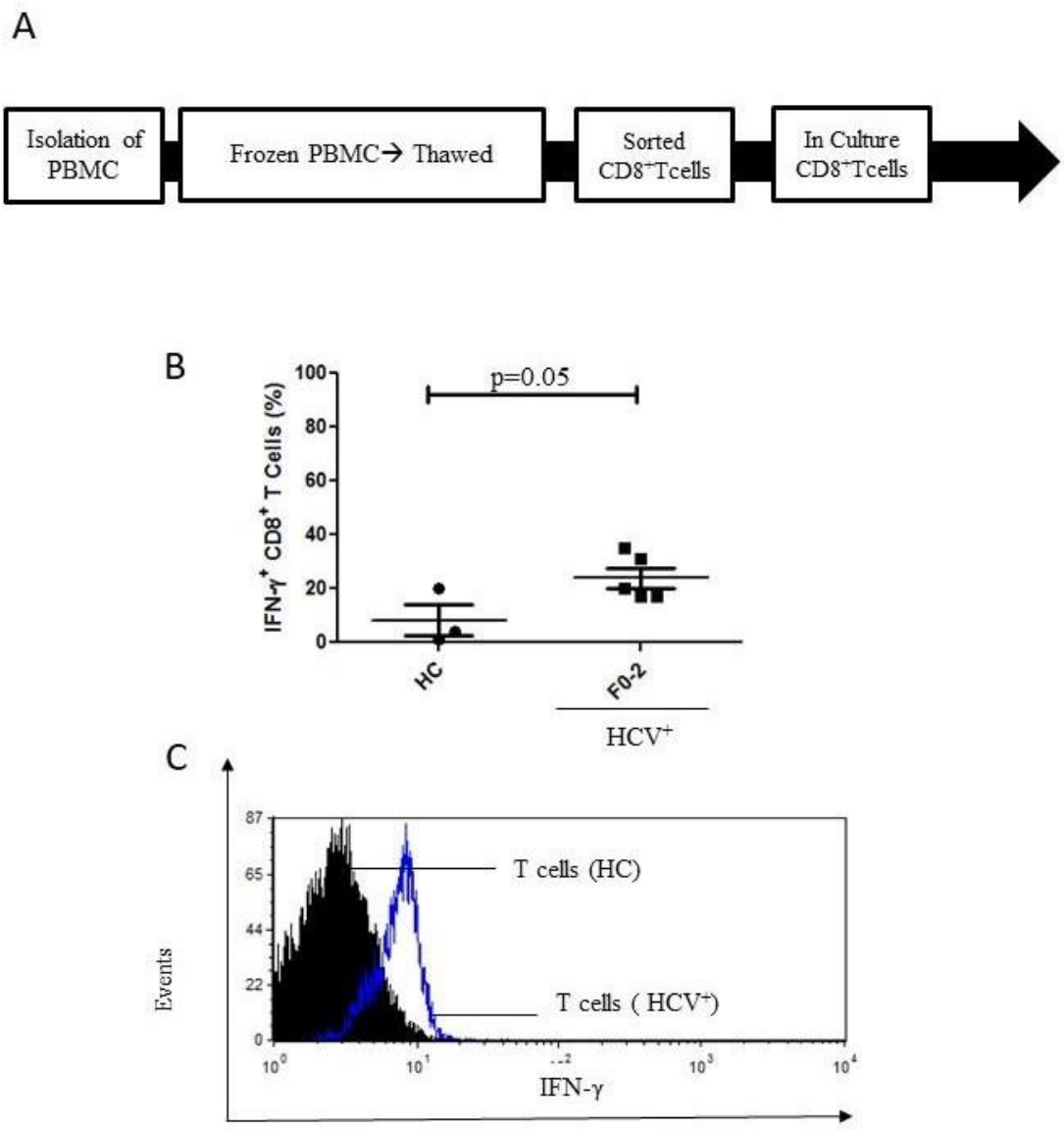

A
Isolation of PBMC
Frozen PBMC→ Thawed
Sorted CD8⁺Tcells
In Culture CD8⁺Tcells
B
p=0.05
IFN-γ⁺ CD8⁺ T Cells (%)
100
80
60
40
20
0
HC
F0-2
HCV⁺
C
87
65
44
22
0
Events
T cells (HC)
T cells (HCV⁺)
10⁰
10¹
10³
10⁴
IFN-γ

Figure 25

Figure 25: $CD8^+$T cells derived from HCV-infected patients with early fibrosis have increased percentage of $CD8^+$ IFN-γ^+ cells. (A) Method map indicates that **i**solated and frozen PBMC were thawed and $CD8^+$ T cells isolated from HCV-infected donors (F0-2) and healthy controls (HC). $CD8^+$ T cells were then cultured for 24 hours and analyzed for intracellular IFN-γ. **(B)** Percentage of IFN-γ^+ $CD8^+$ T cells from each group (HC n=3, F0-2 n=5). Each symbol on the graph (dot/square/triangle) indicates a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant. **(B)** Representative donor histogram indicates IFN-γ expression in $CD8^+$T cells from healthy controls and HCV-infected patients in early fibrosis stage.

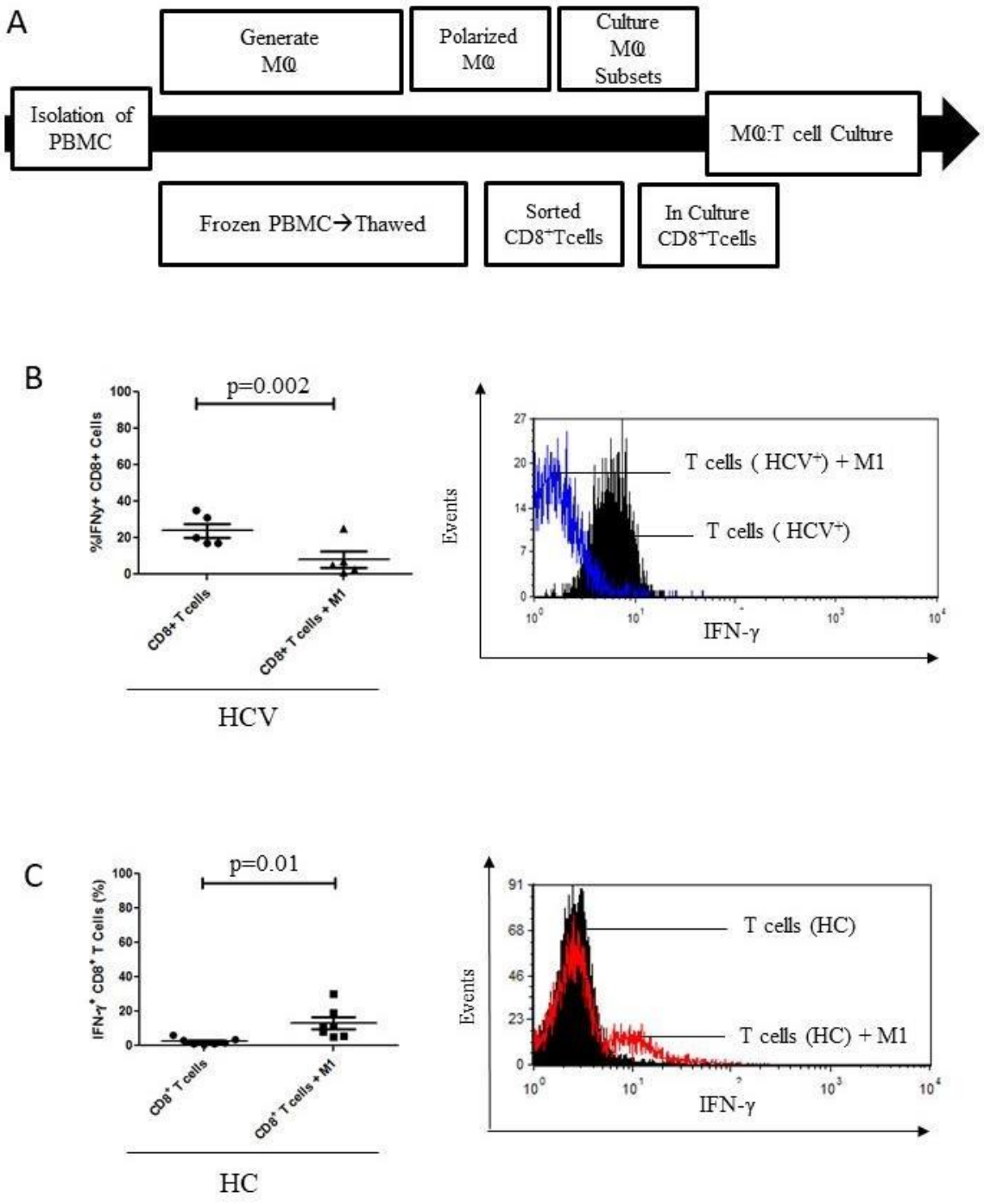

A
Generate MΦ
Polarized MΦ
Culture MΦ Subsets
Isolation of PBMC
MΦ:T cell Culture
Frozen PBMC→Thawed
Sorted CD8+Tcells
In Culture CD8+Tcells
B
p=0.002
%IFNγ+ CD8+ Cells
CD8+ T cells
CD8+ T cells + M1
HCV
Events
T cells (HCV+) + M1
T cells (HCV+)
IFN-γ
C
p=0.01
IFN-γ+ CD8+ T Cells (%)
CD8+ T cells
CD8+ T cells + M1
HC
Events
T cells (HC)
T cells (HC) + M1
IFN-γ

Figure 26

Figure 26: M1 macrophages following culture with T cells from HCV-infected patients decrease percentage of IFN-γ^+ T cells. (A) Method map indicates, macrophage subsets were generated from PBMC, polarized to subsets and cultured for 72 hours, while in parallel, $CD8^+$ T cells were isolated from frozen-thawed PBMCs and cultured for 24 hours leading up to the co culture. Following that, $50X10^3$ macrophages were cultured with $200X10^3$ $CD8^+$ T cells for 24 hours. Non-adherent cells were harvested and analyzed for intracellular IFN-γ. **(B)** Percentage of IFN-γ^+ $CD8^+$ T cells among $CD8^+$ T cells alone and those in culture with M1 macrophages in HCV-infected patients with early fibrosis (n=5). Representative donor histogram showing IFN-γ expression among $CD8^+$ T cells alone and those cultured with M1 macrophages. **(C)** Percentage of IFN-γ^+ $CD8^+$ T cells from healthy controls among T cells alone and those in culture with M1 macrophages (n=7) are shown again from **Figure 19** for clarity and comparison. Representative donor histogram from healthy controls indicate IFN-γ expression in $CD8^+$ T cells alone and those cultured with M1 macrophages. Each symbol (dot/square/triangle) on the graph represents a unique donor. P values where significant are indicated. A value of $p \leq 0.05$ was considered to be statistically significant.

Chapter 4: Discussion

Herein, I have presented data that identifies the importance of M1 macrophages in inducing cytolytic and immunomodulatory functions of normal human $CD8^+$ T cells and highlight their inability to induce such functions in chronic HCV infection. I have provided evidence that M1 macrophages in normal, healthy donors, induced the expression of IFN-γ and the degranulation marker CD107a. I have also shown the various phenotypic alterations that M1 macrophages displayed in advanced stages of liver fibrosis identified as the F3-4 stage. I showed how M1 macrophages lost some of its pro-inflammatory characteristics at F3-4 stage by its decreased production of TNF-α and increased production of IL-10. Simultaneously, M1 macrophages showed increased expression of CD86 and IL-12, resulting in an altered phenotype. In M2 macrophages, I showed a decrease in proportion of $CD206^+$ cells (in M2c macrophages) and an increase in IFN-γ, IL-10 and IL-12 production in M2a, M2b and Mc macrophages. This suggests an alteration in M2 phenotype where it gains some M1-like characteristics through increased production of IFN-γ and IL-12 and decrease in $CD206^+$ cells. In normal, healthy controls, M1 macrophages cultured with autologous $CD8^+$ T cells could increase the proportion of IFN-γ^+ $CD8^+$ T cells. In contrast, M1 macrophages cultured with $CD8^+$ T cells in chronic HCV infection reduced the proportion of IFN-γ^+ $CD8^+$ T cells. These findings were novel in the context of chronic HCV infection, showing that altered phenotypes and functionality of macrophages may impact $CD8^+$ T cells, thereby contributing to immune impairment in such conditions.

4.1 Phenotypic characterizations of macrophage subsets in HCV infection

As my first objective, I assessed the phenotypes of macrophage subsets and compared them between healthy, normal donors and HCV-infected donors, using a combination of three surface receptors (CD86, CD163 and CD206) and five secreted cytokines (IL-6, TNF-α, IL- 10, IL-12, and IFN-γ).

Surface Receptors: For CD86, I observed that proportions of $CD86^+$ cells were significantly higher among M0 (unpolarized control) and M1 macrophages (Figure 6B, 6C) when derived from advanced fibrosis compared to healthy controls. Not only that, the expression of CD86 per cell was also significantly higher for M1 in this group compared to the health group (Figure 7B). This suggests an activated M1 phenotype in chronic HCV infection. The impact of increased CD86 expression on M1 macrophages in chronic HCV patients on the development of immune responses is poorly understood. Other studies have observed increased CD86 expression in a similar chronic, hepatotropic infection with HBV, where liver lobules were found to have a higher total count and percentage of $CD86^+$ macrophages, compared to $CD80^+$ or $PD\text{-}L1^+$ macrophages (227). While CD80 is known to drive a Th1 response, CD86 is expected to drive a Th2 response in murine $CD4^+$ T cells (202, 227-229). In fact, CD86 (B7-2) was unable to activate $CD8^+$ T cells effectively unlike CD80 (B7-1) (230).

It has been shown that a small subset of circulating blood monocytes from individuals with chronic HCV infection co-expressed CD206 and collagen, suggesting a

possible correlation between fibrosis and the M2 macrophage family (212). M2 macrophages are predominant in the livers of chronic HCV patients (183, 231), and they are known to activate HSCs, which play a key role in inducing liver fibrosis (183, 212). However, my observation showed decreased CD206 expression in M2c macrophages at F3-4 stage (Figure 10E).

Studies reported an increased expression of CD163 in the monocytes of HCV-infected individuals compared to healthy controls (212). However, I did not find any significant differences in the expression of CD163 in any of the macrophage subsets between normal, healthy controls and HCV-infected patients (Figure 8, 9). The soluble form of CD163, sCD163 (a marker of inflammation and macrophage activation), was found in abundance in the serum of individuals with chronic HCV infection; this was diminished upon successful viral clearance (232). Others have reported a correlation between the extent of liver fibrosis and cirrhosis with the levels of the sCD163 in serum (219). I did not observe any alterations in their expression on the surface of macrophage subsets. It must be noted that CD163 found on cell surface of monocytes and macrophages can be 'shed' resulting in the soluble protein sCD163 in quick response to certain TLR stimulations such as LPS (233-235). However, it is known to recover to a high level on the surface of cells in 24 hours (233-235). Since my experiments did not assess the sCD163 and only investigated the surface CD163 at 72 hours after culture. Further studies on CD163 on monocytes and macrophages and sCD163 at various times after culture can resolve this issue.

Cytokines: Macrophages are one of the main producers of the pro-inflammatory cytokine TNF-α (236). Since they also express the TNF-α receptor, macrophages can respond to TNF-α in an autocrine fashion (236). In fact, in tumor microenvironments, a tightly regulated pathway exists where change in TNF mRNA expression and Type I TNF receptor

signaling can modulate the switch from M1 to the M2 subset (237). I found a significant reduction in spontaneous production of TNF-α from M1 macrophages in HCV-infected individuals with advanced liver fibrosis compared to normal, healthy controls (Figure 12B). The reduction in TNF-α production by M1 macrophages may point to the loss of a pro-inflammatory characteristic.

IL-6 produced by macrophages plays a key role in driving inflammation (238). M2b macrophages (polarized using LPS and IL1β) are the main producer of IL-6 in our model. There was no significant change in IL-6 production between HCV-infected donors and normal, healthy controls (Figure 13A-E). Although M1 macrophage s produce low levels of IL-6 in healthy controls (15-30pg/mL) there was a pattern of decrease in IL-6 with increasing levels of liver fibrosis (Figure 13B). Although not statistically significant, this decrease suggests M1 macrophages producing lower levels of IL-6 with increasing liver fibrosis. IL-6 in combination with IL-2 is known to play an important role in activation of immature $CD8^+$ T cells to cytotoxic cells (205, 239). It is possible that change in IL-6 concentration alone (or in conjunction with other cytokines) may influence the activity of $CD8^+$ T cells in HCV infection.

Increased expression of IFN-γ mRNA has been shown in liver tissue samples from chronic HCV patients, especially in areas with inflammation and fibrosis (240). Similar results were shown by another group who reported elevated levels of IFN-γ within sections of liver tissue, but low levels in serum of patients with chronic HCV infection (241). Moreover, the patients who responded well to treatment were able to produce high level IFN-γ upon PBMC stimulation with a variety of viral peptides compared to non-responding patients (241, 242). Another study reported that IFN-γ-producing HCV-specific $CD8^+$ T cells in the liver of chronic HCV-patients are correlated with lower fibrosis and reduced progression of liver damage (243). I have focused my cross-sectional studies on analyzing IFN-γ production by various macrophage subsets and $CD8^+$ T cells in chronic HCV infection. My results show in advanced fibrosis, an increase in IFN-γ from M0 and M2 macrophage subsets (M2a, M2b, M2c) (Figure 14). Although I observed increased IFN-γ production from certain macrophage subsets, their possible correlation with disease prognosis is unclear.

Finally, I quantified the two of the subset-defining cytokines, IL-10 and IL-12. IL-10 is a classic inhibitory cytokine with the capacity of shutting down Th1 responses from cells such as NK (Natural Killer cells), Th1 cells as well as activated macrophages (244). I observed an increase in the production of IL-10 in all macrophage subsets at advanced fibrosis groups, compared to those in healthy controls (Figure15). Not only can IL-10 inhibit pro-inflammatory cytokines production by macrophages and monocytes through autocrine suppression, but it can also reduce IFN-γ production by $CD4^+$ T cells through a reduction in IL-12p40 and the co-stimulator protein IL-1β in macrophages and monocytes (245). The increased IL-10 production may be responsible for the autocrine inhibition of TNF-α production, as observed in my results (245).

IL-12 is a strong inducer of IFN-γ from NK and $CD4^+$ T cells, which also plays a role in promoting functionality and maturation of $CD8^+$ T cells (246). I observed an increase in IL-12 production by all macrophage subsets in patients with chronic HCV at advanced liver fibrosis (Figure 16). For M1 macrophages, the increase in IL-12 was observed in the HCV-infected patients with both early and advanced fibrosis. Conventionally, IL-12 is expected to rise in the early stages of an infection as part of the activating immune response (247). At later stages, we expect to see increased IL-10, produced to inhibit and regulate IL-12 (247). In my results, increased levels of IL-12 is observed at both early and late stage of fibrosis among infected patients (Figure 16). However, the inhibitory IL-10 appears in the advanced stage of fibrosis only (Figure 15). It is possible that since the infection is persistent and chronic, the decline of IL-12 commonly observed during late stages of viral infection and clearance may not be detectable.

My results show an impaired or dysregulated differentiation of macrophage subsets in terms of surface markers and cytokine production in chronic HCV infection. The possible

mechanism underlying the observed impairment remains unknown. *In vitro* studies have reported that certain viral proteins (particularly HCV core and NS3), alone or in combination, are able to trigger an increased production of TNF-α, IL-12, IL-10 with no impact on IL-6 levels in blood monocytes (248, 249). In fact, HCV core protein has been implicated in preventing complete differentiation of monocytes to M1 or M2 macrophages by modulating the STAT signaling pathway (248). Therefore, the possible role of viral proteins such as HCV core in mediating the observed dysfunction in monocyte-derived macrophages needs to be explored further.

In summary, my results suggest that both M1 and M2 macrophages are altered in chronic HCV infected patients. Most of the phenotypic alterations are observed in advanced stages of liver fibrosis. M1 macrophages appear to lose some of their M1-like characteristics by a decreased TNF-α production and increased IL-10 production. Although, the increased production of IL-12 by M1 macrophages indicates that they are able to produce both pro- and anti-inflammatory cytokines at this dysfunctional state. Similarly, M2 macrophages were also altered with a decrease in proportion of $CD206^+$ cells and an increase in IFN-γ, IL-10 and IL-12 production. The IFN-γ and IL-12 increase on all M2 family (M2a, M2b and M2c) suggest a gain of M1-like characteristics.

4.2 Role of M1 macrophages in the induction of $CD8^+$ T cell functions in normal, healthy donors

It is well established that macrophages, as APCs activate T cells by presenting antigen peptides (250-252). Conventionally, $CD8^+$ T cells activation requires cytokine-mediated

support from $CD4^+$ T helper cells (194, 253). In case of specialized APCs, such as DC, they can activate naïve $CD8^+$ T cells by a process known as cross-presentation (254). When these activated virus-specific $CD8^+$ T cells proliferate and travel to the site of infection, they encounter the infected cells and induce targeted cell lysis (254). Alternatively, activation of $CD4^+$ and $CD8^+$ T cells is also possible via a combination of cytokine stimulants independent of antigen activation (255, 256). To the best of my knowledge, my results suggested for the first time that $CD8^+$ T cells from normal, healthy donors can be activated upon culture with M1 macrophages in a cytokine- and antigen-independent manner. My preliminary observations suggested an increased in production of IFN-γ following culture of M1 macrophages with $CD8^+$T cells in culture supernatants (Figure 17A). Subsequently, I confirmed through intracellular IFN-γ staining that M1 and M2b macrophages induce the expression of IFN-γ on $CD8^+$ T cells (Figure 19). Furthermore, M1 macrophages induce a subset of $CD107a^+$ $CD8^+$ T cells when cultured with $CD8^+$ T cells (Figure 22). As previously discussed (in the introduction), CD107a is a marker of degranulation of cytolytic granules in T cells (257). These exocytosed granules released by the $CD8^+$ T cells are known to contain perforin, granzymes and serine esterases (134). Surprisingly, there was no difference in the percentage of $CD8^+$ T cells expressing perforin or the production of perforin after culture with M1 and other macrophage subsets (Figure 20 and 21). It was of interest to study the mechanism by which the M1 macrophages induce $CD8^+$ T cell activation in healthy controls. My results show that the induction of IFN-γ^+ $CD8^+$ T cells (Figure 23) and $CD107a^+$ $CD8^+$ T cells (Figure 24) following culture with M1 macrophages is contact-dependent. The precise mechanism by which macrophage-$CD8^+$ T cell contact causes IFN-γ and CD107a expression needs to be addressed further.

Since the degranulation marker CD107a is upregulated, it is expected that the cytolytic molecule- perforin would be increased as well. However, my results indicate no significant difference in perforin expression and production following culture of macrophage subsets and $CD8^+$ T cells. This may be attributed to a variety of reasons. Perforin and granzymes are generally found together within vesicles in activated $CD8^+$ T cells (135, 258). However, each vesicle may contain different combinations of granzymes with and without perforin (135, 258-261). It is possible that increased CD107a expression may be accompanied by a release in granzymes but not perforin. Alternatively, M1 macrophages may prime the $CD8^+$ T cells but require a second stimulating signal to release cytolytic molecules like perforin. Further investigations are needed to understand why IFN-γ and CD107a are upregulated but not perforin.

This induction of $CD8^+$ T cell activation is contact-dependent but cytokine- and antigen-independent. A somewhat similar antigen-independent induction of $CD8^+$ T cell proliferation by DC has been reported in murine models of lymphopenia (262). DC co-cultured with $CD8^+$ T cells resulted in T cell proliferation because of the self-peptides presented by the DC and the endogenously produced IL-15 (262). Whether similar presentation of self-peptides by M1 macrophages is involved in their induction of IFN-γ and CD107a in $CD8^+$ T cells should be investigated.

4.3 Role of M1 macrophage in modulating $CD8^+$ T cell function in chronic HCV infection

My results show that $CD8^+$ T cells from patients with chronic HCV infection (at early fibrosis stage) had higher percentages of baseline IFN-γ^+ $CD8^+$ T cells compared to healthy controls (Figure 25). These results suggested that similar to healthy controls, M1 macrophages may significantly enhance IFN-γ expressing $CD8^+$ T cells in chronic HCV infection. Surprisingly, myresults showed that M1 macrophages derived from HCV-infected patients at early fibrosis significantly reduced the proportion of IFN-γ^+ $CD8^+$ T cells following co-culture (Figure 26B).

It is not clear why M1 macrophages reduce the proportion of IFN-γ^+ $CD8^+$ T cells following co-culture in chronic HCV infection compared to their enhancing effect in normal, healthy donors. This suggests that M1 macrophages may have some negative regulatory effects on the development of the IFN-γ^+ $CD8^+$ T cells. For instance, there could be an increased expression of inhibitory ligands (such as PD-L1) on M1 macrophages in chronic HCV infection, resulting in contact-mediated exhaustion of the IFN-γ^+ $CD8^+$ T cells. There could also be a suppressive macrophage population that forms in chronic HCV infection. Therefore, exhaustion expression marker analysis needs to be performed. The immunoregulatory cytokines such as IL-10, TGF-β that are produced in M1 macrophages in chronic HCV infection may mediate the negative regulatory effects. Attempts need to be made to elucidate the involvement of various immunoregulatory cytokines in the inhibition of IFN-γ^+ $CD8^+$ T cells.

Limitations: An important limitation of the project is that the co-culture of M1 macrophages and $CD8^+$ T cells could not be repeated with cells derived from patients who had advanced liver fibrosis. Most of the phenotypic alterations observed in M1 macrophages were at advanced fibrosis stage but the impact of M1-$CD8^+$ T cell co culture on T cells was observed only at early fibrosis stage. I was unable to assess the impact of phenotypic

alterations at advanced fibrosis on $CD8^+$ T cell function.

The peripheral blood samples and subsequent PBMCs from the infected patients were severely limited in number for the culture experiments. Since the impact on $CD8^+$ T cells were observed M1 macrophages only in healthy donors, we focused out studies on M1 macrophages and their effect on $CD8^+$ T cells in chronic HCV infection. However, it is entirely possible that macrophage subsets other than M1 may also be impaired in HCV infection regarding their effect on $CD8^+$ T cells. Attempts should be made to assess the impact of other subsets on $CD8^+$ T cells. Moreover, I was not able to investigate if M1 macrophages in chronic HCV still increased the proportion of $CD107a^+$ $CD8^+$ T cells as it did with normal, healthy donors and its impact on $perforin^+$ $CD8^+$ T cells. Furthermore, I could not assess if the impact of M1 macrophages on $CD8^+$ T cells in chronic HCV infection remains solely contact-dependent as it was in normal, healthy donors. It is possible that M1 macrophages from chronic HCV infected patients impacted $CD8^+$ T cells in a contact-independent fashion or through soluble factors.

In terms of the clinical limitations of the study, the uninfected blood donors and the chronic HCV-infected patient groups are not age-matched. Although initially intended in the project design, my results reveal that the average age of HCV-infected patients who participated in the study was much higher than the average age of the uninfected, normal donors. Recruiting healthy donors in the same age range as the patients was not possible in my study.

This variation in age among the sample group and the control group may influence the findings. A study that compared the production of cytokine by immune cells among healthy, normal donors in various age groups found that T cells from elderly women (compared to younger women) were more likely to be positive for IFN-γ, IL-2, IL-4, IL-10, and IL-13 (263). They also reported that lower proportions of monocytes from these elderly

women were able to produce TNF-α and IL-6 (263). Further studies are needed to address this issue with age-matched donors between patients and control groups.

A large sample size is ideal in such studies that focus on human populations due to the high degree of genetic variation that exists in the population. Unfortunately, the rarity of the HCV^+ population who matched all the sample-inclusion criteria, prevented me from achieving the ideal sample size. As mentioned earlier, the recruitment criteria of these patients included: HCV infection for 6 months or more, being untreated for HCV at time of blood draw, being negative for HBV and HIV infections.

As a result of my small population using random-sampling method, the study findings may have failed to properly represent the genetic variation of the population in Ottawa that are living with chronic HCV infection. As indicated in Table 1 (Chapter 2), the sample patient population was overwhelmingly Caucasian white, which may not be accurate for the Ottawa population. This is another limitation of the project.

4.4 Summary, Conclusion & Future Directions

In summary, I have shown multiple alterations and dysfunctions of macrophages derived from HCV-infected patients. These observations suggest that M1 macrophages lost some M1-like characteristics while M2 macrophages appeared to gain some M1-like characteristics. I also investigated the impact of each macrophage subset on $CD8^+$ T cells in normal, healthy donors. Notably, M1 macrophages induced $CD8^+$ T cell functions such as degranulation and IFN-γ production, upon close cell-to-cell contact. This impact of M1 macrophages on $CD8^+$ T cells was reversed when the macrophages and T cells were derived from chronic HCV-infected patients. Expression of IFN-γ^+ $CD8^+$ T cells in chronic HCV infected patients was reduced upon contact with M1 macrophages when similar co-cultures

were established.

I have not investigated the molecular mechanism underlying various phenotypic alterations identified among macrophage subsets in chronic HCV infection. Whether monocytes from these patients were harbor a productive HCV infection needs to be investigated, since infection of non-hepatocytes by HCV is controversial. If indeed monocyte-derived macrophages allow viral replication, the possibility of viral proteins impacting $CD8^+$ T cell function upon culture with these macrophages may present a possible mechanism. It has been suggested that viral proteins may alter monocytes function {Tacke, 2011 #522}{Brady, 2003 #523}. By preventing the viral protein-induced dysfunction of monocytes, we may be able to prevent their subsequent dysfunction at the macrophage level as well. In the long-run, therapeutic attempts can be made to prevent the binding and impact of such viral proteins with monocytes, although more investigation would be needed in this aspect. My research only investigated three surface receptors and four cytokines produced by the macrophage subsets. Further research should be focused on observing a wide range of other receptors (such as CD80, sCD163, PD-1, MHC I and II), cytokines and chemokines (such as IL-1β, TGF-β, CCL1, CCL17, CCL22, and CCL24) produced by these macrophage subsets and investigate their alterations in early and advanced fibrosis levels in chronic HCV patients.

It is not clear how 'bulk' $CD8^+$ T cells would get activated by M1 macrophages in an environment where no exogenous cytokines or antigens were introduced. Conversely, why such functions would be altered when $CD8^+$ T cells from chronic HCV infected individuals

were cultured with M1 macrophages derived from HCV infected patients is not known. With regards to the induction of $CD8^+$ T cell functions in healthy donors upon close contact with M1 macrophages, more experiments need to be conducted to identify the molecules responsible for the contact-dependent activation. After identifying some target proteins on the surface of M1 macrophages, they can be blocked and the co-cultures repeated to assess if the impact on $CD8^+$ T cells is diminished. Apart from some of the functional markers of $CD8^+$ T cells which I assessed (such as IFN-γ, CD107a, and perforin), other experiments can be conducted to check for production of granzymes and T cell target lysis assays among others. These will add to the understanding of which functions of a $CD8^+$ T cell can be induced by M1 macrophages and to what extent it leads to functional lysis by the T cell. My research was only able to investigate the impact of M1 macrophages on $CD8^+$ T cell function in chronic HCV patients with early fibrosis. Therefore, future work can extend the study to look at patients with advanced fibrosis and the impact of other macrophage subsets from patients as well.

In recent years, the innate lymphoid cells (ILC) have been identified as source of effector cytokines which play an important role in inflammation {Spits, 2012 #524}. In a cancer model, they were able to suppress the function of anti-tumor T cells {Crome, 2017 #525}. In fact, the ILC1 subset has been implicated in mediating liver immune-tolerance during HCV infections {Liu, 2017 #526}. The inhibitory molecule NGK2A expressed by this subset of ILC was able to induce a cascade of suppression, eventually preventing potent $CD8^+$ T cell responses to a viral hepatitis in mice {Krueger, 2017 #527}. Based on these reports, it is promising to explore the role of ILC subsets in the immune dysfunction of bulk $CD8^+$ T cells as observed in chronic HCV infection as well.

Many therapeutic attempts have been made to circumvent the lack of a potent IFN-γ response in the livers of chronic HCV infected patients. For instance, gene transfer therapy in HCV-infected chimpanzees was carried out to increase intrahepatic IFN-γ mRNA (263). Although this impact was transient, an increased frequency of HCV-specific T cells was observed both in the circulation and in the liver (263). However, attempt must be made to induce IFN-γ from cellular sources such as $CD8^+$ T cells and NK cells for a consistent supply of this essential, antiviral cytokine. My findings regarding the ability of M1 macrophages to induce IFN-γ^+ $CD8^+$ T cells in healthy donors and the loss of this function in chronic HCV infection is significant with a view to gain a better understanding of the immunopathogenesis of chronic HCV infection.

Chapter 5: References

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