Understanding Immune Suppression in Patients with Chronic Hepatitis C Virus Infections

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ABSTRACT

Hepatitis C Virus (HCV) is a small RNA virus that progresses to chronicity in 50-80% of infected individuals. Direct-acting antivirals (DAAs) are revolutionary treatments for HCV with 90-98% cure rates. However, over time, chronic HCV infections can result in advanced liver disease, including cirrhosis. Patients with advanced fibrosis experience a poor response to vaccination, recurrent infections and increased risk for hepatocellular carcinoma (HCC). These outcomes are, in part, a consequence of immune dysfunction. Increased inhibitory receptor and Galectin-9 (GAL-9) expression is a possible mechanism promoting lymphocyte dysfunction.

In this study, blood samples were collected from chronic HCV patients with different degrees of liver fibrosis. I conducted a 13-parameter flow stain on the peripheral blood mononuclear cells (PBMC) of these patients. Next, I measured the expression of inhibitory receptors (PD-1, CTLA-4, LAG-3, TIGIT and TIM-3) and GAL-9 on bulk T cell and NK cells of 15 chronic HCV patients with no to moderate fibrosis (F0-F2) and 15 with advanced fibrosis (F3-F4). To analyze receptor co-expression, I employed t-distributed stochastic neighbor embedding (t-SNE) analysis to dimensionally reduce the multi-parametric data.

Notably, I found that F3-F4 patients had higher frequencies of >3 inhibitory receptor co-expression on NK cells. Moreover, t-SNE analysis of bulk T cells revealed that F3-F4 patients manifest a higher frequency of cells in the clusters with $CD25^{+}TIGIT^{med\text{-}hi}$ $CD4^{+}$ T cells and $PD\text{-}1^{med}LAG\text{-}3^{med\text{-}hi}GAL\text{-}9^{med\text{-}hi}$ $CD4^{+}$ T cells. t-SNE analysis of NK cells also showed that F3-F4 patients manifest a higher frequency of cells in the cluster with $CD25^{+}TIGIT^{med\text{-}hi}TIM\text{-}3^{med\text{-}hi}$ $CD56^{Dim}$ NK cells and $CCR7^{+}$ $PD\text{-}1^{med}LAG\text{-}3^{med\text{-}hi}GAL\text{-}9^{med\text{-}hi}$ $CD56^{Dim}$ NK cells. Lastly, the frequency of cells in these clusters was found to positively correlate with patient's extent of liver damage. In **conclusion**, I identified phenotypes of immune dysregulation that could explain the

increased susceptibility to infection and HCC in chronic HCV patients with advanced fibrosis. These phenotypes could identify targets for combinatorial checkpoint blockade therapy to potentially improve immune function in these patients.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
ALT	Alanine Aminotransferase
APC	Antigen Presenting Cell
AST	Aspartate Transaminase
Bat3	HLA-B Associated Transcript 3
CARD	Carbohydrate Recognizing Domains
CCL5	C-C Motif Chemokine Ligand 5
CD	Cluster of Differentiation
CEACAM-1	Carcinoembryonic Antigen Cell Adhesion Molecule
CM	Central Memory
CTL	Cytotoxic T Lymphocytes
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
DAA	Direct Acting Antiviral
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
eIF2	Eukaryotic Initiation Factor 2
EM	Effector Memory
EMRA	Terminal Effector
EphA2	Ephrin Receptor A2
ER	Endoplasmic Reticulum
FDA	Food & Drug Administration

FGL	Fibrinogen-Like Protein
FoxP3	Forkhead Box P3
FSC-A	Forward Scatter Area
GAL	Galectin
GATA-3	GATA Binding Protein 3
HBV	Hepatitis B virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HMGB1	High Mobility Group Protein B 1
HPRT1	Hypoxanthine-Guanine Phosphoribosyl Transferase 1
HPSC	Hematopoietic Stem Cells
HSC	Hepatic Stellate Cells
IFN	Interferon
IFNAR	IFN-α/β Receptor
IL	Interleukin
IQR	Interquartile Range
IRES	Internal Ribosome Entry site
IRF	Interferon Regulatory factor
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
ITIM	Immunoreceptor Tyrosine-Based Inhibitory Motif
ITT	Immunoreceptor Tail Tyrosine
KIR	Killer Cell Immunoglobin-like Receptor

KIRG1	Killer Cell Lectin-Like Receptor Subfamily G Member 1
kPa	Kilopascals
LAG-3	Lymphocyte-Activation Gene 3
LCMV	Lymphocytic Choriomeningitis Virus
LDL	Low Density Lipoprotein
LSECtin	Liver Sinusoidal Endothelial Cell Lectin
LSM	Liver Stiffness Measure
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
MSM	Men Who Have Sex with Men
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NF-κb	Nuclear Factor -κb
NK	Natural Killer
NKG	Natural Killer Group
NS	Non-Structural Protein
NTR	Non-Translated Region
p-NK	Primary NK
PAMP	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Cell Death Ligand 1
PEG-IFN	Pegylated-IFN

PI3K	Phosphoinositide 3-Kinase
PtdSer	Phosphatidylserines
PTK	Protein Tyrosine Kinases
PWID	People Who Inject Drugs
RIG-I	Retinoic Acid-Inducible Gene I
RNA	Ribonucleic Acid
SHIP1	SH2 Domain-Containing Inositol-5-Phosphatase 1
SSC-A	Side Scatter Area
STAT	Signal Transducer and Activator of Transcription
SVR	Sustained Virologic Response
T-bet	T-box expressed in T cells
t-SNE	t-Distributed Stochastic Neighbor Embedding
TCR	T cell Receptor
T_{effs}	T Effector Cells
TIGIT	T Cell Immunoreceptor with Ig and ITIM Domains
TILs	Tumour Infiltrating Lymphocytes
TIM-3	T Cell Immunoglobulin Mucin 3
TGF-β	Transforming Growth Factor-Beta
Th	T Helper
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TRAIL	Tumour Necrosis Factor Related Apoptosis Induced Ligand

T_{regs}	T Regulatory Cells
UTR	Untranslated Region
VLDL	Very Low-Density Lipoprotein
ZAP-70	Zeta-Chain-Associated Protein Kinase 70

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1. INTRODUCTION

1.1 Hepatitis C Virus (HCV) Infection.

Epidemiology. Hepatitis C Virus (HCV) is a blood-borne virus that has infected >185 million people and currently, over 71 million people are chronically infected[1, 2]. Although HCV is a worldwide pathogen, North Africa- Middle East, Central and East Asia are the regions with the highest prevalence with the virus affecting >3.5% of the region's populations[2]. The highest prevalence of HCV is among individuals aged 55-64 years old with prevalence higher in men[2]. In a national study conducted in 2011, it was found that ~ 0.6% of the Canadian population were chronically infected with HCV, but the diagnosis rate is on the decline with a decrease of 10.1 cases per 100,000 Canadians between 2005 and 2015[3]. Prior to the identification of HCV, it was described as a non-A, non-B hepatitis and the primary mode of transmission was through the transfusion of blood or blood-related products, but this risk has greatly reduced following the anti-HCV screening of blood donations[4].

People who inject drugs (PWID) are a high-risk group for HCV infection and ~2 million new HCV infections each year are due to unsafe injections[5]. Additionally, the seroprevalence of HCV among PWID was ~52.3% as of 2011[6]. Although the availability of antiviral therapy has likely decreased this seroprevalence, ~90% of individuals infected with HCV are unaware of their infection status[7, 8]. Another prominent risk group is men who have sex with men (MSM) with a prevalence of 1-7% among those who do not inject drugs, and 25-50% among those who have a history of drug injection[9]. Other risk groups of HCV infection are the prison population, as well as those who are infected with the human immunodeficiency virus (HIV)[1].

HCV Genotypes and Chronic Infection. There are currently 6 known HCV genotypes that differ in 30-35% of their genomes[4]. The type, success and duration of HCV treatment can be highly

dependent on the HCV genotype of an infected individual[10]. The most prevalent HCV genotype is genotype 1 and this accounts for 46.2% of HCV cases. Next is genotype 3 accounting for 30.1% of cases, genotype 2, 4 and 6 account for 22.8% of cases, while genotype 5 accounts for only <1% of cases[4].

New HCV infections are often asymptomatic, but if untreated, acute HCV progresses to chronic infection in 50-80% of infected individuals[11]. As previously stated, chronic HCV affects ~71 million people worldwide and it is the leading infectious cause of cirrhosis, hepatocellular carcinoma (HCC), liver transplantation, and liver-related deaths[2, 12, 13]. The chronic stage of HCV infections begins when a person has been infected for more than 6 months and patients with chronic HCV have a high risk of then developing liver cirrhosis[1]. Chronic HCV patients can produce up to 10^{12} viral particles a day[14].

Life Cycle. HCV is a single-stranded, hepatotropic enveloped ribonucleic acid (RNA) virus in the *Flaviviridae* family and, it was cloned in 1989 and originally identified as the cause of non-A, non-B hepatitis[15]. The virus is approximately 9600 nucleotides in length and exhibits extraordinary genetic diversity due to the high error rate of its RNA polymerase[16]. The high mutation rate of the virus contributes to its ability to successfully evade the host's immune response and develop drug resistance[16]. The HCV genome can be broken down into 4 major parts (**Fig.1**). The untranslated region (UTR)/non-translated region (NTR) at the 5' end of the RNA is 341 nucleotides long, folded into a pseudoknot and many stem loops[17-19]. The 5' UTR contains an internal ribosome entry site (IRES), and this allows for a cap-independent translation of the HCV genome utilizing both viral and host proteins[20]. Directly downstream of this 5' UTR is a continuous open reading frame ranging from 9024 to 9111 nucleotides depending on the HCV genotype[17]. The polyprotein generated from this reading frame encodes 4 structural and 6 non-structural proteins. The C

structural protein forms the viral nucleocapsid, which encloses and protects the viral genome during transmission[21]. The capsid is also responsible for lipid and RNA binding, as well as immune modulation[17, 22]. The E1 and E2 proteins are envelope glycoproteins that form a noncovalently linked heterodimer and surround the viral particle[17, 23]. The last structural protein is the viroporin, p7, downstream of the E2 sequence. This portion encodes a transmembrane small ion channel essential for viral particle assembly, envelopment and release[24, 25]. The non-structural proteins: NS2 and NS3 are viral proteases that cleave the single translated virus polyprotein and NS4A, which forms a stable complex with NS3. Additionally, NS4B plays a role in the modulation of the virus' RNA polymerase, NS5B, while NS5A is an important mediator of the virus' genome replication[26, 27].

HCV is present in the host as either free viral particles or associated with low-density lipoprotein (LDL)/very low-density lipoprotein (VLDL), which are called lipoviral particles[28-30]. The virus' infection cycle begins upon binding of the HCV to its target surface receptors. The exact receptor recognized by HCV on hepatocytes to allow for cellular entry has been an area of extensive debate. The most likely candidate has been identified to be cluster of differentiation (CD) 81 as it has been shown to directly interact with soluble E2 protein[31, 32]. Furthermore, antibody blocking studies showed that CD81 plays a critical role in HCV pH-dependent viral entry[33]. Even so, studies have also shown that CD81 does not interact with mature HCV virions, thus suggesting, binding of HCV to the host cell alters its membrane conformation, revealing the CD81 binding site[34, 35]. Binding to entry factors, epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) and recognition of CD81 allows for target cell entry via endocytosis in clathrin-coated vesicles[36, 37]. Subsequently, the genome is released into the cytoplasm after a decrease in pH fuses the viral membrane with the entry vesicle[38]. The viral genome is translated via a cap-independent

mechanism to produce a polyprotein of about 3000 amino acids in length[39]. This polyprotein is then processed using both cellular and viral proteins to yield the 10 HCV proteins mentioned above. The assembly and release of the virus are closely interwoven with the lipid metabolism of the host cell. Although the exact mechanism of HCV assembly and release is poorly understood, the viral proteins are known to accumulate in the endoplasmic reticulum (ER) lumen where the core is formed at the site of viral budding and the virions escape the cell through secretory pathways[18]. Given that humans and chimpanzees are the only known natural hosts of HCV, there is a certain challenge with developing both cell culture-based and animal-based models to study HCV chronicity and liver disease. Currently the most utilized small animal model to study HCV infection is a human-liver xenograft mouse model. Human hepatocytes are injected into immunodeficient mice with liver damage. Due to the high regeneration ability of the liver, the inserted hepatocytes are able to proliferate and integrate into the mouse liver, creating a human-liver chimeric mouse[40]. HCV replicons and HCV pseudo-particles are among the systems used to generate cell culture models to study the virus' infectious cycle[41, 42].

HCV Therapy. The first treatment approved by the Food & Drug Administration (FDA) for the treatment of chronic HCV infection was the interferon (IFN) alpha-2b (α-2b) monotherapy, which takes advantage of the body's natural defence mechanism against viral infections[43]. Type 1 IFNs; IFN-α and IFN-β, are a class of proteins produced by a wide range of cells, including hepatocytes[44]. IFN binding to cellular receptors both activates the transcription of IFN stimulated genes and suppresses the transcription of other genes. During a viral infection, the first immune reactions are initiated by the virus binding to pathogen-associated molecular patterns (PAMPs) receptor such as protein kinase R (PKR)[45]. PKR will recognize double-stranded viral RNA, dimerize and autophosphorylate to be activated[46]. This will subsequently lead to the arresting of

cap-dependent translation through the inactivation of eukaryotic initiation factor 2 (eIF2)[46]. Degradation of viral RNA by RNA specific adenosine deaminase, 2',5'- oligoadenylate synthetase (OAS), and ribonuclease L as well as alteration of cellular vesicle trafficking via a guanosine triphosphatase is among the cellular processes which culminate in shifting the cell into an anti-viral state[45]. Engagement of other PAMP receptors such as toll-like receptor (TLR) and retinoic acid-inducible gene I (RIG-I) leads to the downstream activation of transcription factors such as nuclear factor (NF)-κb and IFN regulatory factors (IRFs)[46]. These transcription factors control the expression of Type I IFNs, tumour necrosis factor (TNF), and other inflammatory cytokines[45].

IFN-α/β binds to IFN-α/β receptor (IFNAR) and stimulates the synthesis of IFN responsive genes, which ultimately leads to the activation of NK cells and $CD8^+$ T cells[45]. Although IFNs are produced in the host upon viral infection, HCV has evolved to disrupt their synthesis. NS3/4A cleaves TLR3 and RIG-I, and this hinders the activation of IRFs, thus, IFN synthesis cannot be initiated[45]. Therefore, by administering IFN therapy to the patient, the goal is to provide and/or supplement the immune system with the IFN it needs to initiate the anti-viral immune response[47].

The first generation of IFN treatment showed a 15-20% sustained virological response (SVR) rate, lack of HCV RNA in the blood 12 weeks after completing treatment, in patients with chronic HCV infections[48]. Moreover, it led to adverse side effects such as neutropenia, myalgia as well as asthenia, making it less than desirable[49, 50]. Although subsequent waves of IFN treatment were able to improve the SVR rate, (38% in patients treated with IFNα-2b and ribavirin and 54-56% in patients treated with either Pegylated-IFN (PEG-IFN)α-2a or PEG-IFNα-2b), the long duration of treatment (6 to 12 months) and significant side effects created a necessity for the development of better treatment options for chronic HCV.

The current therapies to treat chronic HCV are direct-acting antivirals (DAAs) treatments, molecules that target specific non-structural proteins of the virus resulting in the disrupting of viral replication and infection. In 2011, the first wave of DAAs were NS3/4A inhibitors (telaprevir and boceprevir) and, in combination with PEG-IFNα, they showed a 75% SVR rate in patients with genotype 1[51, 52]. However, these DAAs showed significant side effects, creating a need for a second wave DAAs which were introduced in 2014. These DAAs include NS5A inhibitors, NS5B inhibitors, as well as a new generation of protease inhibitors and they have shown a 90-98% SVR rate in all HCV genotypes[53-55].

The introduction of a single pill combining an NS5B inhibitor with a high barrier to resistance, sofosbuvir, and an NS5A inhibitor, ledipasvir, ushered in the era of well tolerated DAAs[56]. Sofosbuvir is a nucleotide prodrug which when active, is an analogue for the RNA nucleotide, uridine. The incorporation of this uridine analogue by NS5B, into the HCV RNA during genome replication, effectively stops the replication process[56]. By impeding this step of the HCV viral cycle, this drug can safely stop the infection. This sofosbuvir and ledipasvir combination is administered for 12 weeks to patients with very few adverse effects. The most common adverse effects include: nausea, fatigue and headaches[56]. This combination treatment is usually used for patients infected with HCV genotypes 1, 4, 5 and 6 with no cirrhosis or with compensated cirrhosis[56, 57].

Glecaprevir and pibrentasvir can also be administered to all patients infected with HCV regardless of the genotype for a shorter treatment course of 8 weeks[57]. Other DAA treatments include elbasvir and grazoprevir as well as sofosbuvir and velpatasvir[57]. Special considerations are made for patients with decompensated cirrhosis and for those who have previously failed treatment. For patients with decompensated cirrhosis who are ribavirin ineligible, sofosbuvir with

either ledipasvir or velpatasvir is recommended for 24 weeks. For those who have failed prior treatment with sofosbuvir, this treatment is recommended in combination with ribavirin[57]. Additionally, HCV co-infection with HIV should not impact the prescription of DAA therapy[58].

Amazingly, this new era of DAAs has seen a decline in the morbidity and mortality from HCV-related liver disease. In Australia, there has been a decline in the diagnosis of HCV-related decompensated cirrhosis[59]. Despite the effectiveness of DAAs, a 2018 survey found that only 38% of Canadian infectious diseases physicians prescribed DAAs for the treatment of HCV[60]. Furthermore, 45% were not interested in prescribing DAAs within the next 12 months. This study found that physicians not prescribing DAAs felt they were not sufficiently trained for this[60]. This will most likely change the longer DAAs are around and become more readily available. Another prominent barrier to HCV treatment with DAAs is cost. In 2019, the average cost of DAAs was USD 41,046 per treatment[61]. This shows that there is still work to be done before DAA therapy is truly accessible.

HCV Vaccine. The main focus on HCV prevention thus far has been through educating individuals, specifically members of the HCV high-risk groups. However, the road to developing an HCV vaccine for both treatment and prophylactic measures remains a challenge. In addition to the high mutation rate of the virus, there is large genetic diversity across the 6 HCV genotypes thus, developing a single HCV vaccine is difficult[62]. Targeted delivery of the vaccine based on the incidence of HCV genotypes worldwide is likely the most effective strategy[62].

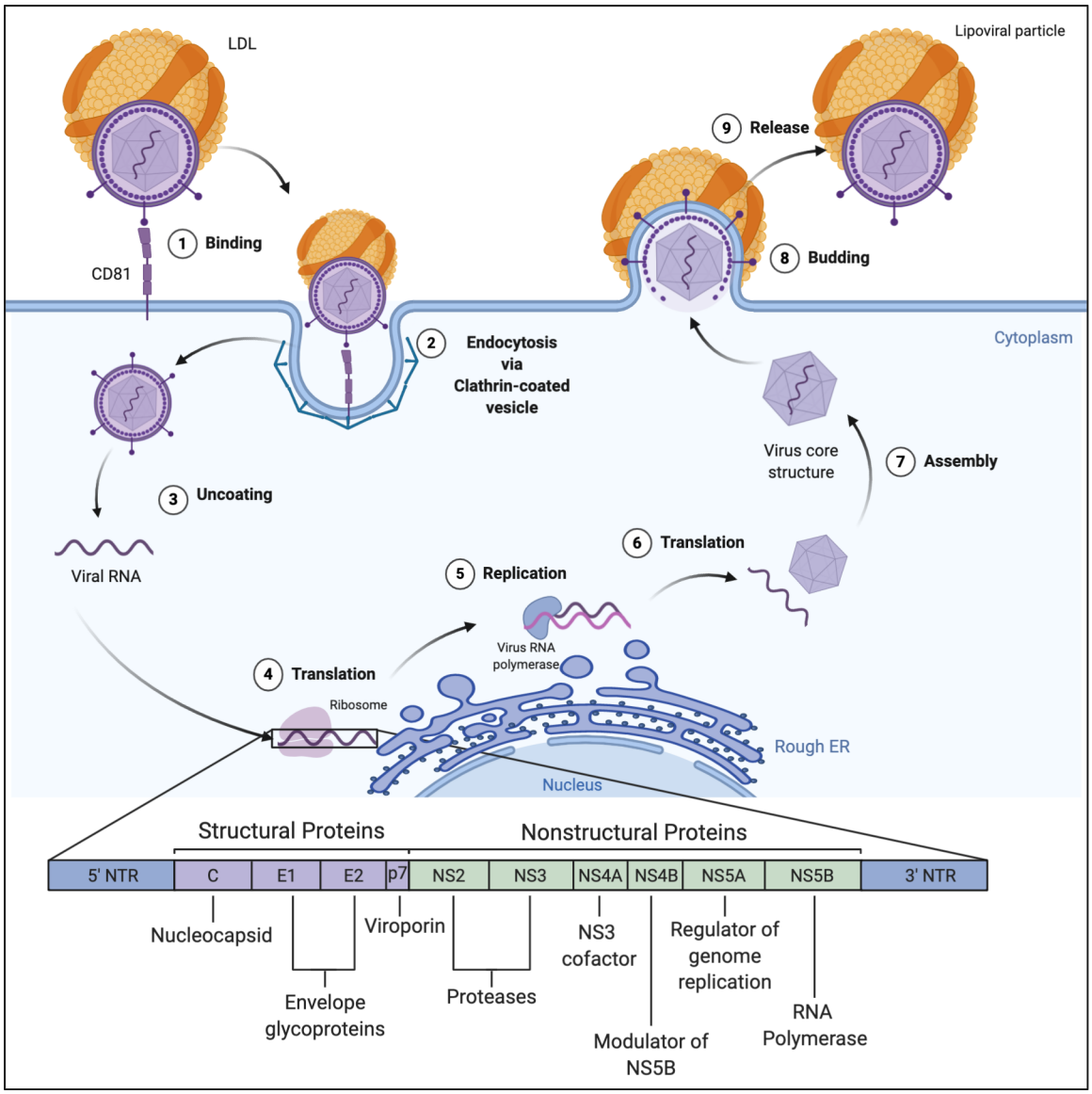

LDL
Lipoviral particle
9 Release
1 Binding
CD81
8 Budding
Cytoplasm
2 Endocytosis via Clathrin-coated vesicle
Virus core structure
7 Assembly
3 Uncoating
Viral RNA
6 Translation
5 Replication
Virus RNA polymerase
4 Translation
Ribosome
Rough ER
Nucleus
Structural Proteins
Nonstructural Proteins
5' NTR
C
E1
E2
p7
NS2
NS3
NS4A
NS4B
NS5A
NS5B
3' NTR
Nucleocapsid
Envelope glycoproteins
Viroporin
Proteases
NS3 cofactor
Modulator of NS5B
Regulator of genome replication
RNA Polymerase

Figure 1. Schematic of the HCV life cycle. HCV is a single-stranded, hepatotropic enveloped RNA virus in the *Flaviviridae* family that primarily infects hepatocytes. It can be present in the host as either free viral particles or associated with low-density lipoprotein (LDL). This figure was generated using Biorender.

1.2 Liver Fibrosis and Hepatocellular Carcinoma (HCC).

Epidemiology. Chronic HCV is the leading infectious cause of cirrhosis, hepatocellular carcinoma (HCC), liver transplantation, and liver-related deaths[12, 13]. Each year, 390,000 people die from HCV-related liver complications[1]. During chronic HCV infections, hepatic inflammation triggers wound-healing, and continuous tissue regeneration leads to the accumulation of scar tissue, and therefore liver fibrosis[63]. Extensive liver fibrosis leads to cirrhosis and this accounts for a majority of the HCV-related morbidities and mortalities[64]. About 20% of those with chronic HCV will develop liver cirrhosis over a 20-year period, and the risk of developing HCC once at the cirrhotic stage is 3-4% per year[64, 65] (**Fig. 2**). HCV infections are responsible for half of all HCC cases, and cirrhosis due to HCV infection accounts for 35-40% of liver transplantation in the United States[64, 66].

Fibrosis Development. One of the major drivers of liver fibrosis, is the inflammation triggered by HCV replication and spread. Inflammation is a protective mechanism by the immune system in response to harmful stimuli such as damaged cells or a viral infection[67]. Although inflammation plays an essential and beneficial role during viral infections, chronic HCV infection results in liver fibrosis through hepatocellular damage and continual inflammation. Chronic HCV infection causes an increase in the production of pro-inflammatory cytokines such as Interleukin (IL)-1β and IL-18 by hepatocytes, liver resident macrophages (Kupffer cells) and other liver-resident immune cells[68, 69]. Transforming growth factor-beta (TGF-β) is a profibrogenic cytokine produced by HCV infected cells, and its production activates quiescent hepatic stellate cells (HSCs) (**Fig. 2**)[70, 71]. Activated HSCs differentiate into myofibroblasts, and they are the major producers of the extracellular matrix (ECM) which are deposited in the liver to repair the damaged tissue[72].

Continual scar tissue accumulation causes chronic HCV patients to develop cirrhosis, portal hypertension, and HCC[70, 71].

MicroRNAs (miRNAs) have also been shown to play a role in fibrosis development during chronic HCV infections. miRNAs are short non-coding RNAs that play a role in post-transcriptional regulation of gene expression through messenger RNA (mRNA) degradation, or translational suppression by binding to the mRNAs[73]. Expression of miRNA-27a and miRNA-7b in HSCs have been shown to induce their proliferation by inhibiting the action of retinoid X receptors, which are essential to inhibit HSC activation. HSCs with a downregulation of these two miRNAs were shown to exhibit decreased proliferation, and were restored to their quiescent state[74]. On the other hand, upregulation of miRNA-29 expression in HSC has been shown to reduce their proliferation and suppress their collagen production[75]. Furthermore, miRNA-29 expression has been found to be significantly lower in patients with chronic HCV[69].

Fibrosis Scoring. In clinical practice, various scoring systems have been used to classify the different stages of liver fibrosis in chronic HCV patients with one of the most widely used ones being the METAVIR scoring system[69, 76, 77]. In the METAVIR scoring system, F0 is when there is no fibrosis in the liver and is typically the starting point of patients with chronic HCV; F1 is portal fibrosis without septa/bridges, and this is mild fibrosis; F2 is periportal fibrosis with few bridges, and the fibrosis has spread beyond the area containing the blood vessels. This stage denotes the point considered to be moderate fibrosis[78]. F3 is portal central fibrosis where there are now numerous bridges to other areas with fibrosis, and this is known as severe fibrosis[69]. Lastly, the F4 stage is cirrhosis, and clinically, it is further divided into compensated liver disease when patients show no liver complications and decompensated liver disease when patients begin to develop liver complications[69]. F3 and F4 are classified as the advanced stages of fibrosis, and at these stages,

patients with chronic HCV begin to experience symptoms of immune dysregulation such as a poor response to vaccination, recurrent infections and an increased risk of developing HCC[79-81].

Determination of fibrosis stages can be achieved through both invasive and non-invasive techniques. The gold standard to determine the fibrosis stage of patients with chronic HCV is the evaluation of patient liver biopsies[82]. However, this method has significant pitfalls: (1) obtaining liver biopsies are both invasive and costly, (2) the biopsy obtained is only a small section of the liver and thus might not be an accurate description of the state of the entire liver, (3) the evaluation of the liver biopsy to determine the fibrosis stage is carried out by a pathologist and thus their level of expertise could impact the accuracy of the results, and (4) there is a risk of pain and bleeding[69, 83].

Fibroscan (transient elastography) is a non-invasive test used to determine the extent of liver fibrosis in patients with chronic HCV infection. These machines work by passing a 50-MHz wave, via a transducer, through the liver and subsequently measuring the shear wave velocity. This velocity is then converted into a liver stiffness measure (LSM) which is expressed in kilopascals (kPa)[83]. It is a quick and cheap test, and to date, has not been associated with any side effects. The Fibroscan is performed on the right lobe of the liver in the intercostal position and takes about 5-10 minutes[84]. To have the most accurate value, 5-10 different measurements are taken and the outliers are removed before an average is calculated.

Although a Fibroscan has its merits, a major drawback is its inability to be used for all patients. Specifically, this test is highly inaccurate in individuals with a large amount of chest wall fat and those who are morbidly obese[83]. Also, shear waves do not propagate through fluids, thus, obtaining an accurate LSM of patients with fluid buildup in their abdomen (ascites) is difficult[84]. The cutoff Fibroscan values that dictate the METAVIR classification of patients depend on their

type of liver disease. The METAVIR stages based on Fibroscan ranges for patients with chronic HCV infections are ≤7 kPa for F0-F1, 7kPa - 9kPa for F2, 9kPa - 12.5kPa for F3 and >12.5kPa for F4 patients[85]. For patients with non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH), a LSM of ≤7 kPa denotes F0-F1, 7kPa - 10kPa for F2, 10kPa - 14kPa for F3 and >14kPa for F4 patients[86]. For patients with cholestatic liver disease, the F4 cutoff starts at 17kPa[86]. This is most likely due to the edema and inflammation seen in these patients from obstructed bile flow[87]. Thus, although Fibroscan measures are beneficial to patients with liver disease, its readings might be misleading in certain patient populations.

Hepatocellular Carcinoma (HCC). Liver cancer or HCC is the fifth most common cancer and the second leading cause of cancer-related deaths in the world[88]. Individuals infected with HCV have a 15-20 fold higher chance of developing HCC, and over the past decade, deaths from HCC caused by HCV infections increased by 21.1%[89]. As previously mentioned, 3-4% of cirrhotic HCV patients develops HCC every year. The factors that pose an increased risk of developing HCC in patients with chronic HCV infection include the extent of liver damage, patient lifestyle, obesity, HCV genotype, and the presence of any other liver disease[90]. The progression from an HCV infection to the development of HCC can take 20-40 years, with both viral and host factors contributing to its development[91].

A viral factor that can play a role in HCC development is the HCV core, which inhibits the expression of the retinoblastoma protein and p53 protein, which are tumour suppressors[92]. The loss of these proteins is known to lead to increased carcinogenesis and tumour development. Various HCV factors can also promote the progression of liver fibrosis, and this creates an increased risk of HCC development. In terms of host factors, the most significant cause of HCC development is the immune response to HCV infection. Infection with HCV leads to cell damage, and thus the

host goes through a repair process through cell division. This continuous division process requires cells to undergo the cell cycle, and this continual cycle progression creates an increased risk of introducing carcinogenic mutations. Among the most common genetic mutations targets which have been known to contribute to HCC development is p53 protein which, as previously stated, is a tumour suppressor, β-catenin, which is a transcription factor of oncogenes and has also been shown to suppress T cell responses, and telomerase reverse transcriptase which is responsible for the maintenance of the telomerase to prevent oxidative stress in cells[90, 93, 94].

Effect of HCV Therapy on Fibrosis Progression and HCC Development. The balance between the production of new ECM, fibrogenesis, and clearing of old scar tissue, fibrolysis, is finely maintained to ensure optimal wound healing ability in the liver[69]. However, during constant inflammation, it is difficult for the body to maintain this balance, and this is, in effect, what causes scar tissue buildup in the liver leading to fibrosis. The issue of potential fibrosis reversal post viral clearance is an important question currently being investigated. The most significant/main predictor of fibrosis reversal after SVR, is the patient's extent of liver damage. Patients with advanced liver fibrosis, particularly decompensated cirrhosis, have both a lower chance of achieving SVR, and a lower chance of fibrosis reversal post SVR[63].

The risk of HCC occurrence is significantly reduced after patients are cured from HCV and this is true for both IFN and DAA therapy routes. Initially, it was thought that DAA treatment might have a bigger impact on preventing HCC development than IFN therapy but ultimately, studies have found that there is no significant difference in risk reduction depending on the type of cure received[95-99]. Although the risk of HCC occurrence was reduced in patients post DAA treatment, the risk remained high in cirrhotic patients[100, 101]. Due to this high risk of HCC development in chronic HCV patients with advanced liver fibrosis, it is crucial to understand not

only the structural changes in the liver of patients that contribute to the development of cancer, but also the deficiency in the immune system which loses its cancer surveillance function.

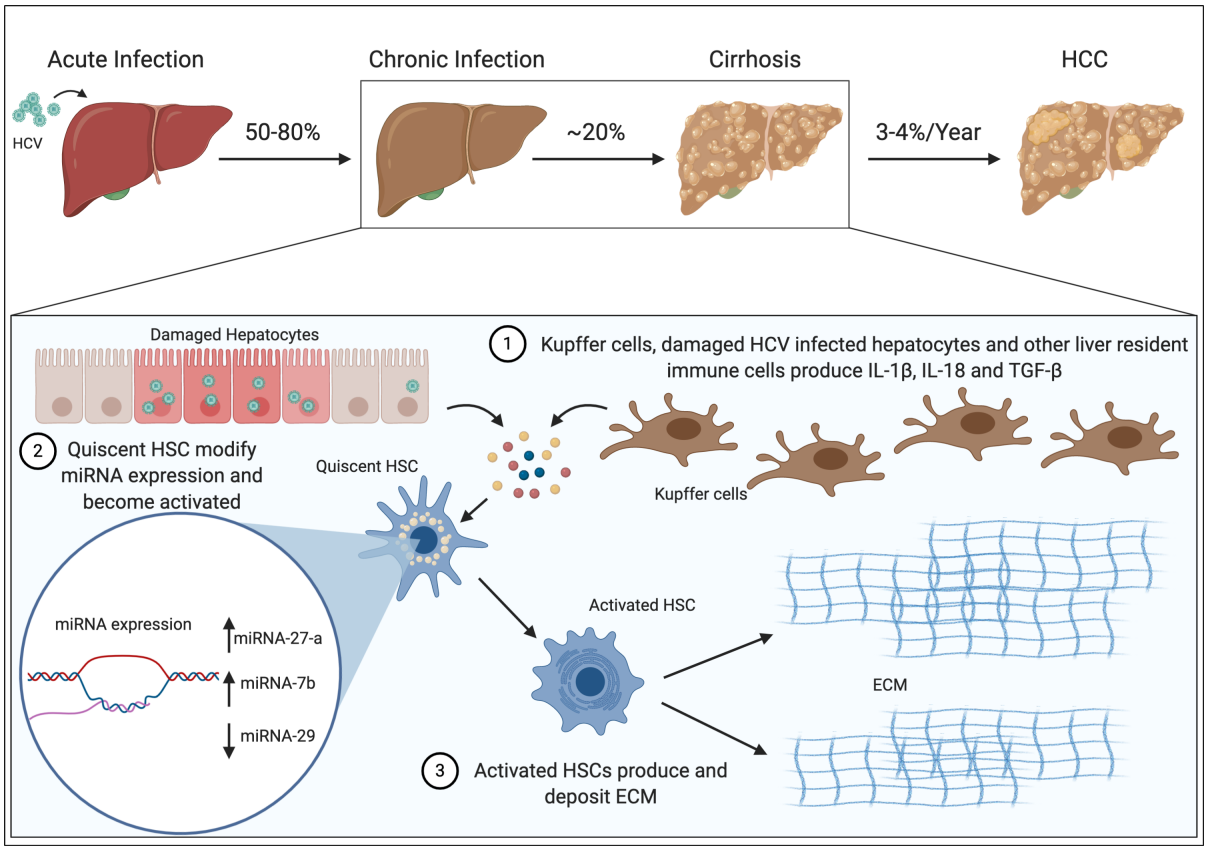

Acute Infection
Chronic Infection
Cirrhosis
HCC
HCV
50-80%
~20%
3-4%/Year
Damaged Hepatocytes
1 Kupffer cells, damaged HCV infected hepatocytes and other liver resident immune cells produce IL-1β, IL-18 and TGF-β
2 Quiscent HSC modify miRNA expression and become activated
Quiscent HSC
Kupffer cells
miRNA expression
miRNA-27-a
miRNA-7b
miRNA-29
Activated HSC
ECM
3 Activated HSCs produce and deposit ECM

Figure 2. The development of fibrosis in chronic HCV patients. Upon HCV infection, 50-80% of individuals will progress to the chronic stage. Chronic infection with HCV leads to liver damage and inflammation which activates quiescent hepatic stellate cells (HSC). Activated HSC will deposit extracellular matrix (ECM) and ECM buildup leads to fibrosis. Extensive fibrosis buildup causes ~20% of chronic HCV patients to develop cirrhosis and subsequently, 3-4% of cirrhotic patients per year develop hepatocellular carcinoma (HCC). This figure was generated using Biorender.

1.3 NK and T Lymphocytes.

Natural Killer (NK) Cell Development. Lymphocytes play a crucial role in anti-viral responses and cancer surveillance at both the innate and adaptive immune levels. NK cells are innate immune cells vital to defense against tumours and virus-infected cells. Unlike T cells, NK cells have the ability to respond quickly during an infection. Their development occurs after the differentiation of hematopoietic stem cells (HPSCs) in the bone marrow and secondary lymphoid tissues such as the lymph nodes[102]. Human NK cells begin to express a very high level of CD56 to become $CD56^{Bright}$, and then downregulate CD56 expression and begin to express CD16 to become $CD56^{Dim}$ and $CD16^{+}$ [103]. $CD56^{Bright}$ NK cells are considered a less mature NK cell subset and they make up only about 5% of the NK cell population[104]. While this subset of NK cells primarily resides in secondary lymphoid tissues, $CD56^{Dim}$, the majority, are mainly found in the peripheral blood. This development of NK cells from $CD56^{Bright}$ to $CD56^{Dim}$ creates a key shift in function. While $CD56^{Bright}$ NK cells are mainly responsible for the production of inflammatory cytokines during an immune response, maturation to $CD56^{Dim}$ cells comes with the acquisition of cytolytic capabilities[105].

NK cell Recognition and Function. NK cells can differentiate between healthy and unhealthy cells, and recognize abnormal or infected cells in order to efficiently target and kill tumours or virus-infected cells. They do not require any prior activation[106]. The induction of an NK cell killing a target cell is based on the balance of signals received from both its activating receptors such as, natural killer group (NKG) 2C, NKG2D and natural cytotoxicity receptors (NCRs); and its inhibitory receptors, such as NKG2A, killer cell immunoglobin-like receptors (KIRs) and killer cell lectin-like receptor subfamily G member 1 (KIRG1)[106]. The three mechanisms of NK cells target cell recognition are immunological-self, missing-self and induced-self[103]. When NK cells

are interacting with their target cell, their activating receptors will recognize activating ligands on the target cell while their inhibitory receptors will recognize inhibitory ligands, such as major histocompatibility complex (MHC) class I.

In the immunological-self mechanism, since the target cell is expressing MHC class I, the NK cells receive inhibitory signals, preventing its activation and target cell death. In most cases, these cells are healthy cells (**Fig. 3**). In the missing-self mechanism, the cell lacks or has severely downregulated the expression of MHC class I on its surface, as is the case for virus-infected cells to prevent antigen presentation to $CD8^+$ T cells. However, by downregulating MHC class I, NK cells now lack an inhibitory signal when interacting with the target cell, and are only receiving activating signals leading to target cell killing. Lastly, in the induced-self mechanism, the target cells begin to heavily express stress-induced ligands, as is the case in cancerous cells which still express MHC class I. In this case, the balance of the inhibitory to activating signals has shifted to increase the activation signal. Thus, the NK cells are activated to kill the target cancer cell.

After recognition of infected and cancerous cells, NK cells are able to proliferate and perform their effector function by releasing cytotoxic granules, producing cytokines, and inducing target cell apoptosis via tumour necrosis factor-related apoptosis-induced ligand (TRAIL) expression[107-110]. Specifically, during degranulation, NK cells release perforin, which forms pores in the target cell, and then release granzymes. Granzymes are proteases which, upon entry into the target cell, will cleave caspases to induce apoptosis[111]. During infections, NK cells also produce a wide range of cytokines and chemokines such as IFN-γ, TNF-α, IL-5, IL-10, IL-13 and IL-8[112]. Inflammatory cytokines such as IFN-γ and TNF-α, are vital for anti-tumour and anti-viral responses as they are able to act on macrophages and dendritic cells to enhance the immune response[113]. IL-10, in the presence of IL-18, is also able to induce NK cell proliferation,

cytotoxicity and IFN-γ production[114]. Lastly, during infections, NK cells can induce the death of the target cell by upregulating their surface expression of TRAIL. TRAIL expressed on the surface of NK cells can bind to TRAIL-receptors on the target cell, and this can lead to the activation of caspase-8, which activates caspase-3 to induce apoptosis[115, 116].

Although NK cells make up 5-15% of the peripheral blood mononuclear cells (PBMCs), they make up 30-50% of liver-resident lymphocytes[110]. This disproportionate representation of NK cells in the liver compared to the peripheral blood suggests an important role of NK cells during HCV infections. As previously mentioned, NK cells are vital for the host's anti-viral response, and NK cells are activated early on during an HCV infection. Strong NK cell responses have been shown to prevent acute infection in healthcare workers accidentally exposed to HCV[117]. Also, spontaneous clearance of HCV is associated with NK cells having stronger cytotoxicity, stronger IFN-γ secretion and higher expression of the activating receptor, NKG2D[117-119]. NK cells have also been shown to aid in the prevention of liver fibrosis, where intrahepatic NK cells with high expression of the activating receptor NKp46 can induce apoptosis of activated HSC, thus preventing liver fibrosis[120].

Despite the importance of NK cells in HCV viral clearance, HCV can interfere with adequate NK cell function, and this could explain the persistence of HCV infection leading to chronicity. During chronic HCV, NK cells in the peripheral blood produce IL-10 and given its immunosuppressive function, the body is unable to sustain an immune response to clear the virus. Furthermore, liver-resident NK cells have reduced degranulation and expression of TRAIL[121]. Lastly, liver-resident NK cells can aid in the development of liver fibrosis by killing infected hepatocytes, which induces inflammation.

T Cell Development and Signal Transduction. T lymphocytes are adaptive immune cells which are activated upon antigen recognition. T cells express T cell receptors (TCRs), responsible for the recognition of antigens presented on MHC class I/II of cells, and either CD4 or CD8, proteins which also associate with MHC proteins on cells[122]. Similar to NK cells, T cells arise from HPSCs and differentiate into pre-T cells in the bone marrow[103]. Pre-T cells then migrate to the thymus and, after further differentiation, begins to express both CD4 and CD8 receptors (double-positive)[123]. During T cell development, a large variation of TCRs, created by recombination of the TCR gene segments, are expressed on naïve cells (cells which have never encountered their corresponding antigen)[124]. These cells subsequently undergo selection to delete autoreactive T cells. In the final stage of development, the double-positive T lymphocytes lose the expression of either CD4 or CD8 to become a single positive $CD8^+$ T cell or a $CD4^+$ T cell. When T cells are first introduced into circulation, they are naive cells, unable to perform their immune function. However, once they encounter an antigen-presenting cell (APC) bearing an antigen-specific to their TCR, these naïve cells are activated to become either effector cells that will perform the immune function, or memory cells that will provide immunological memory, providing a better response to another challenge by the same pathogen[122].

Activation of T cells requires three signals with the first being the recognition by TCR of its specific antigen. Human T cells express the protein CD3, which is essential for the signal transduction leading to their activation. CD3 is comprised of 4 chains, which are non-covalently associated with the TCRs to form the TCR complex[125]. Recognition of an antigen leads to a conformational change in the TCR, which causes the activation of protein tyrosine kinases (PTKs). This activation leads to the phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) domains on CD3, which recruits zeta-chain-associated protein kinase 70 (ZAP-70)[125].

Recruitment of ZAP-70 will further activate the signal cascade, which ultimately results in T cell activation. The second signal needed for T cell activation is the binding of the co-stimulatory receptors on T cells with their ligands on the APCs. The co-stimulatory receptor, CD28, plays a major role in the T cell activation, as it binds to CD80 or CD86 on APCs, amplifying the TCR signal to cause cell proliferation and IL-2 production[126, 127]. The final signal for T cell activation is the binding of inflammatory cytokines produced by the APC. The binding of these cytokines facilitates the differentiation and proliferation of the naïve T cells[128].

$CD8^+$ T cells or Cytotoxic T lymphocytes (CTLs). CTLs play a role in the adaptive immune system and are important for the defence against intracellular pathogens (such as viruses) and tumour immunosurveillance. They express the heterodimer CD8 co-receptor, which recognizes the α3 region of MHC class I molecules present on all nucleated cells[129]. Naïve CTLs are activated in the secondary lymph nodes by the recognition of an antigen that is specific to their TCR[130]. APC expressing a viral or tumour antigen on its MHC class I, will associate with the TCR and CD8 on CTLs. After their activation, the antigen-specific CTLs proliferate, and effector CTLs migrate to the site of infection and begin to perform their effector function (**Fig. 3**). CTLs are able to kill their target cells through the release of cytotoxic granules, perforin and granzyme, similar to NK cells[131]. Other than directly killing the target cells, CTLs produce pro-inflammatory cytokines such as IFN-γ and TNF-α which help to further intensify the immune response[130].

$CD8^+$ T cells also have a regulatory role at the peak of the immune response. They were found to be the major producers of IL-10, an immunosuppressive cytokine, at the site of infection[130]. This IL-10 production by $CD8^+$ T cells is distinct from the IL-10 production by $CD4^+$ T cells, which occurs at the end of the infection. Although IL-10 production by CTLs is aimed at preventing immunopathology, it also does not disrupt the immune response to the infection. Lastly,

CTLs play a key role in the elimination of other immune cells at the end of the immune response and this is mediated through the Fas/FasL pathway, acting as another mechanism CTLs use to kill their target[130]. CTLs can express Fas Ligand (FasL) on their surface, which will then bind to its ligand, Fas, expressed on the target cell. This recognition of Fas leads to the induction of signalling molecules that activate the caspase cascade causing apoptosis[130, 132].

During HCV infections, HCV specific CTLs become detectable in the liver at about 8-12 weeks after infection[133]. This extended time was found to be due to delayed priming of CTLs following HCV infection. Compared to other viral infections, this is a significantly lagged response time. The study of HCV kinetics in chimpanzees found that the speed of CTL migration to the liver is associated with the development of chronic infection thus, the delay in CTL response could explain the high rate of chronic infection seen in HCV[134]. Similar to NK cells, spontaneous clearance of HCV is associated with a robust HCV-specific CTL response to multiple HCV epitopes in patients[135]. Although in the acute phase of HCV infection, CTLs show a strong response to the virus, the host is plagued with continual mutation of the viral epitopes. CTLs are unable to keep up with the continual mutation and become overwhelmed, leading to viral persistence and thus chronicity[136, 137]. While CTLs are essential for an adequate immune response, they can induce an excessive immune response, and this contributes to immunopathology. During chronic HCV infections, excessive CTL response contributes to the continuous hepatic inflammation and this, as previously stated, ultimately leads to liver fibrosis.

$CD4^+$ T cells. $CD4^+$ T cells act to maintain and boost immune cell functions, including those of CTLs, in order to orchestrate an effective response to infections. They do this by performing 4 major functions: (1) directly communicating with B cells to promote antibody production, (2) communicating with macrophages to enhance their function, (3) helping recruit eosinophils and

basophils to the site of infection, and (4) producing cytokines and chemokines which will further activate CTLs and NK cells and improve their functions[138]. $CD4^+$ T cells develop as 3 distinct lineages, namely, natural T regulatory cells (nT_{regs}), NKT cells and naïve $CD4^+$ T cells (Th0 cells)[138]. Th0 cells, depending on the signals received during the antigen presentation/activation step, further differentiates into 4 subpopulations. They are T helper (Th) 1, Th2, Th17 and induced Tregs (iT_{regs}), and these populations go on to perform different functions.

Th1 cells are important for the host's immunity against intracellular micro-organisms, and their differentiation and function are controlled by the transcription factors T-bet (T-box expressed in T cells) and STAT-4 (signal transducer and activator of transcription 4) respectively[139-141]. These cells produce; IL-2, important for memory formation in both $CD4^+$ and $CD8^+$ T cell, IFN-γ, activates macrophages to increase their response, as well as several other cytokines[142, 143]. Th2 cells, on the other hand, are implicated in the immune response to large extracellular pathogens and their differentiation and function are controlled by the transcription factors GATA-3 (GATA binding protein 3) and STAT-6[139]. Some of the major cytokines produced by Th2 cells are IL-4, which regulates B cell differentiation and proliferation, IL-5, which recruits eosinophils and IL-25, which amplifies Th2 responses[138, 144-146]. IL-25 can also induce the production of chemokines such as CCL5 (C-C motif chemokine ligand 5) to recruit other immune cells to the infection site.

Th17 cells are the most recently identified population and in addition to their essential role in the immune response against pathogens such as bacteria and fungi, they are also involved in autoimmunity[138, 147]. Their expansion is promoted by IL-23, and they produce IL-17A and IL-21 among other cytokines. IL-17A is crucial in inducing inflammatory responses by promoting the release of inflammatory cytokines, while IL-21 acts on CTLs, B cells, NK cells and dendritic cells to modulate their proliferation, apoptosis and differentiation (**Fig. 3**)[148, 149]. T_{regs} are $CD4^+$ T cells

with constitutive expression of CD25, IL-2 receptor α (IL-2Rα), and the Forkhead Box P3 (FoxP3) transcription factor[138]. They maintain immune homeostasis and tolerance by regulating and suppressing the activity of other immune cells to prevent immunopathology and autoimmunity[150]. These cells produce TGF-β and IL-10, cytokines known to play an immunosuppressive role (**Fig. 3**)[151].

During acute HCV infections, $CD4^+$ T cells and their IL-21 production is essential for CTL mediated HCV clearance[152, 153]. Unsurprisingly, spontaneous clearance of HCV is linked to a robust $CD4^+$ T cell response compared to patients that progressed to the chronic stage[154]. Typically, during a viral infection, virus-specific $CD4^+$ T cells mostly differentiate into the Th1 population, this is not necessarily true in HCV infections. A study found that although HCV-specific Th1 cells are produced during acute HCV infection, they rapidly decline after the infection progresses to the chronic stage[155]. These cells become barely detectable during chronic infection, and the depletion of these cells could play a role in the CTL dysfunction seen in patients with chronic HCV infections. With regards to T_{regs}, the number and functionality of bulk T_{regs} has been shown to increase during chronic HCV infection[156]. They have also been shown to accumulate in the liver and suppress HCV specific T cell proliferation and function[157, 158].

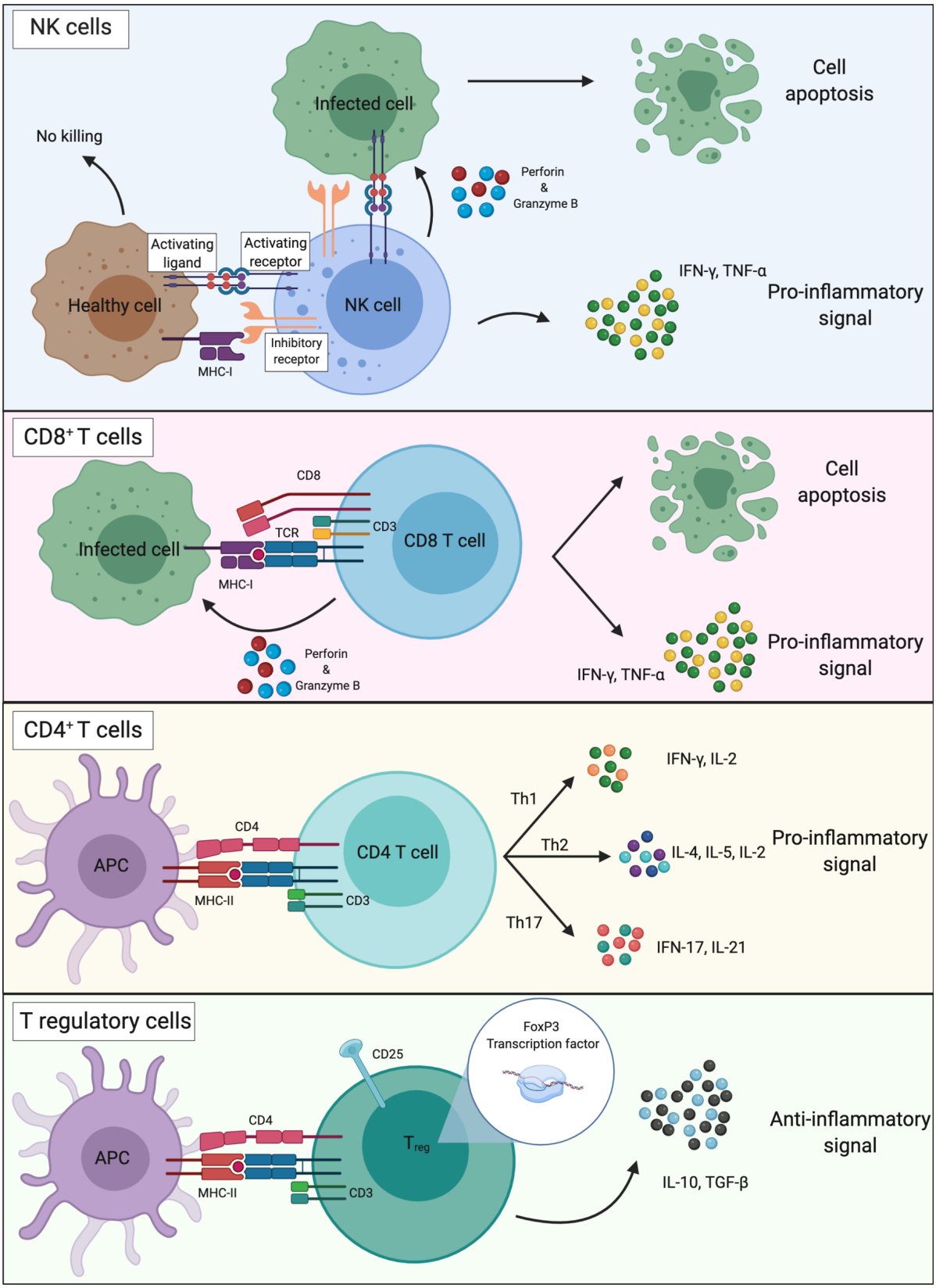

NK cells
Infected cell
Cell apoptosis
No killing
Perforin & Granzyme B
Activating ligand
Activating receptor
Healthy cell
NK cell
IFN-γ, TNF-α
Pro-inflammatory signal
Inhibitory receptor
MHC-I
CD8+ T cells
CD8
CD3
TCR
CD8 T cell
Infected cell
MHC-I
Cell apoptosis
Perforin & Granzyme B
IFN-γ, TNF-α
Pro-inflammatory signal
CD4+ T cells
APC
CD4
CD4 T cell
MHC-II
CD3
Th1
IFN-γ, IL-2
Th2
IL-4, IL-5, IL-2
Pro-inflammatory signal
Th17
IFN-17, IL-21
T regulatory cells
FoxP3 Transcription factor
CD25
APC
CD4
Treg
MHC-II
CD3
Anti-inflammatory signal
IL-10, TGF-β

Figure 3. Summary of NK cell and T cell function during infections. NK cells recognize infected cells lacking expression of MHC class I and induce their apoptosis by releasing perforin and granzymes as well as producing inflammatory cytokines. $CD8^+$ T cells recognize antigens presented on MHC class I of infected cells and induce their apoptosis as well as produce inflammatory cytokines. $CD4^+$ T cells recognize antigens presented on antigen-presenting cells (APCs) and produce either Th1, Th2 or Th17 cytokines to further facilitate the immune response. T regulatory cells (T_{regs}) are a subset of $CD4^+$ T cells, which produce anti-inflammatory cytokines to dampen the immune response. This figure was generated using Biorender.

1.4 Inhibitory Receptors and Ligands.

Regulatory Role of Inhibitory Receptors. Immune checkpoint inhibitors are receptors whose primary roles are to inhibit the overactivation of immune cells. These inhibitory receptors have a critical role in regulating immune responses to infections, thereby limiting autoimmunity and/or immunopathology[159, 160]. In activated and proliferating T and NK cells, the expression of inhibitory receptors is used to control the expansion of these populations. Among these inhibitory receptors are programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and T cell immunoglobulin and ITIM domain (TIGIT). PD-1 and CTLA-4 are among the most well-explored inhibitory receptors, and they play vital roles at different stages of the immune response.

CTLA-4 is thought of as the leader among the inhibitory receptors as it plays an inhibitory role in the lymph node during the activation of naïve T cells[161]. This prevents the activation of potentially autoreactive T cells, which lead to autoimmunity[162]. As previously stated, the recognition of CD80/CD86 by CD28 is a vital step for T cell activation. Specifically, this co-stimulatory step leads to the proliferation, differentiation, survival and IL-2 production of these cells[161]. CTLA-4 is a homolog of CD28 but with a stronger binding affinity for CD80[163]. However, the binding of CTLA-4 to CD80 triggers inhibitory signals that directly counteract the TCR and CD28 activation signals[164]. CTLA-4 binding also inhibits the IL-2 production and cell cycle progression[165]. CTLA-4 is regulated through intracellular storage in the cell and is expressed on the surface following T cell activation[166]. Stronger TCR signaling causes more CTLA-4 to be translocated to the cell surface. CTLA-4 expression on T_{regs} is another pathway for the immunomodulatory role of this receptor. Animal models have shown that CTLA-4 deficiency in

T_{regs} hinders their suppressive function, therefore highlighting the importance of its expression in T_{reg} mediated immunosuppression[167]. Although there is limited data on CTLA-4 expression on NK cells and specifically human NK cells, its expression was shown to be upregulated on IL-2 activated mouse NK cells and it inhibited their IFN-γ production[168]. More prominent though is the indirect impacts of CTLA-4 inhibition of NK cell activation through suppression of T cell IL-2 production, and the role of CTLA-4 on T_{reg} mediated immune suppression.

On the other hand, PD-1 inhibition of T cell activation occurs in the peripheral tissues at the later stages of the immune response[162]. In the effector phase of T cell response, PD-1 regulates T cell activation following recognition of its ligands programmed death-ligand 1 (PD-L1) and PD-L2 on the APC[169]. PD-1 binding leads to the early termination of TCR signals by preventing the phosphorylation of signaling intermediates[170, 171]. Similar to CTLA-4, PD-1 binding leads to an inhibition of T cell IFN-γ production, IL-2 production, proliferation and decreased cell survival[169]. PD-L1 and PD-L2 are widely expressed on leukocytes, non-hematopoietic cells, nonlymphoid tissues as well as many types of tumours[161]. Likewise, in NK cells, PD-1 is a negative regulator of their function. In cancer patients, NK cells expressing PD-1 were found to express markers of apoptosis sensitivity, have reduced degranulation, and have reduced IFN-γ release[172].

LAG-3 (CD223) is a receptor, which is structurally similar to the CD4 co-receptor but with a higher binding affinity for MHC class II[173]. Some other LAG-3 ligands include Galectin (GAL)-3, liver sinusoidal endothelial cell lectin (LSECtin), and fibrinogen-like protein (FGL)-1[174-176]. After recognition of an antigen by the TCR, engaged LAG-3 can associate with CD3 through cross-linking, and this inhibits signal transduction leading to decreased cell proliferation and cytokine production[177]. LAG-3 expression on T cells is induced by cell activation[178]. However, due to the lack of a definable signaling motif on the LAG-3 cytoplasmic tail, the exact signal

transduction leading to inhibition remains largely unknown[160]. On T_{regs}, LAG-3 expression has been found to correlate with increased IL-10 production[179]. Moreover, loss of LAG-3 in T_{regs} reduces their immunosuppressive capabilities[180]. The role of LAG-3 on NK cells remains less defined and open to exploration as limited studies have investigated the role of LAG-3 expression on NK cell cytotoxicity and cytokine production. Notably, LAG-3 was among the inhibitory receptors with low expression on NK cells of HIV patients who were able to achieve viral control[181]. Furthermore, LAG-3 deficient mice showed reduced tumour elimination abilities[182].

TIGIT is among the most recently identified inhibitory receptors. It structurally resembles CD226, an activating receptor, and binds to its ligand CD155 with a higher affinity[160, 183]. It also binds, albeit weakly, to CD112, and both of these ligands are expressed on APCs. The exact signaling at the protein level involved in TIGIT inhibition of T cells is still under investigation, but emerging evidence suggests that TIGIT engagement inhibits T cell TCR signaling, proliferation and cytokine production[184]. In NK cells, TIGIT binding leads to the phosphorylation of tyrosine residues on its immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tail tyrosine (ITT)-like motifs[185]. This allows SH2 domain-containing inositol-5-phosphatase 1 (SHIP1) recruitment, which prematurely terminates the activity of NF-κB, PI3K (phosphoinositide 3-kinase) and MAPK (mitogen-activated protein kinase), inhibiting NK cytotoxicity and cytokine secretion[185, 186].

Lastly, TIM-3 was originally identified as a marker of Th1 cells, and its expression is controlled by T-bet[187, 188]. Its expression has now been confirmed on T_{regs}, CTLs, NK cells, monocytes, macrophages and dendritic cells[160]. The most studied ligands of TIM-3 are GAL-9 and carcinoembryonic antigen cell adhesion molecule 1 (CEACAM-1). Other ligands include high mobility group protein B 1 (HMGB1) and phosphatidylserines (PtdSer) exposed by apoptotic

cells[189]. The cytoplasmic tail of TIM-3 lacks any classical inhibitory signaling motifs and instead contains conserved tyrosine residues which, when unphosphorylated, bind to Bat3 (HLA-B associated transcript 3)[190]. During T cell activation, the tyrosine residue becomes phosphorylated, leading to the release of Bat3, which allows TIM-3 to deliver inhibitory signals to the T cell. Ultimately, TIM-3 engagement induces cell death in Th1 cells[191]. Furthermore, co-expression of CEACAM-1 and TIM-3 on the CTLs of patients with colorectal cancer led to decreased IFN-γ production[192]. Unlike T cells, $CD56^{Dim}$ NK cells highly express TIM-3[193]. Though $TIM\text{-}3^{+}$ NK cells show increased IFN-γ production and cytotoxicity, this outcome seems to shift upon chronic antigen availability[194, 195]. In patients with advanced melanoma, TIM-3 expression on NK cells led to decreased cytotoxicity and cytokine production. Although inhibitory receptor expression plays an important regulatory role on T and NK cell activation, they also play a major role in lymphocyte exhaustion.

Lymphocyte Exhaustion. Increased and sustained expression of inhibitory receptors, often found in patients with chronic viral infections and malignancies, is a principal mechanism priming lymphocytes to be dysfunctional. The suppressive role of PD-1, CTLA-4, LAG-3, TIM-3 and more recently, TIGIT, is relatively well understood[159, 160, 196, 197]. The expression of these receptors under healthy conditions, acts as a brake after adequate lymphocyte activation, but during chronic infections, their expression quickly becomes a marker of exhausted cells. With chronic antigen availability, the surface expression of the inhibitory receptor is significantly upregulated and maintained on T and NK cells[159, 160]. It leads to T and NK cell dysfunction, which manifests as an inability to effectively perform their effector function. Moreover, the sustained expression of these inhibitory receptors creates an environment permitting the persistence of infection, as well as the development of advanced stages of cancer as dysfunctional immune cells are unable to conduct

tumour immunosurveillance. Notably, T cells highly expressing inhibitory receptors showed impaired effector functions[198]. For this reason, the targeting of inhibitory receptors is actively being explored in cancer immunotherapies[199].

A study showed that the progression of acute to chronic HCV infection is associated with high PD-1 expression on HCV-specific CTLs[200]. During chronic HCV infections, HCV-specific CTLs in the liver have also been shown to co-express PD-1 and CTLA-4, but this phenotype was not observed in the peripheral blood lymphocytes[201]. Additionally, prior to the revolutionary impact of DAA therapy on the treatment of chronic HCV infection, anti-PD-1 therapy showed positive potential as a means to persistently suppress HCV replication in chronic patients[202]. TIM-3 has also been found to be over-expressed on HCV-specific CTLs and $CD4^+$ T cells, and its blockade restored their proliferative and IFN-γ production capacities[203]. A summary of the tested receptors and some of their known ligands are outlined in **Table 1**.

The majority of studies exploring the role of inhibitory receptors on T cell exhaustion during chronic infection, focus on antigen-specific T cells. What is less well defined is the exhaustion that exists in the bulk T cell population during chronic infections. Information on bulk T cell exhaustion provides a better understanding of considerable wide-spread immune suppression seen in chronically infected patients. A study found that mice chronically infected with lymphocytic choriomeningitis virus (LCMV) showed an upregulation of PD-1 and LAG-3 expression on not only LCMV-specific CTLs, but also the bulk $CD8^+$ T cell population[204]. In particular, both LCMV-specific and non-LCMV-specific CTLs had a higher expression of inhibitory receptors in mice with chronic infections (LCMV clone-13 strain) compared to acute infections (LCMV Armstrong strain). This indicates there is bulk T cell exhaustion during chronic infections.

Galectin-9 (GAL-9). Recently, the role of GAL-9 surface expression in immune cell dysfunction has been identified. GAL-9 is a member of the galectin family, carbohydrate-binding proteins that have carbohydrate recognizing domains (CARD), and control many biological functions such as adhesion, migration and cell proliferation or death[205-207]. GAL-9 is one of the ligands of TIM-3 and is widely expressed in various tissues, but is abundant in the liver[208]. The TIM-3/GAL-9 interaction can induce Th1 death, but can also induce NK cell IFN-γ production[194, 209]. Additionally, GAL-9 has been shown to have TIM-3 independent regulatory functions. GAL-9 suppression of Th17 cell development and GAL-9 control of CD40 signaling on APCs is done in a TIM-3 independent manner[210, 211].

During chronic viral infections, specifically HCV, the circulating levels of GAL-9 and its expression in the liver are increased, primarily by GAL-9 producing macrophages and monocytes[196, 212]. A study showed that the presence of soluble GAL-9 during NK cell stimulation impairs their cytokine production and cytotoxic ability[196]. More recently, NK and bulk T cells with surface expression of GAL-9 (GAL-9$^+$ cells) in HIV patients were shown to have impaired cytotoxicity due to reduced expression of granzyme B and perforin[213, 214]. A study has also shown that HCC tumours express GAL-9, and currently, the potential use of GAL-9 expression as a biomarker of HCC is being explored[215].

Receptor	Ligand	Reported role in HCV	Reported role in HCC
PD-1	PD-L1, PD-L2	x	x
CTLA-4	CD80, CD86	x	x
LAG-3	MHC class II, GAL-3, LSECtin	x	x
TIGIT	CD155, CD112		x
TIM-3	GAL-9, CEACAM-1, HMGB1, PtdSer	x	x

Table 1. Inhibitory receptors, their known ligands and role in HCV and HCC. Some known ligands of the studied inhibitory receptors and identification of receptors which have been implicated in HCV infections, and hepatocellular carcinoma (HCC).

1.5 Rationale and Aim.

Rationale: As previously mentioned, the introduction of DAAs transformed the HCV treatment landscape as most therapies show a 90-98% cure rate in all HCV genotypes[216]. However, chronic HCV patients with advanced liver fibrosis face immune dysregulation that persists after viral clearance. Bulk $CD8^+$ T cells of HCV-infected individuals with advanced liver disease were dysregulated and showed sustained activated status compared to those with minimal liver fibrosis, and this persisted post-DAA treatment[217]. Importantly, this differential nature of immune suppression is present not only in chronic HCV patients relative to healthy individuals but specifically in patients with advanced fibrosis compared to those with minimal or no fibrosis. The risk of developing HCC as patients progress from varying stages of fibrosis (F0-F2) to cirrhosis (F3-F4) increases substantially[218, 219]. Therefore, it is imperative to characterize the immune dysregulation associated with advanced liver fibrosis. This will help to develop targeted strategies for restoring effective T and NK cell immune function, in order to treat or reduce the risk of adverse clinical outcomes of cirrhosis in HCV^+ individuals. To do this, I examined the patterns of GAL-9 expression alongside inhibitory receptors on bulk T cells and NK cells, and identified immune phenotypes associated with immune dysregulation in chronic HCV patients with advanced liver fibrosis.

AIM: To investigate the nature of inhibitory receptor/ligand expression and co-expression on bulk T cells and NK cells of patients with chronic HCV infection with advanced liver fibrosis (F3-F4) compared to those with no to moderate fibrosis (F0-F2).

1. *I analyzed the expression of PD-1, CTLA-4, LAG-3, TIGIT and TIM-3, as well as GAL-9 on the surface of T and NK cells to answer the following questions:*

a) Which inhibitory receptors have a differential expression on the surface of T and NK cells of patients with advanced liver fibrosis (F3-F4) compared to those with no to moderate fibrosis (F0-F2)?

b) Is GAL-9 expression different on the surface of T and NK cells of patients with advanced liver fibrosis (F3-F4) compared to those with no to moderate fibrosis (F0-F2)?

2. *I analyzed the co-expression of inhibitory receptors and GAL-9 on the surface of T and NK using both Boolean gating and t-SNE analysis to answer the following questions:*

a) What inhibitory receptors and/or GAL-9 are co-expressed together on the various T and NK cell populations?

c) Is there a different degree of inhibitory receptor and/or GAL-9 co-expression on T and NK cells of patients with advanced liver fibrosis (F3-F4) compared to those with no to moderate fibrosis (F0-F2)?

b) Is there a correlation between inhibitory receptor and/or GAL-9 co-expression with patients' LSM?

3. *I further analyzed GAL-9 expression on T and NK cells to answer the following questions:*

a) What are the phenotypic characteristics of GAL-9^{+} lymphocytes in patients with chronic HCV infection?

b) What is the nature of GAL-9 expression on NK-92 and primary-NK cell lines on the cell surface and at the mRNA level?

2. MATERIALS AND METHODS

The methods are summarized in **Fig. 4**.

2.1 Study Subjects.

Blood samples from chronically infected HCV^+ individuals (i.e., > 6 months HCV RNA-positive) who were DAA treatment-naive were collected in collaboration with Dr. Curtis Cooper at The Ottawa Hospital (**Table 2**). Heparin-treated green capped tubes (Vacutainer; BD, Franklin Lakes, NJ, USA) were used for the collection and transportation of the blood samples. The study cohort was composed of 2 groups with 30 patients: 15 chronic HCV patients in fibrosis stage F0-F2 and 15 chronic HCV patients in stage F3-F4. 27 of these patients were interferon treatment naïve, while 3 patients had previously received interferon treatment. In addition to fibrosis stages, I collected patients' age, sex, ethnicity, alanine aminotransferase (ALT) and aspartate transaminase (AST) levels, as well as liver stiffness measures (LSM). Written informed consent was obtained from all participants, and the study was approved by The Ottawa Health Science Network Research Ethics Board (Ottawa Health Science Network REB 2010-0009, REB attached in appendix).

Characteristics[a]	**F0-F2**[b]	**F3-F4**[c]
Age (years)	46.8 ± 2.0	55.3 ± 2.4
Male/Female (N)	11/3	13/2
Race (N) -Caucasian	 15	 15
LSM[d] (kPa)	6.05 ± 0.5	27.8 ± 4.9
AST[e] (U/L)	48 ± 8	97 ± 19
ALT[f] (U/L)	75 ± 10	104 ± 19
AST/ALT	0.62 ± 0.04	0.99 ± 0.15
HCV genotype (N) -1a -2 -3 -4	 10 0 2 1	 6 3 5 1
METAVIR stage (N) -F0 -F1 -F2 -F3 -F4	 4 6 3 - -	 - - - 3 11

[a] All data is presented as mean ± SEM.

[b] Two patients within this group could not be identified in the clinic post analysis but at the time of sample collection, were placed in this group by the clinician. Thus, I am lacking AST, ALT, HCV genotype and METAVIR stage for the two patients and the gender of one patient.

[c] One patient within this group did not have a definitive METAVIR classification due to lack of liver stiffness measurement or liver biopsy but was placed in this group after evaluation by the clinician.

[d] Five patients are excluded due to lack of LSM data

[e] AST- Aspartate transaminase

[f] ALT- Alanine transaminase

Table 2. Study subjects characteristics. The study cohort was composed of 30 patients with chronic HCV infection divided into 2 groups: 15 patients in fibrosis stage F0-F2 and 15 patients in stage F3-F4.

2.2 Fibrosis Scoring.

During chronic HCV infections, in an effort to repair the damage caused by virus replication in the liver, collagen is continually deposited, leading to the development of fibrosis (liver scarring). The continual deposition of collagen and spreading of fibrosis causes chronic HCV patients to progress along with fibrosis, and if not addressed, patients develop cirrhosis. At this stage, there is a significant loss of liver function. Fibrosis stages of study subjects were determined by transient elastography (FibroScan, Echosens) and/or liver biopsies and were grouped in accordance with the METAVIR stages (F0: no fibrosis, F1: minimal fibrosis, F2: spreading of fibrosis to other areas of the liver including blood vessels, F3: spreading and presence of fibrosis network in the liver, F4: cirrhosis). Fibrosis evaluation was performed at The Ottawa Hospital Viral Hepatitis Clinic and in cases where a Fibroscan was not done, the classification of fibrosis stage was left to the discretion of the physician. Based on LSM, patients with chronic HCV infections can be classified into their corresponding METAVIR stages, with F0-F1 patients having a value of 7 kPa or less, F2 patients being between 7kPa and 9kPa, F3 patients between 9kPa and 12.5kPa and F4 patients being greater than 12.5kPa.

2.3 Extraction and Storage of PBMC from Blood Samples.

PBMCs were isolated by Ficoll gradient density centrifugation (Ficoll-Paque PLUS; GE Healthcare, Mississauga, ON, Canada). Blood samples were overlayed on Ficoll and centrifuged at 515 relative centrifugal force (RCF) for 35 minutes with an acceleration of 1 and brake of 0. PBMCs were subsequently aliquoted in freezing media consisting of 10% DMSO (Fisher BioReagents, Fisher Scientific) and 90% FBS (Gibco; Life Technologies, Burlington, Ontario, Canada), and stored at -80^{o}C for future use. The cells were thawed in complete RP-10 media,

consisting of RPMI 1640 (HyClone, GE Healthcare) supplemented with 10% FBS, 2mM L-glutamine (HyClone), 10mM HEPES buffer (HyClone), 1% penicillin/streptomycin (HyClone), and 55μM 2-Mercaptoethanol (Gibco), before assessing cell surface and intracellular protein expression.

2.4 Flow Cytometry Analysis.

Anti-human fluorophore-conjugated antibodies were purchased from BD Bioscience, Biolegend and Invitrogen. The total list of antibodies used is outlined in **Table 3**. Near IR fluorescent reactive dye (Life Technologies, California, USA) was used to exclude dead cells. PBMC were incubated with surface stain markers in BD Horizon™ Brilliant Stain Buffer to avoid Brilliant Violent (BV) dye interactions. Cells were fixed using paraformaldehyde (2%) and acquired using BD LSRFortessa. For T_{reg} analysis, cells were fixed using eBioscience™ FoxP3/Transcription factor staining buffer set and acquired using Attune™ NxT Flow cytometer. All flow cytometry data was analyzed using FlowJo v.10 software.

Antigen	Conjugate	Clone	Supplier	Catalog Number
CD3	BV421	SK7	BD Biosciences	563798
	BV510	HIT3a		564713
CD8	BV786	RPA-T8	BD Biosciences	563875
CD4	BV650	SK3	BD Biosciences	563823
CD56	BV786	NCAM 16.2	BD Biosciences	564058
CD16	BV650	3G8	BD Biosciences	563692
CD45RO	BV605	UCHL1	BD Biosciences	562791
CCR7	PE-CF594	2-L1-A	BD Biosciences	566768
CD25	BB700	M-A251	BD Biosciences	566447
	PE			555432
FoxP3	APC	236A/E7	eBioscience	17-4777-42
CD14	APC-Cy7	MΦP9	BD Biosciences	557831
CD19	APC-Cy7	SJ25C1	BD Biosciences	363010
PD-1	FITC	MIH4	BD Biosciences	557860
CTLA-4	PE-Cy7	14D3	eBioscience	25-1529-42
LAG-3	APC-R700	T47-530	BD Biosciences	565774
TIGIT	APC	MBSA43	eBioscience	17-9500-42
	PerCP-efluor710			46-9500-42
TIM-3	BV421	7D3	BD Biosciences	565562
GAL-9	PE	9M1-3	BD Biosciences	565890

Table 3. List of antibodies used for flow cytometry analysis. For T cell analysis, CD3, CD8, CD4, CD45RO, CCR7, CD25 and FoxP3 were utilized to define T cell subsets. PD-1, CTLA-4, LAG-3, TIGIT, TIM-3 and GAL-9 were used to identify T cell phenotypes. For NK cell analysis, CD56, CD16, CCR7 and CD25 were utilized to define NK cell subsets. PD-1, CTLA-4, LAG-3, TIGIT, TIM-3 and GAL-9 were used to identify NK cell phenotypes. In the NK cell analysis, CD19 was used to exclude B cells and CD14 to exclude monocytes.

2.5 t-SNE Analysis of T Cells.

t-SNE analysis was performed using FlowJo v.10 t-SNE plugin. Each subject's T cells were down-sampled to randomly select 50 000 cells for t-SNE analysis. Each 50 000 cell down-sample was then concatenated into a single file. Prior to analysis, I excluded double negative ($CD4^-CD8^-$) T cells and ultimately performed t-SNE analysis on 1 402 236 cells. Analysis of T cells was done using the surface expression of CD8, CD4, CD45RO, CCR7, CD25, PD-1, CTLA-4, LAG-3, TIGIT, TIM-3 and GAL-9. T cell clusters were defined by qualitatively accessing expression of subset markers, inhibitory receptors and GAL-9. The analysis was done using: 1 000 iterations, 100 perplexity, 98 156 learning rate (eta), Exact (vantage point tree) KNN algorithm and Barnes-Hut gradient algorithm.

2.6 t-SNE Analysis of NK Cells.

For t-SNE analysis on NK cell, each subject's $CD3^-CD14^-CD19^-$ lymphocytes were down-sampled to randomly select 15 000 cells for t-SNE analysis. Each 15 000 cell down-sample was then concatenated into a single file. Prior to analysis, we selected $CD56^+$cells and ultimately performed t-SNE analysis on 317 727 cells. Analysis of NK cells was done using the surface expression of CD56, CD16, CCR7, CD25, PD-1, CTLA-4, LAG-3, TIGIT, TIM-3 and GAL-9. NK cell clusters were defined by qualitatively accessing expression of subset markers, inhibitory receptors and GAL-9. The analysis was done using: 2 000 iterations, 100 perplexity, 22 240 learning rate (eta), Exact (vantage point tree) KNN algorithm and Barnes-Hut gradient algorithm.

2.7 Statistical and Correlation Analysis.

Statistical analyses were performed using GraphPad Prism 8. Unpaired nonparametric Mann-Whitney U test was used for all analysis between the F0-F2 and F3-F4 groups. Results are presented as median with IQR with a p-value < 0.05 being considered statistically significant. Regression analysis was performed following a linear regression model and using LSM, AST/ALT, AST and ALT of chronic HCV patients. R^2 and p-values are reported for all regression analyses. LSM regression analysis was done using 25 chronic HCV patients due to the lack of LSM of 5 patients. AST/ALT, AST and ALT regression analyses were done using 28 chronic HCV patients due to the lack of values for 2 patients.

2.8 Figures.

Schematics and summary figures were created using Biorender.

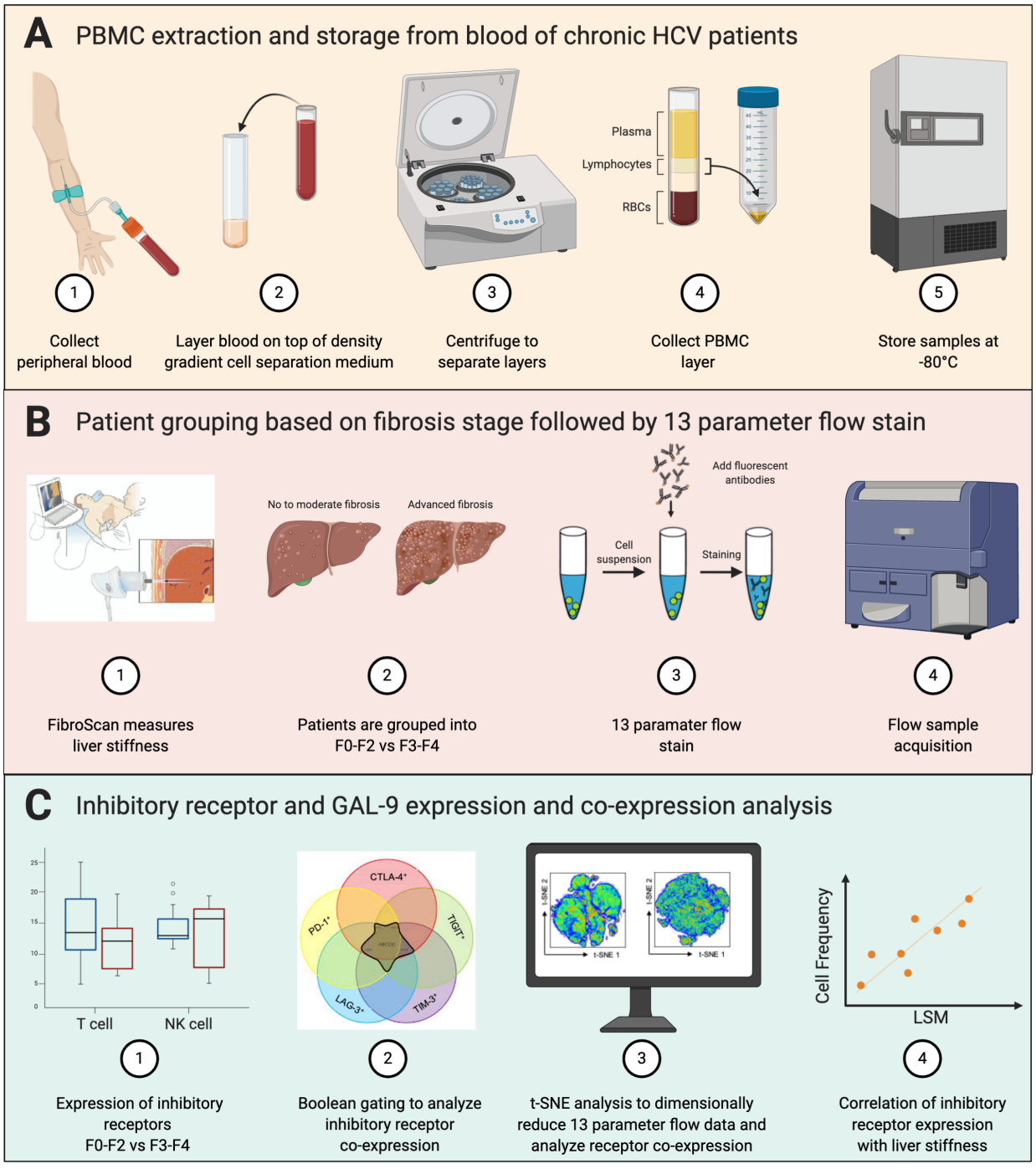

A
PBMC extraction and storage from blood of chronic HCV patients
Plasma
Lymphocytes
RBCs
1
Collect peripheral blood
2
Layer blood on top of density gradient cell separation medium
3
Centrifuge to separate layers
4
Collect PBMC layer
5
Store samples at -80°C
B
Patient grouping based on fibrosis stage followed by 13 parameter flow stain
No to moderate fibrosis
Advanced fibrosis
Add fluorescent antibodies
Cell suspension
Staining
1
FibroScan measures liver stiffness
2
Patients are grouped into F0-F2 vs F3-F4
3
13 paramater flow stain
4
Flow sample acquisition
C
Inhibitory receptor and GAL-9 expression and co-expression analysis
T cell
NK cell
CTLA-4+
PD-1+
TIGIT+
LAG-3+
TIM-3+
t-SNE 2
t-SNE 1
Cell Frequency
LSM
1
Expression of inhibitory receptors F0-F2 vs F3-F4
2
Boolean gating to analyze inhibitory receptor co-expression
3
t-SNE analysis to dimensionally reduce 13 parameter flow data and analyze receptor co-expression
4
Correlation of inhibitory receptor expression with liver stiffness

Figure 4. Summary of materials and methods. (A) Extraction and storage of peripheral blood mononuclear cells (PBMC) from the blood of chronic HCV patients. (B) Classification of chronic HCV patients into F0-F2 or F3-F4 groups based on their liver stiffness measure (LSM) followed by 13 parameter flow stain and acquisition. (C) Inhibitory receptor and GAL-9 expression and co-expression analysis. This figure was generated using Biorender.

3. RESULTS

To characterize the nature of immune dysregulation associated with advanced liver fibrosis during a chronic viral infection, I recruited chronic HCV patients and placed them into two groups based on their stage of fibrosis. The characteristics of study participants are summarized in **Table 2,** with F0-F2 patients having no to moderate fibrosis (n=15) and F3-F4 patients having advanced fibrosis, including cirrhosis (n=15). F3-F4 patients are older than F0-F2 patients, presumably due to the years required to develop liver fibrosis.

3.1 Proportions of T and NK cell Subsets in HCV Patients with Liver Fibrosis.

Given the pivotal role that T and NK cells play in immunosurveillance, I examined the bulk T cells and NK cells in PBMC samples to detect any changes in subset frequencies associated with liver fibrosis severity (**Fig. 5A &B**). Advanced liver fibrosis was not associated with altered frequencies of $CD8^+CD4^-$ ($CD8^+$), $CD8^-CD4^+$ ($CD4^+$), $CD8^+CD4^+$ (double-positive) and $CD8^-CD4^-$ (double-negative) T cell frequencies compared to frequencies in minimal fibrosis (**Fig. 6A**). T cell subsets were identified using CD45RO and CCR7 markers with $CD45RO^- CCR7^+$ denoting naïve cells (T_{Naive}), $CD45RO^- CCR7^-$, terminal effector cells (T_{EMRA}), $CD45RO^+ CCR7^-$, effector memory cells (T_{EM}), and $CD45RO^+ CCR7^+$, central memory cells (T_{CM}) (**Fig. 6B**). Although CD8 and CD4 subsets were not significantly different between the HCV-infected groups, there was evidence of a lower T_{Naive} frequency and a higher T_{CM} frequency in CD4 subsets of F3-F4 patients when compared to F0-F2 patients (**Fig. 6C & D**).

In the NK cell population, advanced liver fibrosis was not associated with altered frequencies of $CD56^{Bright}$ and $CD56^{Dim}$ NK cell frequencies compared to frequencies in minimal fibrosis (**Fig. 7A**). CD25 is a cellular activation marker, and notably, the F3-F4 patients showed a

significantly higher proportion of $CD25^{+}$ $CD56^{Dim}$ NK cells compared to F0-F2 patients (**Fig. 7B & C**). Lastly, the expression of CCR7 on $CD56^{Dim}$ NK cells was higher in the F3-F4 group compared to the F0-F2 group (**Fig. 7D & E**).

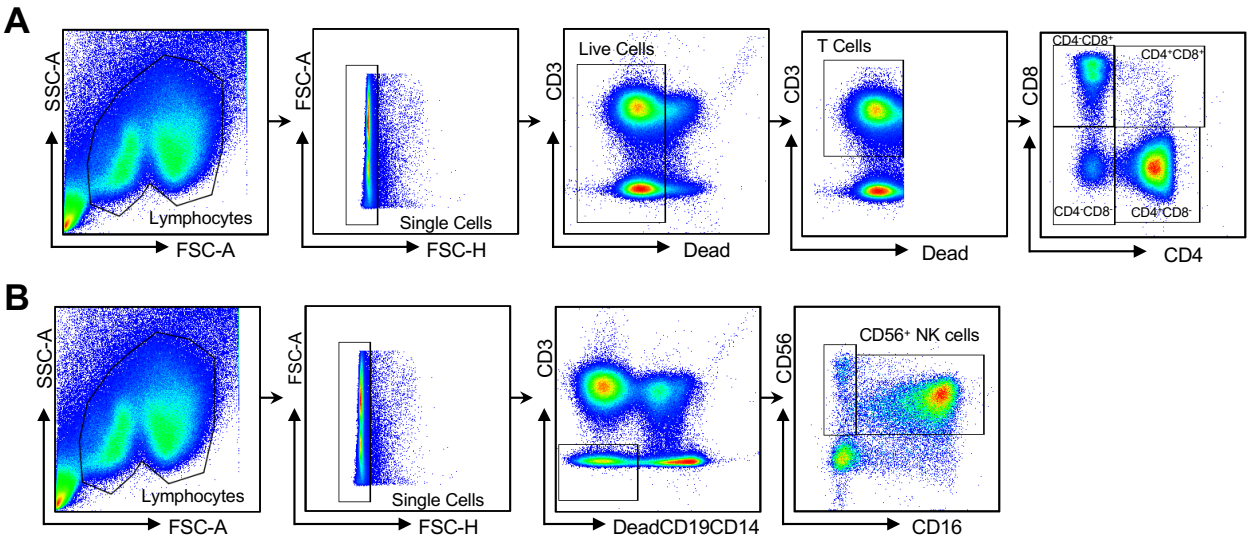

A
SSC-A
Lymphocytes
FSC-A
FSC-A
Single Cells
FSC-H
CD3
Live Cells
Dead
CD3
T Cells
Dead
CD8
CD4⁻CD8⁺
CD4⁺CD8⁺
CD4⁻CD8⁻
CD4⁺CD8⁻
CD4
B
SSC-A
Lymphocytes
FSC-A
FSC-A
Single Cells
FSC-H
CD3
DeadCD19CD14
CD56
CD56⁺ NK cells
CD16

Figure 5. Gating strategy to identify and analyze T and NK cell subsets. I gated on lymphocytes based on SSC-A and FSC-A and subsequently performed doublet discrimination. (A) I gated on T cells following gating on live cells. CD4 and CD8 was used to identify T cell subsets: $CD4^{-}CD8^{+}$, $CD4^{+}CD8^{+}$, $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{-}$ T cells. (B) I gated on Live $CD3^{-}CD19^{-}CD14^{-}$ cells and, using CD56 and CD16, identified $CD56^{+}$ NK cells.

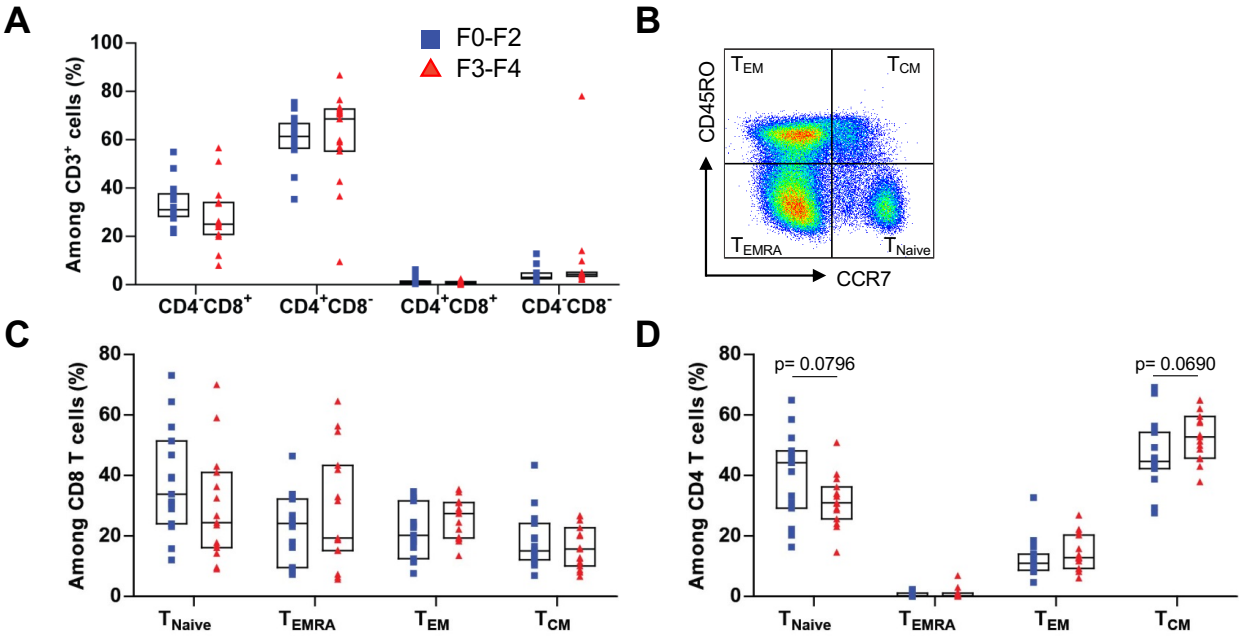

A
F0-F2
F3-F4
Among CD3+ cells (%)
100
80
60
40
20
0
CD4-CD8+
CD4+CD8-
CD4+CD8+
CD4-CD8-
B
CD45RO
TEM
TCM
TEMRA
TNaive
CCR7
C
Among CD8 T cells (%)
80
60
40
20
0
TNaive
TEMRA
TEM
TCM
D
Among CD4 T cells (%)
p= 0.0796
p= 0.0690
80
60
40
20
0
TNaive
TEMRA
TEM
TCM

Figure 6. Proportions of T cell subsets in HCV patients with liver fibrosis. Freshly thawed PBMCs of F0-F2 (n=15), and F3-F4 (n=15) chronically infected HCV patients were stained for surface expression of T cell population markers. (A) Cumulative data showing the percentage of T cell subpopulations ($CD4^-CD8^+$, $CD4^+CD8^-$, $CD4^+CD8^+$ and $CD4^-CD8^-$) of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. (B) Representative plot showing 4 T cell subsets identified using the expression of CD45RO and CCR7. (C) Cumulative data showing the percentage of CD8 T cell subsets of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. (D) Cumulative data showing the percentage of CD4 T cell subsets of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. Terminal effector (EMRA), Effector memory (EM) and Central memory (CM). Data are represented as median with interquartile range (IQR) and each point represents an individual.

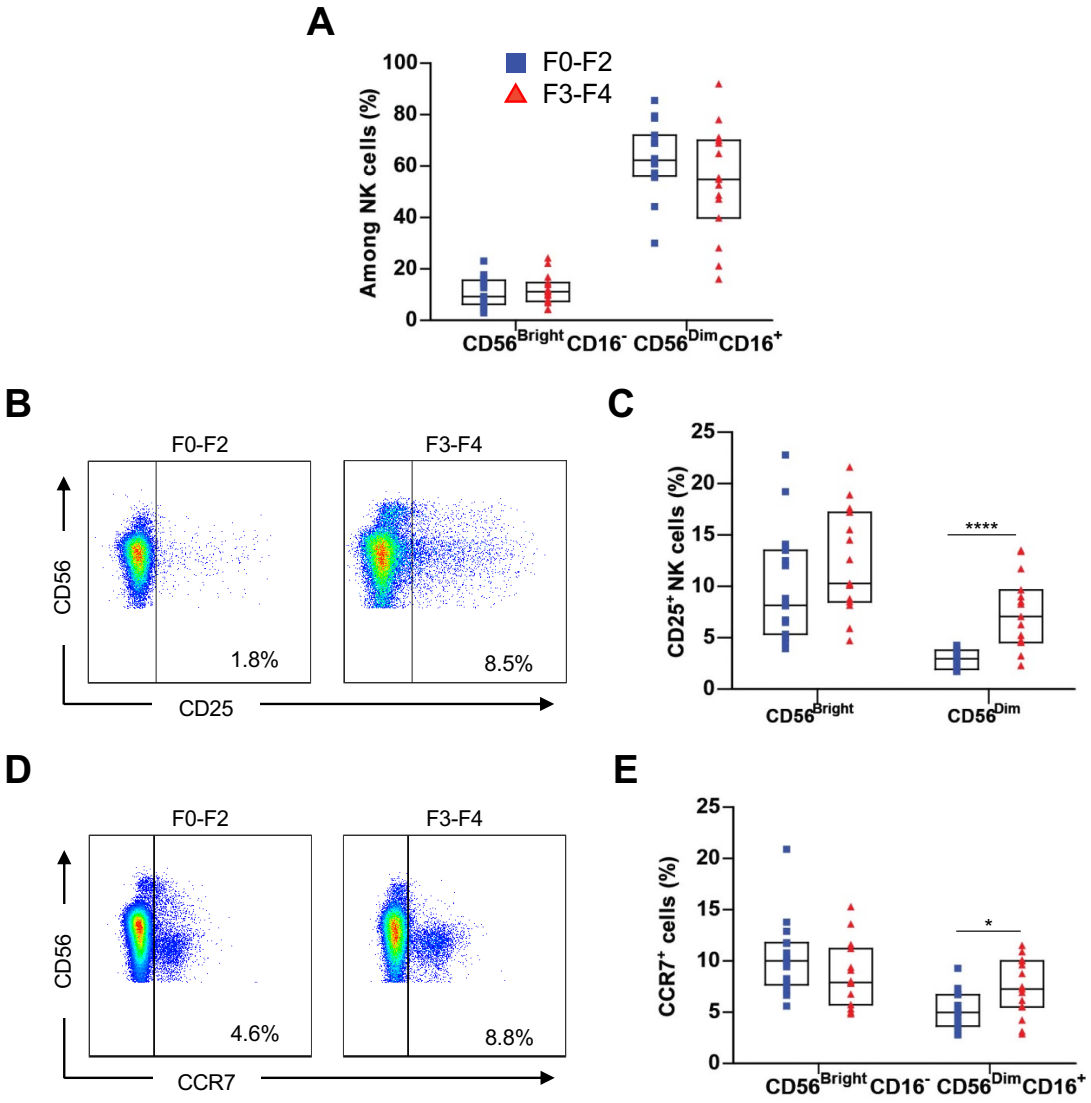

A
F0-F2
F3-F4
Among NK cells (%)
100
80
60
40
20
0
CD56Bright CD16- CD56Dim CD16+
B
F0-F2
F3-F4
CD56
1.8%
8.5%
CD25
C
CD25+ NK cells (%)
25
20
15
10
5
0
****
CD56Bright
CD56Dim
D
F0-F2
F3-F4
CD56
4.6%
8.8%
CCR7
E
CCR7+ cells (%)
25
20
15
10
5
0
*
CD56Bright CD16- CD56Dim CD16+

Figure 7. Proportions of NK cell subsets in HCV patients with liver fibrosis. Freshly thawed PBMCs of F0-F2 (n=15), and F3-F4 (n=15) chronically infected HCV patients were stained for surface expression of NK cell population markers. (A) Cumulative data showing the percentage of NK cell subpopulations ($CD56^{Dim}CD16^{+}$ and $CD56^{Bright}CD16^{-}$) of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. (B) Representative plot showing the expression of CD25 on $CD56^{Dim}$ NK cells of F0-F2, and F3-F4 patients. (C) Cumulative data comparing CD25 expression on NK cells of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. (D) Representative plot showing the expression of CCR7 on $CD56^{Dim}$ NK cells of F0-F2, and F3-F4 patients. (E) Cumulative data comparing the percentage of $CD56^{Bright}$ and $CD56^{Dim}$ NK cells expressing CCR7 of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. Data are represented as median with interquartile range (IQR) and each point represents an individual. *$p<0.05$, ****$p<0.0001$.

3.2 Higher Frequencies of T and NK cells Expressing Multiple Inhibitory Receptors in Patients with Advanced Liver Fibrosis.

Given the critical role of inhibitory receptors and their ligands in modulating T and NK cell function, I measured the surface expression of PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9 on $CD8^+$/$CD4^+$ T cells and $CD56^{Bright}$/$CD56^{Dim}$ NK cells. In the bulk $CD8^+$ T cell population, F0-F2 and F3-F4 patients showed no differences in frequencies of $PD\text{-}1^+$, $LAG\text{-}3^+$, $TIGIT^+$, and $TIM\text{-}3^+$ cells (**Fig. 8A & B**). F3-F4 patients had lower frequencies of $CTLA\text{-}4^+$ cells than F0-F2 patients and a higher frequency of $GAL\text{-}9^+$ cells. To correlate the expression of receptors found to be significantly different between the 2 groups with the extent of a patients' liver damage, I conducted linear regression analyses between patients' LSM and the frequency of $CTLA\text{-}4^+$ cells and $GAL\text{-}9^+$ cells (**Fig. 8C**). I found no significant correlation between the frequency of $CTLA\text{-}4^+$ cells and the LSM of patients with chronic HCV. However, there was a significant positive correlation between the frequency of $GAL\text{-}9^+$ cells and the LSM of patients. This means in addition to F3-F4 patients having a significantly higher frequency of $GAL\text{-}9^+$ cells in their $CD8^+$ T cell population when compared to F0-F2 patients, the frequency of $GAL\text{-}9^+$ cells was also associated with the extent of liver damage.

In the bulk $CD4^+$ T cell population, F0-F2 and F3-F4 patients showed no differences in frequencies of $PD\text{-}1^+$, $CTLA\text{-}4^+$, $LAG\text{-}3^+$ and $TIM\text{-}3^+$ cells (**Fig. 9A & B**). Notably, F3-F4 patients had a higher frequency of $TIGIT^+$, and $GAL\text{-}9^+$ cells than F0-F2 patients. Again, I correlated the expression of receptors found to be significantly different, with liver damage. Thus, I conducted linear regression analyses between patients' LSM and the frequency of $TIGIT^+$ cells and $GAL\text{-}9^+$ cells (**Fig. 9C**). I found a significant positive correlation between both the frequency of $TIGIT^+$ and $GAL\text{-}9^+$ cells in the $CD4^+$ T cell population, and the LSM of patients with chronic HCV.

In the $CD56^{Bright}$ NK cell population, F0-F2 and F3-F4 patients showed no differences in frequencies of PD-1^+ and $TIGIT^+$ cells (**Fig. 10A & B**). Interestingly, F3-F4 patients had a higher frequency of TIM-3^+, CTLA-4^+ and GAL-9^+ cells on $CD56^{Bright}$ NK cells than F0-F2 patients. I also observed evidence of a higher frequency of LAG-3^+ cells, although this did not reach the level of statistical significance (p= 0.0514). Linear regression analyses between patients' LSM and the frequency of CTLA-4^+, LAG-3^+, TIM-3^+ and GAL-9^+ cells showed only a significant positive correlation between the frequency of CTLA-4^+ cells, and patients' liver damage (**Fig. 10C**). I found evidence of a correlation between LAG-3^+ and TIM-3^+ cells and LSM, but this did not rise to the level of statistical significance (p= 0.0583 and p= 0.0740, respectively).

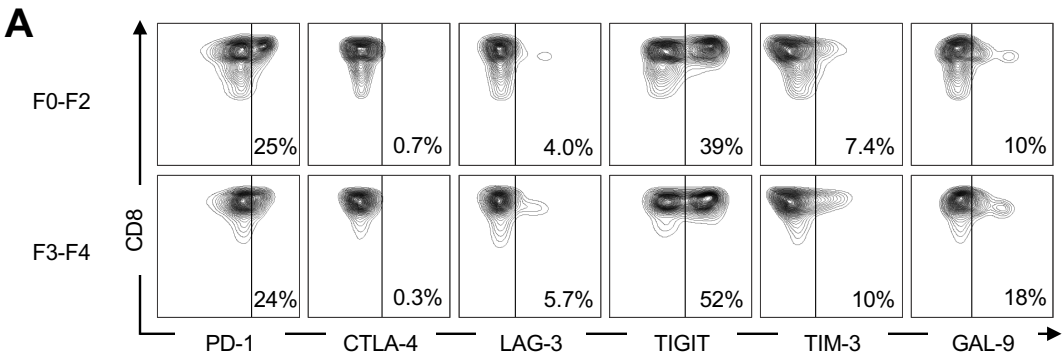
Lastly, in the $CD56^{Dim}$ NK cell population, F0-F2 and F3-F4 patients showed no differences in frequencies of CTLA-4^+, $TIGIT^+$ and TIM-3^+ cells (**Fig. 11A & B**). F3-F4 patients had a higher frequency of PD-1^+, LAG-3^+, and GAL-9^+ cells on $CD56^{Dim}$ NK cells than F0-F2 patients. Notably, the higher expression of GAL-9 was consistent on all tested T and NK cell subsets in F3-F4 patients. The CTLA-4 surface expression was found to be minimal on all tested subset, presumably due to its rapid internalization in the absence of ligand binding[220]. Linear regression analyses found a significant positive correlation between the frequency of LAG-3^+ and GAL-9^+ cells in the $CD56^{Dim}$ NK cell population with the extent of patient liver damage (**Fig. 11C)**.

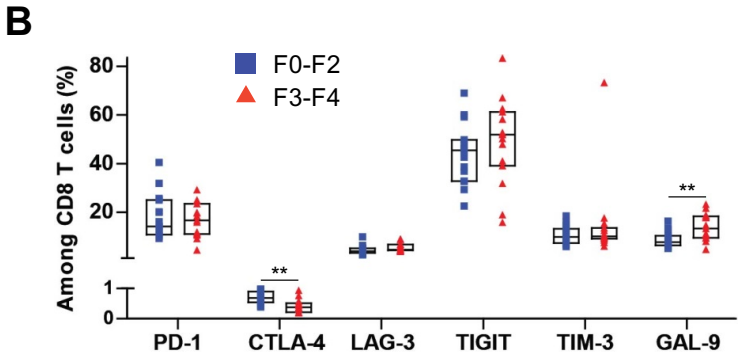
Extensive lymphocyte exhaustion is characterized by the simultaneous expression of multiple inhibitory receptors, and likewise, functionally superior lymphocytes express limited inhibitory receptors[204, 221]. Boolean gating identifies cells based on "AND", "OR" and "NOT" logic. Thus, using Boolean analysis, I compared the frequency of bulk T and NK cells simultaneously expressing more than 3 inhibitory receptors, PD-1, CTLA-4, LAG-3, TIGIT, and TIM-3, (**Fig. 12A**), and the frequency of T and NK cells negative for all five receptors (**Fig. 12B**).

I chose to measure the expression of >3 receptors as the surface expression of CTLA-4 on both T and NK cells was minimal, thus making the number of cells expressing all 5 receptors minimal.

Notably, I observed a higher frequency of both $CD56^{Bright}$ and $CD56^{Dim}$ NK cells positive for >3 (i.e., 4 or 5) inhibitory receptors in F3-F4 patients compared to F0-F2 patients (**Fig. 12A**). No difference was observed in the $CD4^+$ and $CD8^+$ T cell populations. Furthermore, there was evidence of a lower frequency of cells negative for all inhibitory receptors in F3-F4 patients, even though it only reached significance in the $CD4^+$ T cell and $CD56^{Bright}$ NK cell populations (**Fig. 12B**). Next, I included the expression of GAL-9 in the Boolean analysis as GAL-9, similar to the inhibitory receptors, has been shown to modulate bulk T cell and NK cell function[213, 214]. Thus, I determined the frequency of $CD8^+$/$CD4^+$ T cells and $CD56^{Bright}$/$CD56^{Dim}$ NK cells positive for >3 (i.e., 4, 5 or 6) inhibitory receptors or GAL-9. I found that in the $CD8^+$ T cell, $CD4^+$ T cell, $CD56^{Bright}$ NK cell and $CD56^{Dim}$ NK cell populations, F3-F4 patients had a higher frequency of cells positive for >3 inhibitory receptors or GAL-9 than F0-F2 patients (**Fig. 12C**).

Next, I determined the frequency of T and NK cells, which were negative for all inhibitory receptors and GAL-9. I found that in the $CD8^+$ T cell, $CD4^+$ T cell, $CD56^{Bright}$ NK cell and $CD56^{Dim}$ NK cell populations, F3-F4 patients had a lower frequency of cells negative for all inhibitory receptors and GAL-9 when compared to the F0-F2 patients (**Fig. 12D**). Finally, I determined the frequency of bulk $CD8^+$/$CD4^+$ T cells and $CD56^{Bright}$/$CD56^{Dim}$ NK cells positive for >4 (i.e., 5 or 6) inhibitory receptors or GAL-9. I found that in the $CD8^+$ T cell, $CD56^{Bright}$ NK cell and $CD56^{Dim}$ NK cell population, F3-F4 patients had a higher frequency of cells positive for >4 inhibitory receptors or GAL-9 than F0-F2 patients (**Fig. 13**). Altogether, the results suggest that a higher frequency of extensively exhausted T and NK cells and a lower frequency of functionally superior T and NK cells are characteristics of patients with advanced liver fibrosis.


A
F0-F2
F3-F4
CD8
25%
0.7%
4.0%
39%
7.4%
10%
24%
0.3%
5.7%
52%
10%
18%
PD-1
CTLA-4
LAG-3
TIGIT
TIM-3
GAL-9


B
F0-F2
F3-F4
Among CD8 T cells (%)
80
60
40
20
1
0
**
**
PD-1
CTLA-4
LAG-3
TIGIT
TIM-3
GAL-9

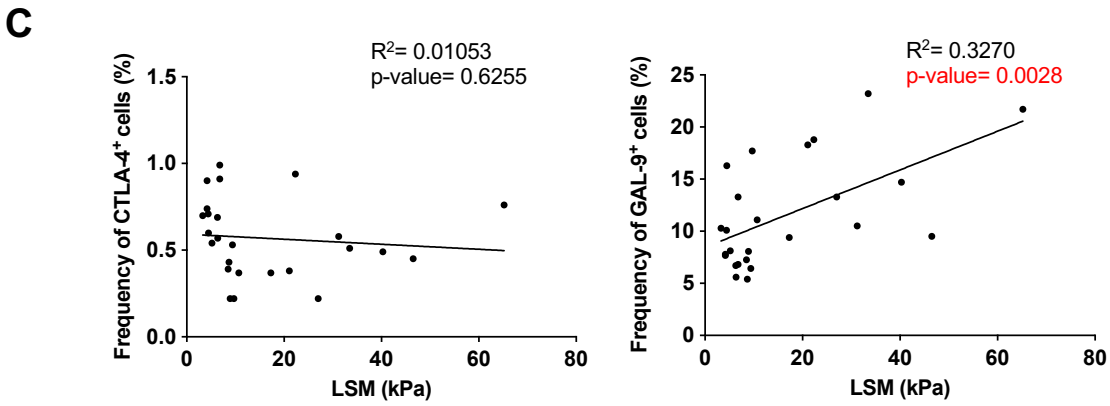
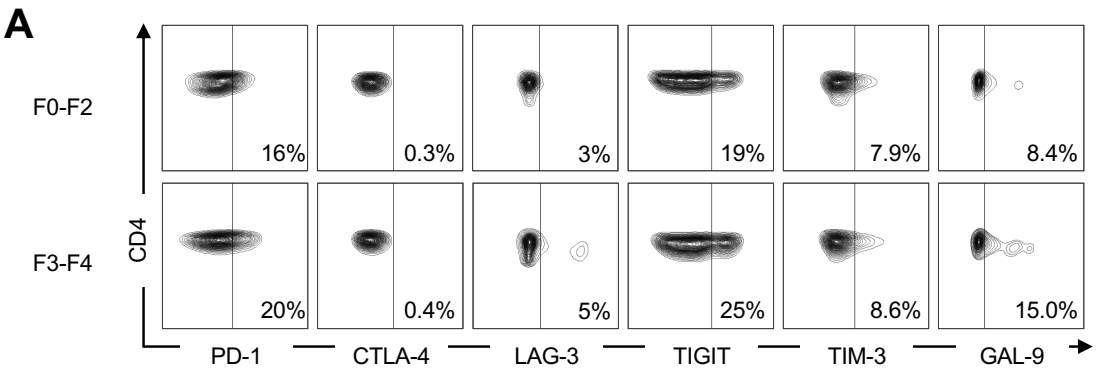
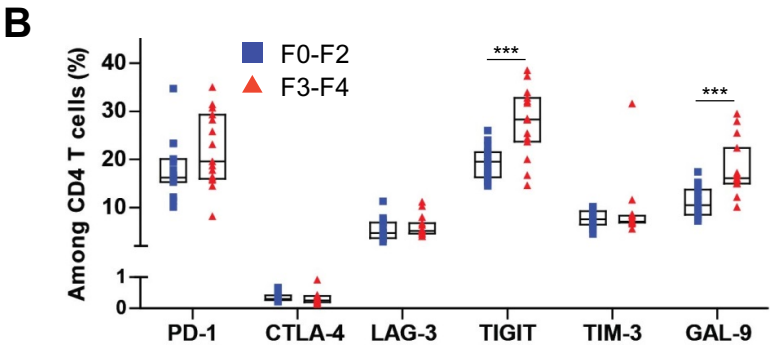

C
R2= 0.01053
p-value= 0.6255
Frequency of CTLA-4+ cells (%)
1.5
1.0
0.5
0.0
0
20
40
60
80
LSM (kPa)
R2= 0.3270
p-value= 0.0028
Frequency of GAL-9+ cells (%)
25
20
15
10
5
0
0
20
40
60
80
LSM (kPa)

Figure 8. The expression of inhibitory receptors and GAL-9 on the bulk $CD8^+$ T cells of HCV patients with liver fibrosis. Freshly thawed PBMCs of F0-F2 (n=15) and F3-F4 (n=15) chronically infected HCV patients were stained for surface expression of PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9. (A) Representative plot of PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9 expression on $CD8^+$ T cells of chronic HCV patients in fibrosis stages F0-F2 and F3-F4. (B) Cumulative data comparing PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9 expression on $CD8^+$ T cells of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. (C) Linear regression analysis of $CTLA\text{-}4^+$ and $GAL\text{-}9^+$ cell frequencies against LSM of chronic HCV patients (n=25). Liver Stiffness Measurement (LSM). R^2 is the coefficient of determination. Data are represented as median with IQR, and each point represents an individual. $^{**}p<0.01$. Statistically significant regression p-values are identified in red.


A
F0-F2
F3-F4
CD4
16%
0.3%
3%
19%
7.9%
8.4%
20%
0.4%
5%
25%
8.6%
15.0%
PD-1
CTLA-4
LAG-3
TIGIT
TIM-3
GAL-9


B
F0-F2
F3-F4
Among CD4 T cells (%)
***
***
PD-1
CTLA-4
LAG-3
TIGIT
TIM-3
GAL-9

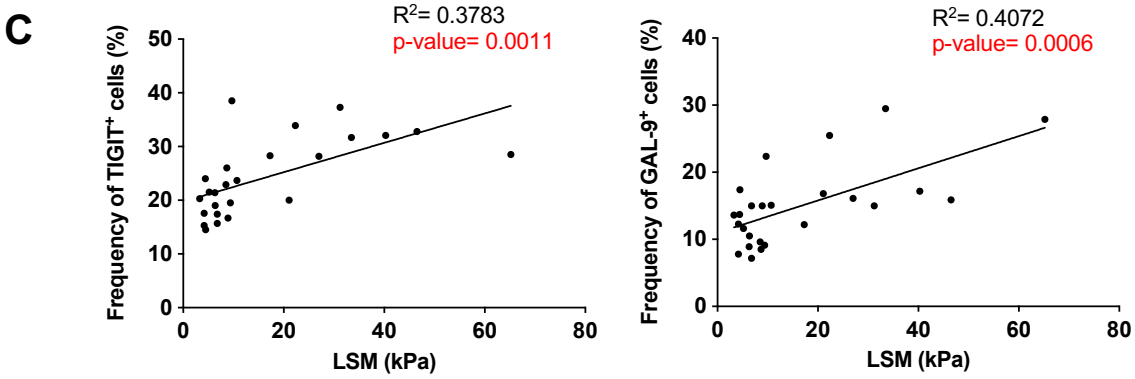

C
R²= 0.3783
p-value= 0.0011
Frequency of TIGIT⁺ cells (%)
LSM (kPa)
R²= 0.4072
p-value= 0.0006
Frequency of GAL-9⁺ cells (%)
LSM (kPa)

Figure 9. The expression of inhibitory receptors and GAL-9 on the bulk $CD4^+$ T cells of HCV patients with liver fibrosis. Freshly thawed PBMCs of F0-F2 (n=15) and F3-F4 (n=15) chronically infected HCV patients were stained for surface expression of PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9. (A) Representative plot of PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9 expression on $CD4^+$ T cells of chronic HCV patients in fibrosis stages F0-F2 and F3-F4. (B) Cumulative data comparing PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9 expression on $CD4^+$ T cells of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. (C) Linear regression analysis of $TIGIT^+$ and $GAL\text{-}9^+$ cell frequencies against LSM of chronic HCV patients (n=25). Liver Stiffness Measurement (LSM). R^2 is the coefficient of determination. Data are represented as median with IQR, and each point represents an individual. ***$p<0.001$. Statistically significant regression p-values are identified in red.

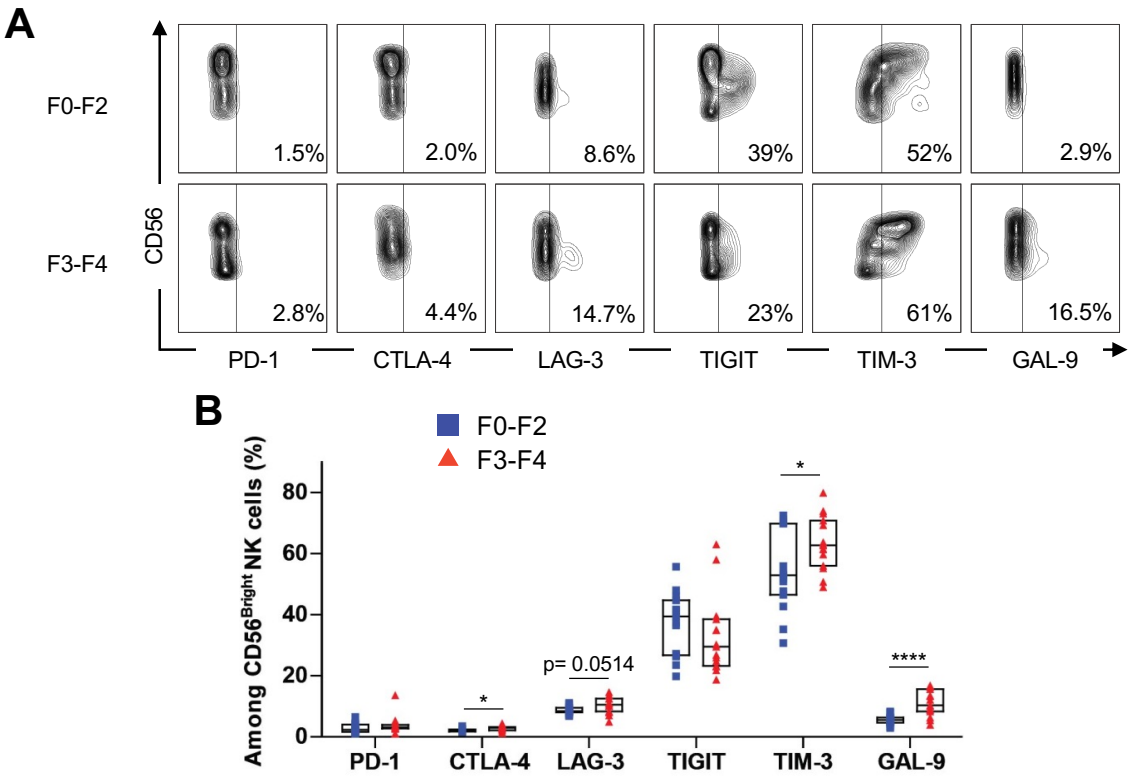

A
F0-F2
F3-F4
CD56
1.5%
2.0%
8.6%
39%
52%
2.9%
2.8%
4.4%
14.7%
23%
61%
16.5%
PD-1
CTLA-4
LAG-3
TIGIT
TIM-3
GAL-9
B
F0-F2
F3-F4
Among CD56Bright NK cells (%)
p= 0.0514
*
*
****
PD-1
CTLA-4
LAG-3
TIGIT
TIM-3
GAL-9
C
Frequency of CTLA-4+ cells (%)
R2= 0.1753
p-value= 0.0372
LSM (kPa)
Frequency of LAG-3+ cells (%)
R2= 0.1472
p-value= 0.0583
LSM (kPa)
Frequency of TIM-3+ cells (%)
R2= 0.1322
p-value= 0.0740
LSM (kPa)
Frequency of GAL-9+ cells (%)
R2= 0.08191
p-value= 0.1586
LSM (kPa)

Figure 10. The expression of inhibitory receptors and GAL-9 on CD56^{Bright} NK cells of HCV patients with liver fibrosis. Freshly thawed PBMCs of F0-F2 (n=15) and F3-F4 (n=15) chronically infected HCV patients were stained for surface expression of PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9. (A) Representative plot of PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9 expression on CD56^{Bright} NK cells of chronic HCV patients in fibrosis stages F0-F2 and F3-F4. (B) Cumulative data comparing PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9 expression on CD56^{Bright} NK cells of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. (C) Linear regression analysis of CTLA-4^{+}, LAG-3^{+}, TIM-3^{+} and GAL-9^{+} cell frequencies against LSM of chronic HCV patients (n=25). Liver Stiffness Measurement (LSM). R^2 is the coefficient of determination. Data are represented as median with IQR and each point represents an individual. *p<0.05, ****p<0.0001. Statistically significant regression p-values are identified in red.

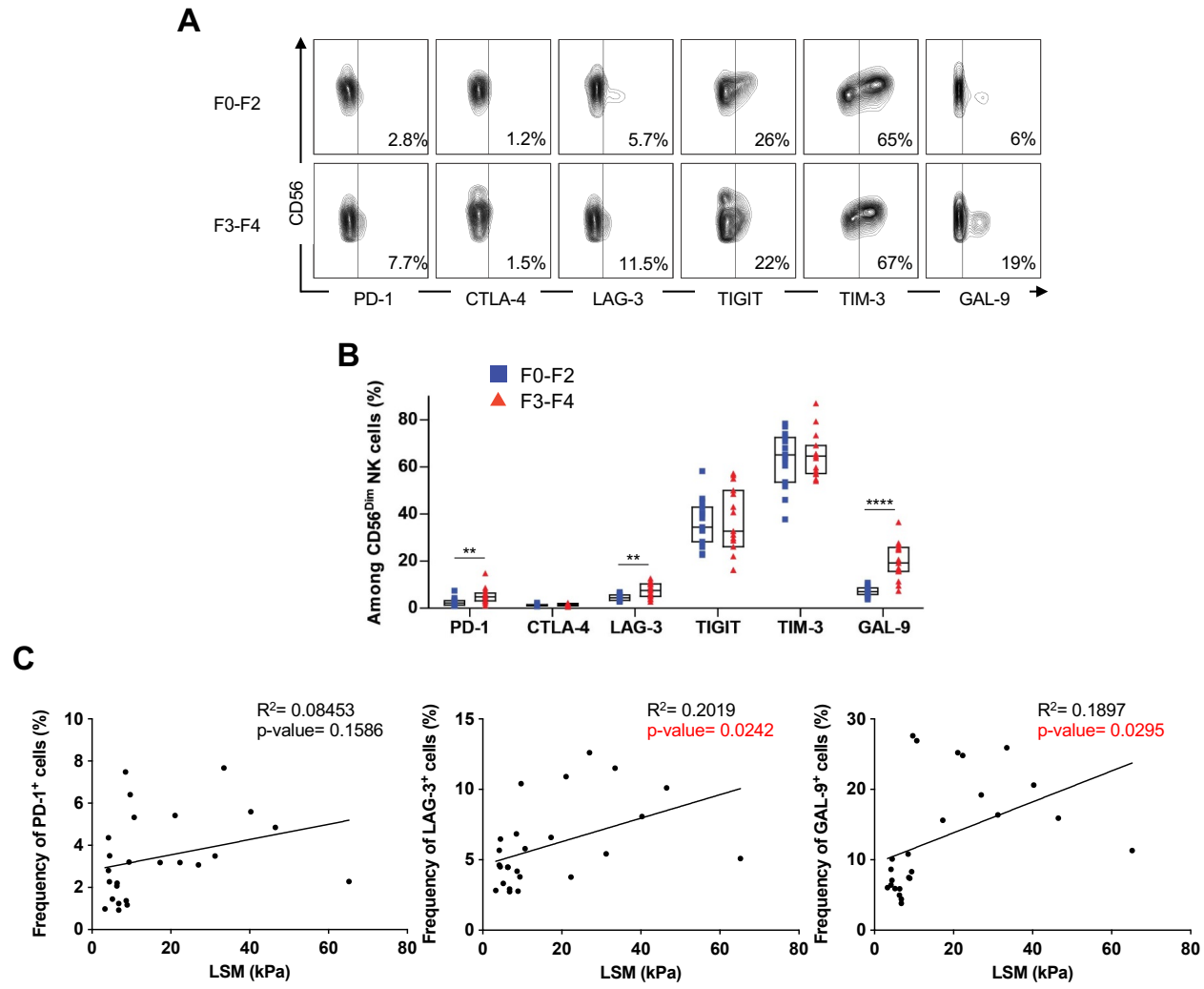

A
F0-F2
F3-F4
CD56
2.8%
1.2%
5.7%
26%
65%
6%
7.7%
1.5%
11.5%
22%
67%
19%
PD-1
CTLA-4
LAG-3
TIGIT
TIM-3
GAL-9
B
F0-F2
F3-F4
Among CD56Dim NK cells (%)
**
**
****
PD-1
CTLA-4
LAG-3
TIGIT
TIM-3
GAL-9
C
R2= 0.08453
p-value= 0.1586
Frequency of PD-1+ cells (%)
LSM (kPa)
R2= 0.2019
p-value= 0.0242
Frequency of LAG-3+ cells (%)
LSM (kPa)
R2= 0.1897
p-value= 0.0295
Frequency of GAL-9+ cells (%)
LSM (kPa)

Figure 11. The expression of inhibitory receptors and GAL-9 on $CD56^{Dim}$ NK cells of HCV patients with liver fibrosis. Freshly thawed PBMCs of F0-F2 (n=15) and F3-F4 (n=15) chronically infected HCV patients were stained for surface expression of PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9. (A) Representative plot of PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9 expression on $CD56^{Dim}$ NK cells of chronic HCV patients in fibrosis stages F0-F2 and F3-F4. (B) Cumulative data comparing PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, and GAL-9 expression on $CD56^{Dim}$ NK cells of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. (C) Linear regression analysis of $PD\text{-}1^{+}$, $LAG\text{-}3^{+}$ and $GAL\text{-}9^{+}$ cell frequencies against LSM of chronic HCV patients (n=25). Liver Stiffness Measurement (LSM). R^2 is the coefficient of determination. Data are represented as median with IQR, and each point represents an individual. **p<0.01, ****p<0.0001. Statistically significant regression p-values are identified in red.

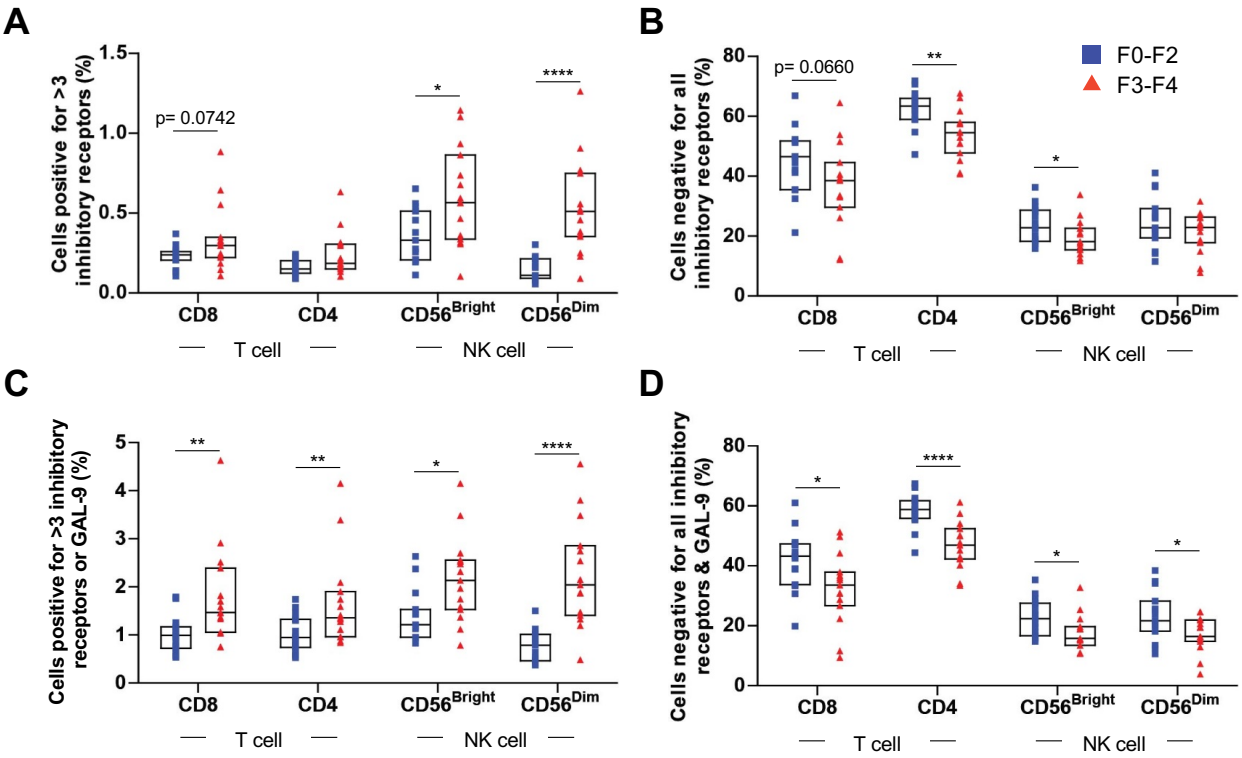

A
Cells positive for >3 inhibitory receptors (%)
p= 0.0742
*
****
CD8
CD4
CD56Bright
CD56Dim
T cell
NK cell
B
Cells negative for all inhibitory receptors (%)
p= 0.0660
**
*
F0-F2
F3-F4
C
Cells positive for >3 inhibitory receptors or GAL-9 (%)
D
Cells negative for all inhibitory receptors & GAL-9 (%)

Figure 12. Boolean analysis of inhibitory receptors and GAL-9 on lymphocytes of HCV patients with liver fibrosis. (A) Cumulative data comparing Boolean analysis of the frequency of T and NK cells expressing >3 inhibitory receptors (PD-1, CTLA-4, LAG-3, TIGIT, TIM-3). (B) Cumulative data comparing Boolean analysis of the frequency of T and NK cells expressing none of the five inhibitory receptors (PD-1, CTLA-4, LAG-3, TIGIT, TIM-3). (C) Cumulative data comparing Boolean analysis of the frequency of T and NK cells expressing >3 inhibitory receptors (PD-1, CTLA-4, LAG-3, TIGIT, TIM-3) or GAL-9. (D) Cumulative data comparing Boolean analysis of the frequency of T and NK cells expressing none of the five inhibitory receptors (PD-1, CTLA-4, LAG-3, TIGIT, TIM-3) and GAL-9. Data are represented as median with IQR and each point represents an individual. *$p<0.05$, **$p<0.01$, ****$p<0.0001$.

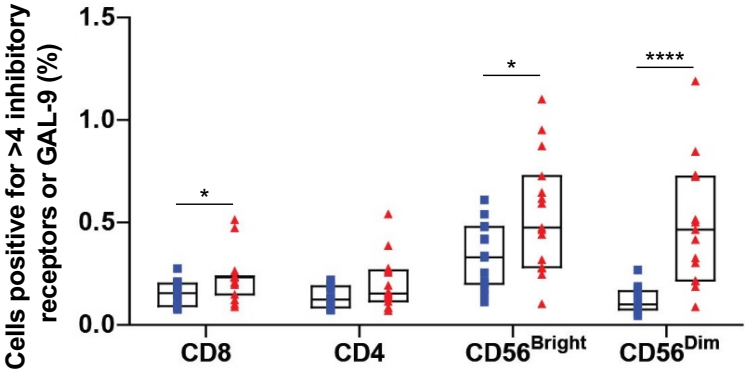

Cells positive for >4 inhibitory receptors or GAL-9 (%)
1.5
1.0
0.5
0.0
*
*
****
CD8
CD4
CD56Bright
CD56Dim

Figure 13. Boolean analysis of inhibitory receptors and GAL-9 on lymphocytes of HCV patients with liver fibrosis. Cumulative data comparing Boolean analysis of the frequency of T and NK cells expressing >4 inhibitory receptors (PD-1, CTLA-4, LAG-3, TIGIT, TIM-3) or GAL-9. Data are represented as median with IQR and each point represents an individual. *$p<0.05$, ****$p<0.0001$.

3.3 Higher Frequencies of CD25^{+}TIGIT$^{med\text{-}hi}$ CD4^{+} T Cells and PD-1^{med} CD4^{+} T Cells Co-expressing LAG-3 and GAL-9 in Patients with Advanced Liver Fibrosis.

I have shown that the expression of multiple inhibitory receptors and GAL-9 is a characteristic of patients with advanced liver fibrosis (**Fig. 12A & C**). Thus, I decided to investigate the co-expression of inhibitory receptors and GAL-9 on the surface of T cells. The t-SNE analysis was employed to dimensionally reduce the expression data of cell surface markers, inhibitory receptors, and GAL-9 from all 30 HCV patients, and to visualize expression patterns in two dimensions[222]. t-SNE analysis of all 30 patients (F0-F4) revealed several clusters, and separating the plot based on the fibrosis scores showed the distribution of cell densities (**Fig. 14A**). I analyzed the expression of T cell subset markers (CD8, CD4, CD45RO, CCR7, and CD25) to characterize T cell clusters (**Fig. 14B**). To visualize the co-expression patterns of the inhibitory receptors and GAL-9, I classified their expressions as either low, medium (med), and high (hi) (**Fig. 14C & Fig. 15**). Through this classification, T cells were found to be either PD-1^{low} or PD-1^{med}; CTLA-4^{low} or CTLA-4^{med}; LAG-3^{low}, LAG-3^{med} or LAG-3^{hi}; TIGIT^{low}, TIGIT^{med} or TIGIT^{hi}; TIM-3^{low}, TIM-3^{med} or TIM-3^{hi} and GAL-9^{low}, GAL-9^{med} or GAL-9^{hi} (**Fig. 14C**).

Prior to cluster analysis, t-SNE plots revealed a high co-expression of GAL-9$^{med\text{-}hi}$ and LAG-3$^{med\text{-}hi}$ on both CD8^{+} and CD4^{+} T cells with PD-1^{med} and TIM-3^{low} expression (**Fig. 14C**). The t-SNE cluster analysis further generated 8 clusters based on the expression of cell subset surface markers, inhibitory receptors and GAL-9 as follows: (1) Naïve CD8 T cells; (2) T_{EM} and T_{CM} CD8 T cells; (3) T_{EMRA} TIGIT$^{med\text{-}hi}$ CD8 T cells; (4) T_{EM} and T_{CM} CD4 T cells; (5) T_{Naive} and T_{CM} CD4 T cells; (6) CD25^{+} TIGIT$^{med\text{-}hi}$ CD4 T cells; (7) LAG-3$^{med\text{-}hi}$GAL-9$^{med\text{-}hi}$ PD-1^{med} CD4 T cells and (8) LAG-3$^{med\text{-}hi}$GAL-9$^{med\text{-}hi}$ PD-1^{med} CD8 T cells (**Fig. 16A**). I next compared the T cell frequency of F0-F2 patients and F3-F4 patients in each cluster (**Fig. 16B**). Notably, I found that

F3-F4 patients had less of their T cells found in cluster 1 (Naïve CD8 T cells), but more found in clusters 6 ($CD25^+$ $TIGIT^{med\text{-}hi}$ CD4 T cells), and cluster 7 ($LAG\text{-}3^{med\text{-}hi}GAL\text{-}9^{med\text{-}hi}$ $PD\text{-}1^{med}$ CD4 T cells) when compared to F0-F2 patients. This data indicates that more advanced stages of fibrosis are associated with a higher frequency of $CD25^+$ CD4 T cells with high TIGIT expression. In addition, it indicates that GAL-9 and LAG-3 are highly co-expressed on $PD\text{-}1^{med}$ cells, and this co-expression is found in both $CD8^+$ and $CD4^+$ T cells. Lastly, F3-F4 patients have an increased frequency of these $LAG\text{-}3^{med\text{-}hi}GAL\text{-}9^{med\text{-}hi}$ $PD\text{-}1^{med}$ $CD4^+$ T cells.

To correlate the identified cell clusters with liver damage, I conducted linear regression analyses between patients' LSM, and the frequency of cells found in significantly different clusters (cluster 1, 6, and 7). I found a significant positive correlation of cluster 6 and 7 frequencies with LSM, but no correlation with cluster 1 (**Fig. 16C**). Similar to LSM, AST, ALT, and AST/ALT are clinical parameters used to identify and measure the extent of liver damage in chronic HCV patients. Increased levels of AST and ALT are indicative of hepatocytic injury. However, a better measure of cirrhosis is AST/ALT. A high AST/ALT is a signal of extensive fibrosis in patients with HCV infections[223]. Thus, I correlated AST/ALT, AST levels and ALT levels with the frequency of cells in cluster 1, 6 and 7 (**Fig. 17A-C**). I found that the only significant correlation was of cluster 6 frequencies with the AST/ALT levels. Taken together, using the t-SNE analysis, I identified high TIGIT expression on $CD25^+$ $CD4^+$ T cells and high LAG-3 and GAL-9 co-expression on $PD\text{-}1^{med}$ $CD4^+$ T cells and these expression patterns are associated with advanced liver fibrosis.

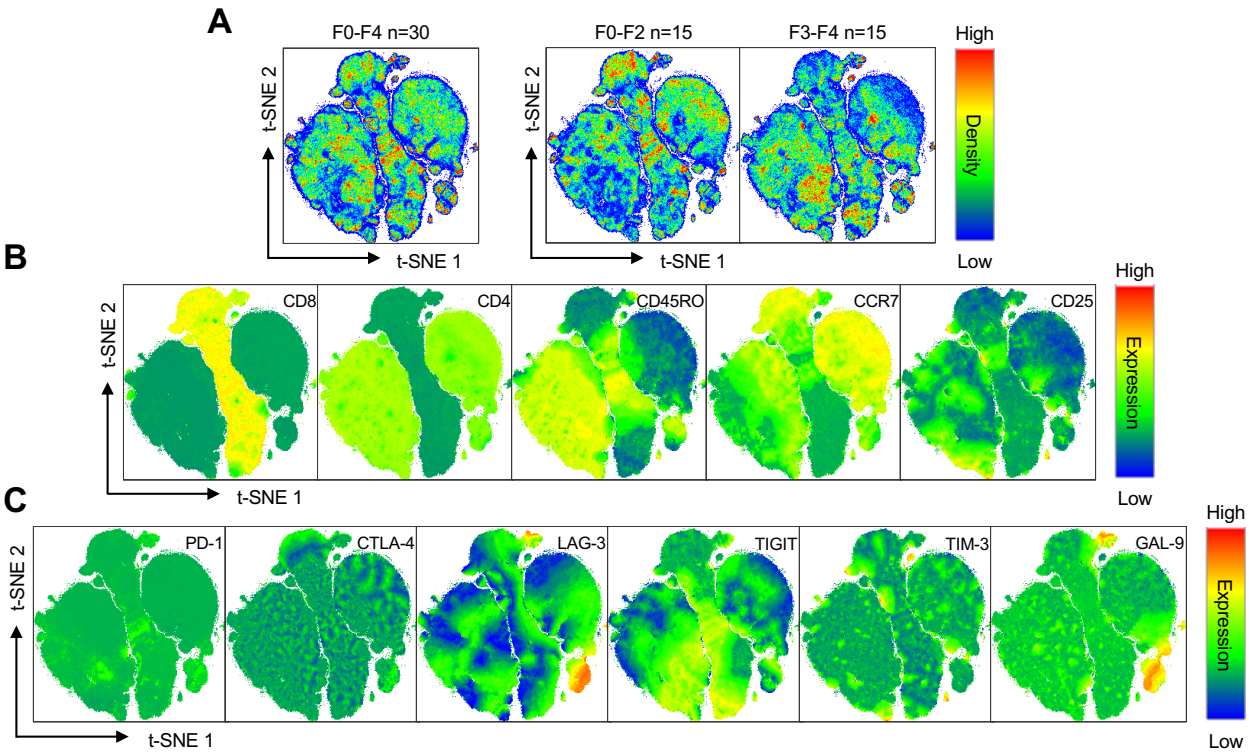

A
F0-F4 n=30
F0-F2 n=15
F3-F4 n=15
t-SNE 2
t-SNE 1
High
Density
Low
B
CD8
CD4
CD45RO
CCR7
CD25
High
Expression
Low
C
PD-1
CTLA-4
LAG-3
TIGIT
TIM-3
GAL-9
High
Expression
Low

Figure 14. t-SNE analysis of bulk T cells of chronic HCV patients. $CD3^+$ cells excluding $CD4^-$ $CD8^-$ cells from F0-F2 (n=15) and F3-F4 (n=15) chronically infected HCV patients, were combined to conduct t-SNE analysis. (A) Merged T cell t-SNE analysis of 30 chronic HCV patients followed by the analysis of F0-F2 and F3-F4 patients separately. (B) Expression of T cell subset markers (CD8, CD4, CD45RO, CCR7, and CD25) in t-SNE analysis. (C) Expression of inhibitory receptors (PD-1, CTLA-4, LAG-3, TIGIT, TIM-3) and GAL-9 in t-SNE analysis.

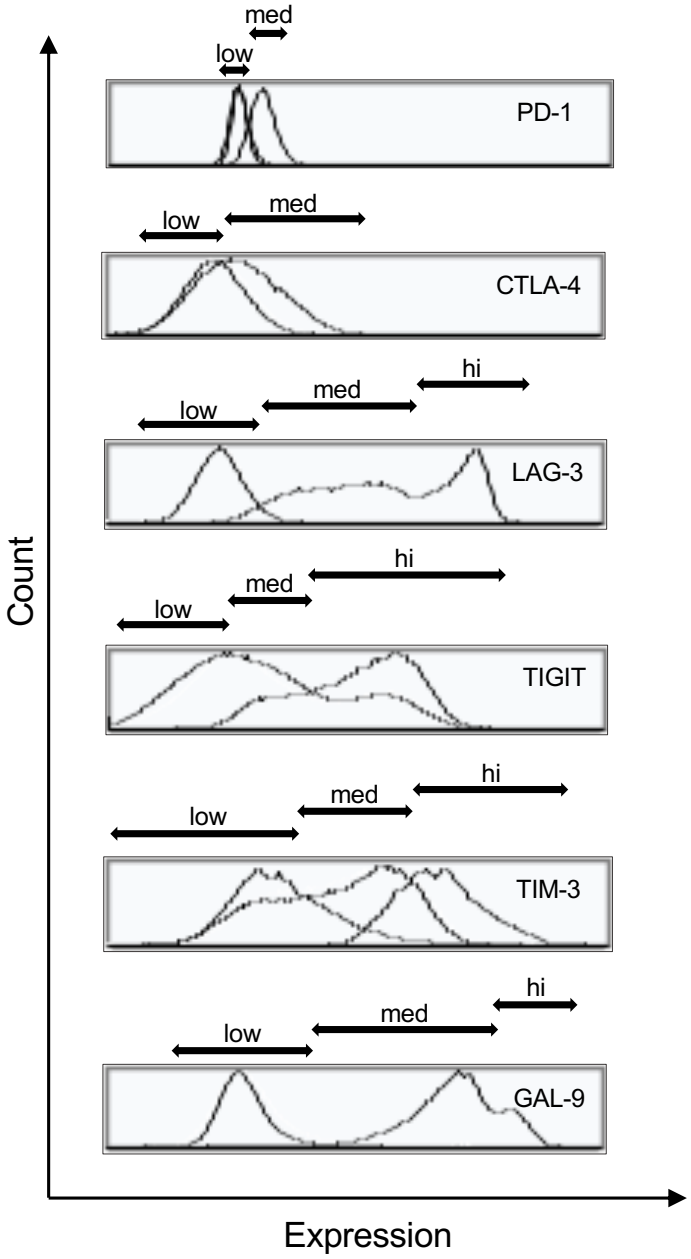

med
low
PD-1
low
med
CTLA-4
hi
med
low
LAG-3
hi
med
low
TIGIT
hi
med
low
TIM-3
hi
med
low
GAL-9
Count
Expression

Figure 15. Representative histograms used to identify and analyze inhibitory receptor and GAL-9 expressions in T and NK cell t-SNE plots. The expression of receptors in each t-SNE cluster was classified as either low, med or hi based on the histogram expression profile of the cluster. Only low and med PD-1 and CTLA-4 expression were identified. Multiple histograms were overlayed to define the differential expression.

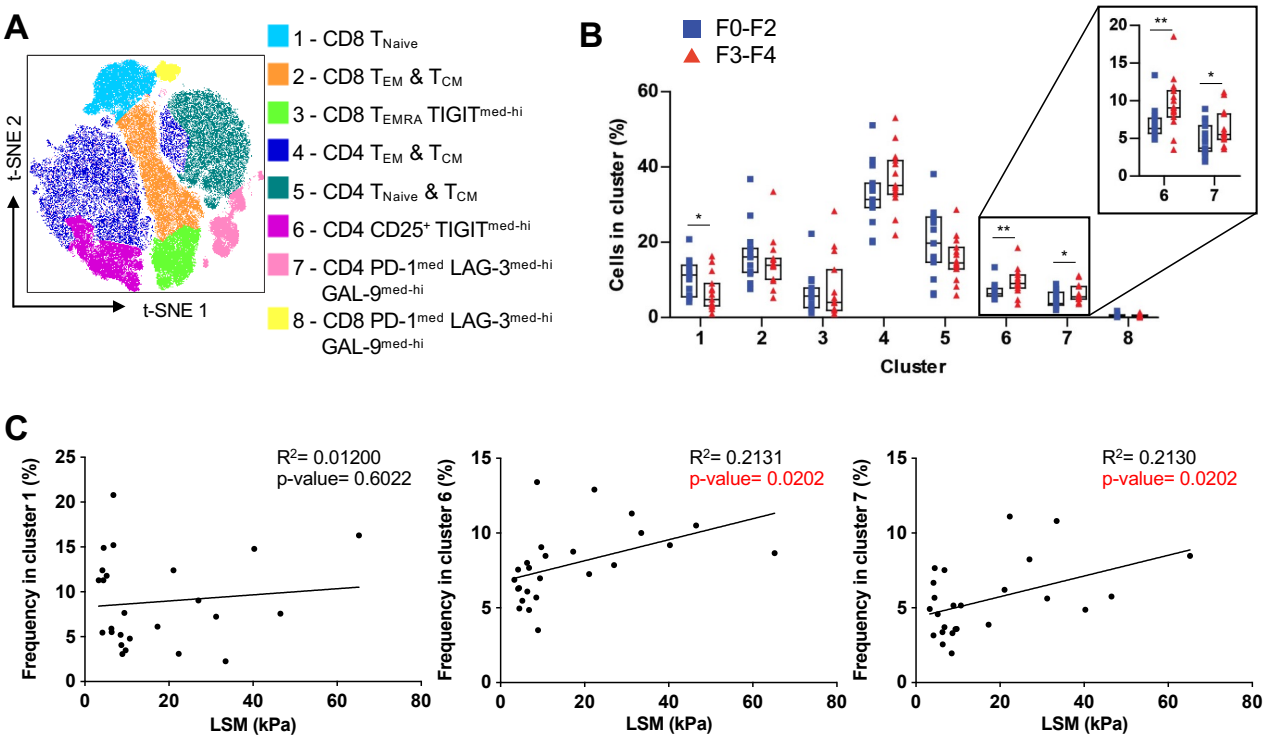

A
t-SNE 2
t-SNE 1
1 - CD8 T Naive
2 - CD8 T EM & T CM
3 - CD8 T EMRA TIGIT med-hi
4 - CD4 T EM & T CM
5 - CD4 T Naive & T CM
6 - CD4 CD25+ TIGIT med-hi
7 - CD4 PD-1 med LAG-3 med-hi GAL-9 med-hi
8 - CD8 PD-1 med LAG-3 med-hi GAL-9 med-hi
B
F0-F2
F3-F4
Cells in cluster (%)
Cluster
C
R2= 0.01200
p-value= 0.6022
Frequency in cluster 1 (%)
LSM (kPa)
R2= 0.2131
p-value= 0.0202
Frequency in cluster 6 (%)
LSM (kPa)
R2= 0.2130
p-value= 0.0202
Frequency in cluster 7 (%)
LSM (kPa)

Figure 16. Cluster analysis of bulk T cells of chronic HCV patients. $CD3^+$ cells excluding $CD4^-$ $CD8^-$ cells from F0-F2 (n=15) and F3-F4 (n=15) chronically infected HCV patients, were combined to conduct t-SNE analysis. (A) Clustering of t-SNE analysis based on subset markers, inhibitory receptor, and GAL-9 expression. (B) Cumulative data comparing the proportion of F0-F2 and F3-F4 patients T cells found in each cluster. (C) Linear regression analysis of cluster 1, 6, and 7 frequencies against LSM of chronic HCV patients (n=25). Liver Stiffness Measurement (LSM). R^2 is the coefficient of determination. Cluster analysis data are represented as median with IQR, and each point represents an individual. *p<0.05, **p<0.01. Statistically significant regression p-values are identified in red.

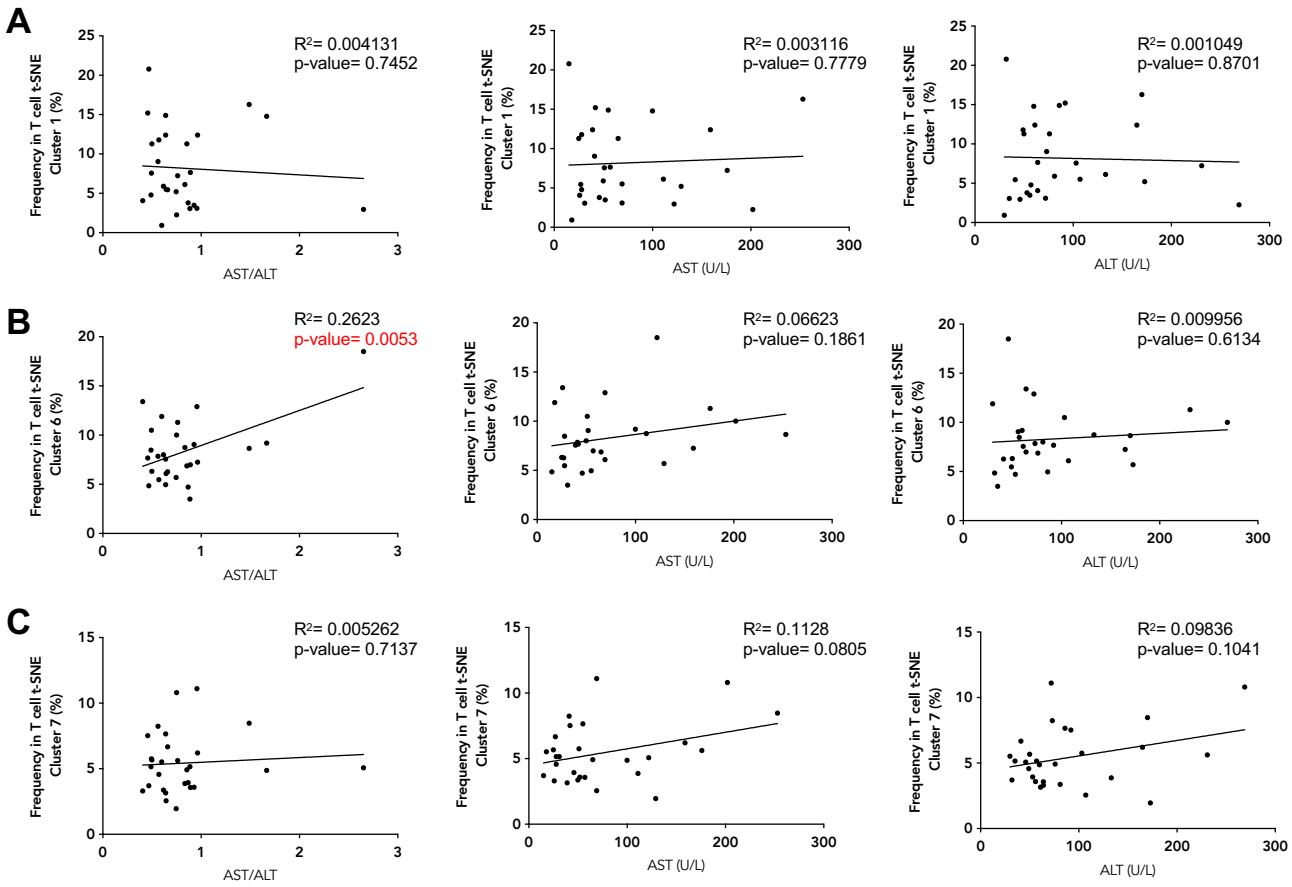

A
Frequency in T cell t-SNE Cluster 1 (%)
R²= 0.004131
p-value= 0.7452
AST/ALT
R²= 0.003116
p-value= 0.7779
AST (U/L)
R²= 0.001049
p-value= 0.8701
ALT (U/L)
B
Frequency in T cell t-SNE Cluster 6 (%)
R²= 0.2623
p-value= 0.0053
AST/ALT
R²= 0.06623
p-value= 0.1861
AST (U/L)
R²= 0.009956
p-value= 0.6134
ALT (U/L)
C
Frequency in T cell t-SNE Cluster 7 (%)
R²= 0.005262
p-value= 0.7137
AST/ALT
R²= 0.1128
p-value= 0.0805
AST (U/L)
R²= 0.09836
p-value= 0.1041
ALT (U/L)

Figure 17. Cluster correlation analysis of bulk T cells of chronic HCV patients. $CD3^+$ cells excluding $CD4^-CD8^-$ cells from F0-F2 (n=15) and F3-F4 (n=15) chronically infected HCV patients, were combined to conduct t-SNE analysis. (A-C) Linear regression analysis of cluster 1, 6, and 7 frequencies against AST/ALT, AST and ALT of chronic HCV patients (n=28). R^2 is the coefficient of determination. Statistically significant regression p-values are identified in red.

3.4 Higher Frequencies of CD56^{Dim} NK Cells Co-expressing LAG-3, GAL-9 and PD-1 as well as TIGIT and TIM-3 in Patients with Advanced Liver Fibrosis.

t-SNE analysis of T cells revealed high co-expression of GAL-9 and LAG-3 on CD4 and CD8 T cells. To determine co-expression patterns of inhibitory receptors on NK cells, I employed t-SNE analysis on CD56^{+} NK cells, first combining the plots derived from all 30 patients, irrespective of fibrosis stage, and then separating plots based on the stage of fibrosis, F0-F2 and F3-F4, (**Fig. 18A**). The expression of subset markers on NK cells was classified as CD56^{Dim} or CD56^{Bright}; CD16^{-}, CD16^{Dim} or CD16^{Bright}; CCR7^{-} or CCR7^{+} and CD25^{-} or CD25^{+} (**Fig. 18B**). The expression of inhibitory receptors was classified as PD-1^{low} or PD-1^{med}; CTLA-4^{low} or CTLA-4^{med}, LAG-3^{low}, LAG-3^{med} or LAG-3^{hi}; TIGIT^{low}, TIGIT^{med} or TIGIT^{hi}, TIM-3^{low}, TIM-3^{med} or TIM-3^{hi} and GAL-9^{low}, GAL-9^{med} or GAL-9^{hi} (**Fig. 18C**). Using this classification, NK cells were divided into 6 clusters (**Fig. 19A**) as follows: (1) CD56^{Bright}CD16^{-} TIM-3^{med-hi} cells, (2) CD56^{Dim}CD16^{-} cells, (3) CD56^{Dim}CD16^{Bright} TIM-3^{med-hi} cells, (4) CD56^{Dim} CD25^{+}TIGIT^{med-hi}TIM-3^{med-hi} cells, (5) CD56^{Dim} CCR7^{+}PD-1^{med}LAG-3^{med-hi}GAL-9^{med-hi} cells, and (6) CD56$^{Bright/Dim}$CD16^{Dim} cells.

I compared the frequency of patients' NK cells in each cluster (**Fig. 19B**) and found that F3-F4 patients had a significantly higher frequency of their NK cells found in clusters 4 (CD56^{Dim} CD25^{+}TIGIT^{med-hi}TIM-3^{med-hi} cells), and cluster 5 (CD56^{Dim} CCR7^{+}PD-1^{med}LAG-3^{med-hi}GAL-9^{med-hi} cells). Interestingly, a higher frequency of CD25^{+} CD56^{Dim} NK cells in F3-F4 patients was observed in Fig. 7C, and t-SNE analysis identified the co-expression of TIGIT and TIM-3 on these CD25^{+} CD56^{Dim} NK cells. Similarly, a higher frequency of CCR7^{+} CD56^{Dim} NK cells in F3-F4 patients was observed in Fig. 7E, and t-SNE analysis identified the co-expression of LAG-3, GAL-9 and PD-1 on these CCR7^{+} CD56^{Dim} NK cells. Linear regression analysis was employed correlating patients' LSM with the frequency of cells found in clusters 4 and 5. I found a significant

positive correlation of cluster 4 frequencies with LSM and a correlation in cluster 5, although this did not rise to the level of statistical significance, p= 0.0863, (**Fig. 19C**). When the frequency of cluster 4 and 5 was correlated with AST/ALT, AST and ALT, there was a significant positive correlation between cluster 4 and AST as well as ALT levels (**Fig. 20 A&B**). Taken together, using t-SNE analysis, I identified $CD25^{+}TIGIT^{med\text{-}hi}TIM\text{-}3^{med\text{-}hi}$ cells, and $CCR7^{+}PD\text{-}1^{med}LAG\text{-}3^{med\text{-}hi}GAL\text{-}9^{med\text{-}hi}$ cells, and these co-expression patterns on $CD56^{Dim}$ NK cells are associated with advanced liver fibrosis.

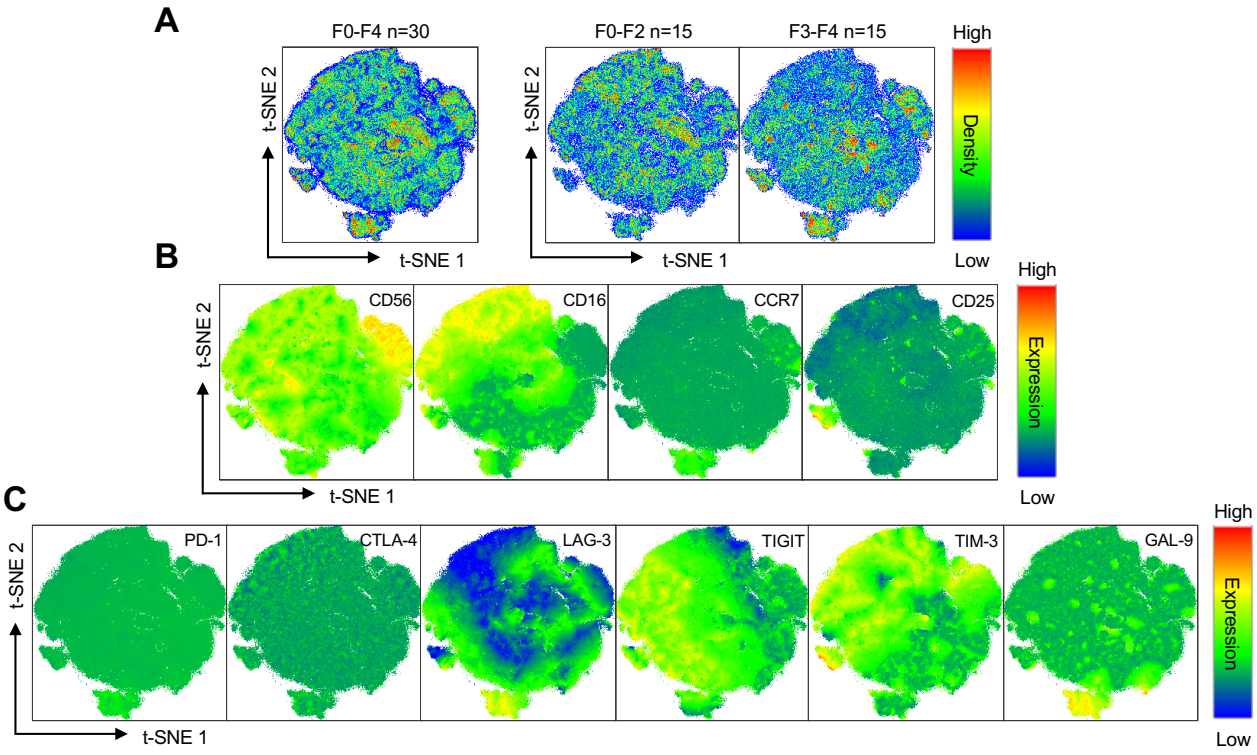

A
F0-F4 n=30
F0-F2 n=15
F3-F4 n=15
High
Density
Low
t-SNE 2
t-SNE 1
B
CD56
CD16
CCR7
CD25
High
Expression
Low
C
PD-1
CTLA-4
LAG-3
TIGIT
TIM-3
GAL-9
High
Expression
Low

Figure 18. t-SNE analysis of NK cells of chronic HCV patients. $CD56^{+}$ NK cells from F0-F2 (n=15) and F3-F4 (n=15) chronically infected HCV patients were combined to conduct t-SNE analysis. (A) Merged NK cell t-SNE analysis of 30 chronic HCV patients, followed by the analysis of F0-F2 and F3-F4 patients separately. (B) Expression of NK cell subset markers (CD56, CD16, CCR7, and CD25) in the t-SNE analysis. (C) Expression of inhibitory receptors (PD-1, CTLA-4, LAG-3, TIGIT, TIM-3) and GAL-9 in the t-SNE analysis.

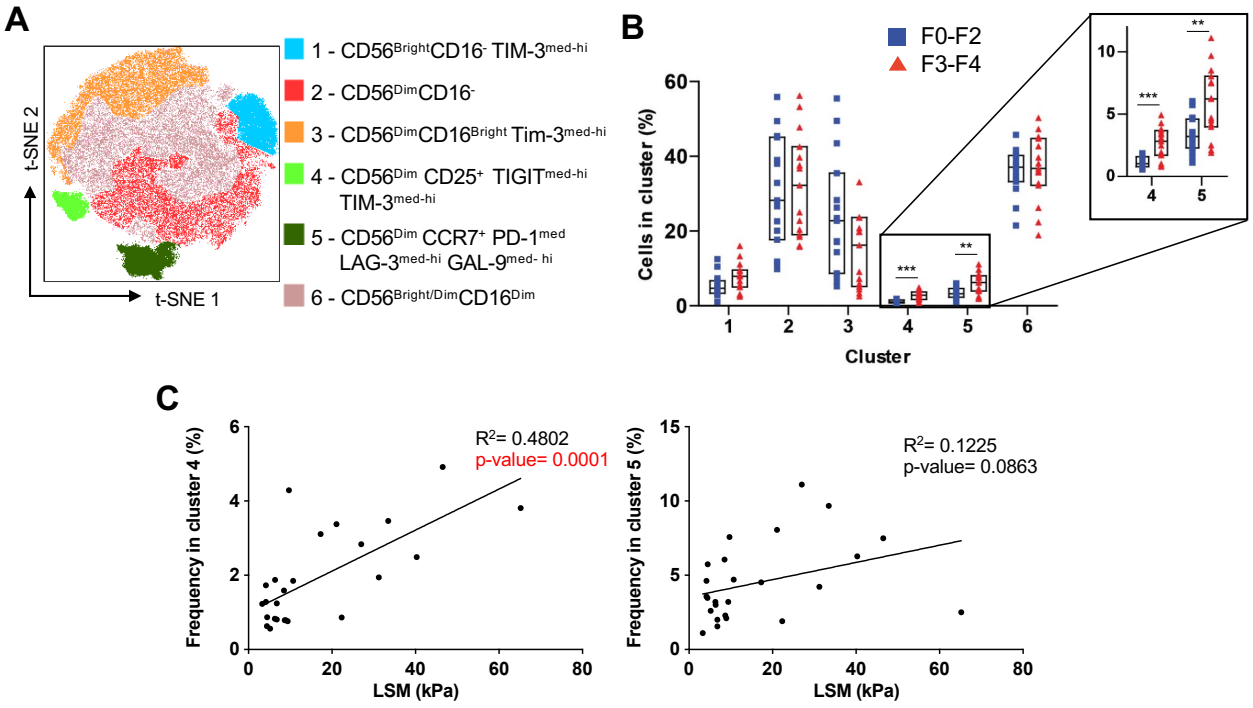

A
t-SNE 2
t-SNE 1
1 - CD56Bright CD16- TIM-3med-hi
2 - CD56Dim CD16-
3 - CD56Dim CD16Bright Tim-3med-hi
4 - CD56Dim CD25+ TIGITmed-hi TIM-3med-hi
5 - CD56Dim CCR7+ PD-1med LAG-3med-hi GAL-9med- hi
6 - CD56Bright/Dim CD16Dim
B
F0-F2
F3-F4
Cells in cluster (%)
Cluster
C
Frequency in cluster 4 (%)
R2= 0.4802
p-value= 0.0001
LSM (kPa)
Frequency in cluster 5 (%)
R2= 0.1225
p-value= 0.0863
LSM (kPa)

Figure 19. Cluster analysis of NK cells of chronic HCV patients. $CD56^+$ NK cells from F0-F2 (n=15) and F3-F4 (n=15) chronically infected HCV patients were combined to conduct t-SNE analysis. (A) Clustering of t-SNE analysis based on subset markers, inhibitory receptor, and GAL-9 expression. (B) Cumulative data comparing the proportion of F0-F2 and F3-F4 patients NK cells found in each cluster. (C) Linear regression analysis of cluster 4 and 5 frequencies against LSM of chronic HCV patients (n=25). Liver Stiffness Measurement (LSM). R^2 is the coefficient of determination. Cluster analysis data are represented as median with IQR and each point represents an individual. **p<0.01, ***p<0.001. Statistically significant regression p-values are identified in red.

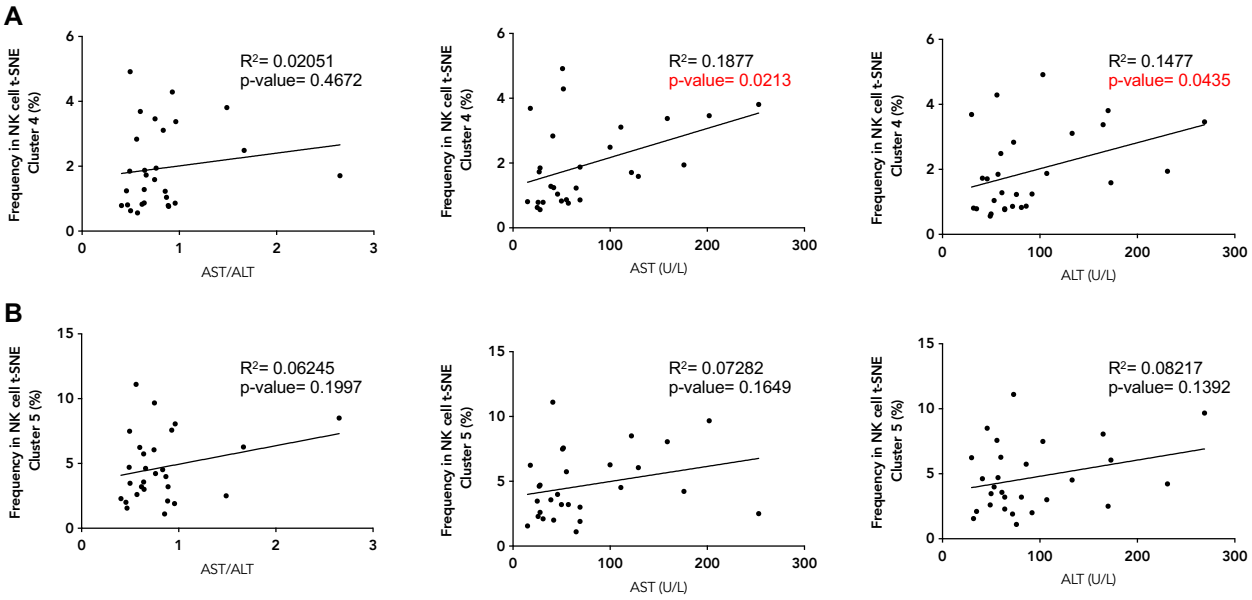

A
Frequency in NK cell t-SNE Cluster 4 (%)
R²= 0.02051
p-value= 0.4672
AST/ALT
R²= 0.1877
p-value= 0.0213
AST (U/L)
R²= 0.1477
p-value= 0.0435
ALT (U/L)
B
Frequency in NK cell t-SNE Cluster 5 (%)
R²= 0.06245
p-value= 0.1997
AST/ALT
R²= 0.07282
p-value= 0.1649
AST (U/L)
R²= 0.08217
p-value= 0.1392
ALT (U/L)

Figure 20. Cluster correlation analysis of NK cells of chronic HCV patients. $CD56^+$ NK cells from F0-F2 (n=15) and F3-F4 (n=15) chronically infected HCV patients were combined to conduct t-SNE analysis. (A & B) Linear regression analysis of cluster 4 and 5 frequencies against AST/ALT, AST and ALT of chronic HCV patients (n=28). R^2 is the coefficient of determination. Statistically significant regression p-values are identified in red.

3.5 Higher Frequency of TIGIT$^+$ Cells among Bulk T_{regs} than T_{effs}.

T_{regs} play an important role in downregulating immune responses and they are the major expressers of the IL-2Rα-chain, CD25, in the CD4 population. I first investigated the expression of CD25 on the bulk CD4 cells in the two groups and found that F3-F4 patients had a higher frequency of CD25$^+$ cells when compared to F0-F2 patients (**Fig. 21A**). This suggested that F3-F4 patients had a higher frequency of T_{regs} than F0-F2 patients. T_{regs} are defined in part by CD25^{High} expression, and the transcriptional factor FoxP3. Conversely, CD25$^+$ cells, which are FoxP3$^-$ are T effector cells (T_{effs}). Thus, I then determined the frequency of bulk T_{regs} in the two patient groups and found no significant difference in frequencies (**Fig. 21B & C)**. Next, I investigated the expression of TIGIT on T_{regs} and T_{effs} as t-SNE analysis of bulk T cells showed F3-F4 patients had a higher frequency of cells in the CD25$^+$TIGIT^{med-hi} CD4 cluster than F0-F2 patients (**Fig. 16B**). What's more, this cluster was found to have a significant positive correlation with the extent of patients' liver damage (**Fig. 16C**).

I found that TIGIT is highly expressed on T_{regs} compared to T_{effs} (**Fig. 21D**). More specifically, about 80% of T_{regs} express TIGIT while only about 15% of T_{effs} express TIGIT. No difference was seen between the F0-F2 patient population and the F3-F4 population. To understand the contribution of T_{regs} to T cell cluster 6, I determined what proportion of CD25$^+$ cells were T_{regs}. T_{regs} were found to make up only about 8% of the CD25$^+$ populations (**Fig. 21E**). However, when I determined what proportion of CD25$^+$TIGIT$^+$ CD4 cells were T_{regs}, I found that T_{regs} comprised over 50% of this population (**Fig. 21F**). This indicates that there is likely an over-representation of T_{regs} in cluster 6.

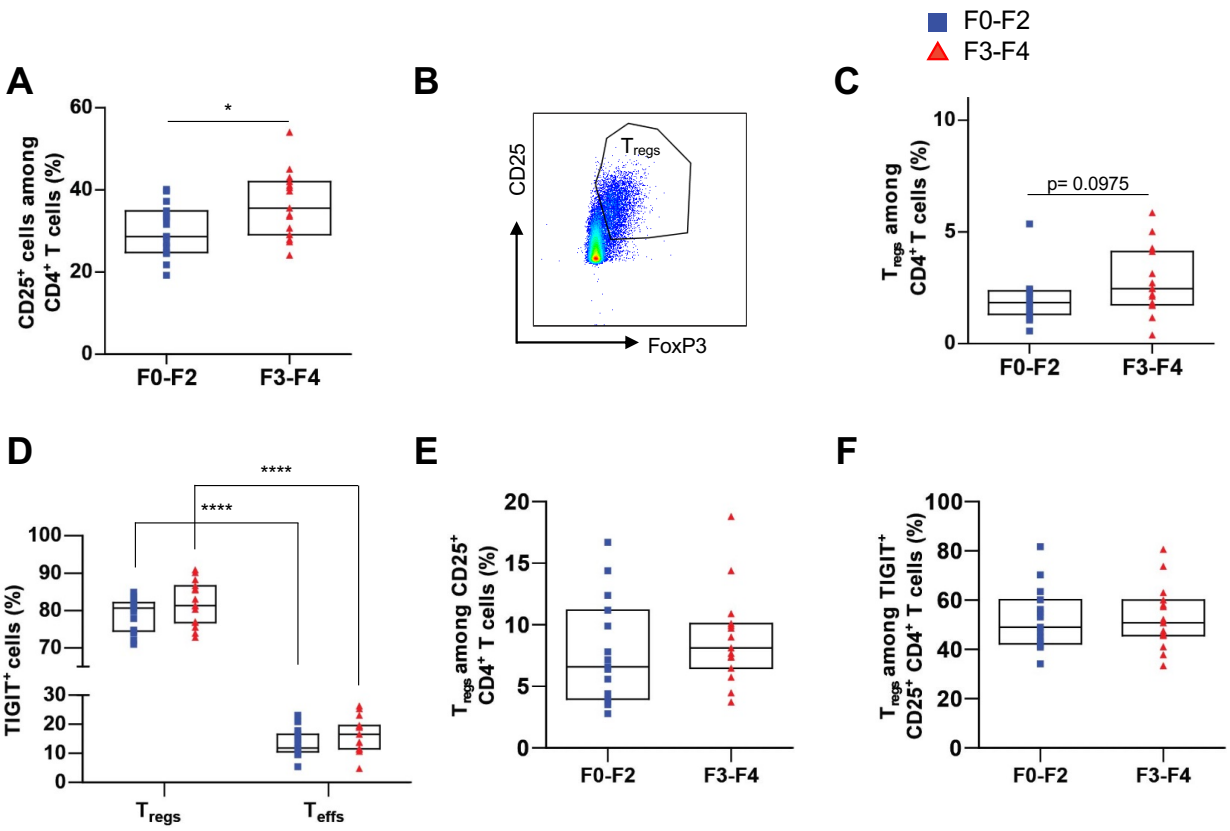

A
B
C
F0-F2
F3-F4
CD25+ cells among CD4+ T cells (%)
*
F0-F2
F3-F4
CD25
FoxP3
Tregs
Treg among CD4+ T cells (%)
p= 0.0975
D
E
F
TIGIT+ cells (%)
****
****
Tregs
Teffs
Treg among CD25+ CD4+ T cells (%)
Treg among TIGIT+ CD25+ CD4+ T cells (%)

Figure 21. TIGIT expression on bulk T_{regs} and T_{effs} of patients with chronic HCV and liver fibrosis. Freshly thawed PBMCs of F0-F2 (n=15), and F3-F4 (n=15) chronically infected HCV patients were stained for surface expression of CD25 and TIGIT, and intracellular expression of FoxP3. (A) Cumulative data comparing the frequency of $CD25^+$ cells among $CD4^+$ T cells of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. (B) Representative plot showing the T_{reg} ($CD25^+FoxP3^+$) population. (C) Cumulative data comparing the frequency of T_{regs} among $CD4^+$ T cells of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. (D) Cumulative data comparing TIGIT expression on T_{regs} and T_{effs} of chronic HCV patients in fibrosis stage F0-F2 and F3-F4. (E) Cumulative data comparing the frequency of T_{regs} among $CD25^+$ CD4 T cells. (F) Cumulative data comparing the frequency of T_{regs} among $CD25^+TIGIT^+$ CD4 T cells. T regulatory cell (T_{reg}) and T effector (T_{effs}). Data are represented as median with IQR and each point represents an individual. *p<0.05, ****p<0.0001.

3.6 Expression of Inhibitory Receptors and Subset Markers on GAL-9^{+} Cells Compared to GAL-9^{-} Cells.

I found that GAL-9 was the only tested inhibitory protein where F3-F4 patients had a higher frequency of positive cells in the bulk CD8/CD4 T cell and CD56^{Bright}/CD56^{Dim} NK populations (**Fig. 8B, 9B, 10B & 11B)**. Also, I found that F3-F4 patients had a significantly higher frequency of NK cells positive for >3 inhibitory receptors simultaneously and a lower frequency of CD4 T cells and CD56^{Bright} NK cells being negative for all inhibitory receptors (**Fig. 12A & B)**. However, by including GAL-9 in the Boolean analysis, I found that in both T cell subsets and both NK cell subsets, F3-F4 patients showed a significantly higher frequency of cells positive for >3 inhibitory receptors or GAL-9 than F0-F2 patients. Furthermore, in both T cell subsets and both NK cell subsets, F3-F4 patients showed a significantly lower frequency of cells negative for all inhibitory receptors and GAL-9 than F0-F2 patients (**Fig. 12C & D)**. Given the undeniable significance of GAL-9 expression on the lymphocytes of patients with advanced liver fibrosis, I sought to better understand GAL-9^{+} cells by exploring their expression of tested inhibitory receptors as well as CD45RO, CCR7 and CD25 compared to GAL-9^{-} cells.

First, I investigated the expression of PD-1, undoubtedly one of the most extensively studied inhibitory receptors, on GAL-9^{+} cells. I found that on both T cells and NK cells, GAL-9^{+} cells had higher PD-1 expression compared to GAL-9^{-} cells (**Fig. 22A**). For CTLA-4, another well studied inhibitory receptor, GAL-9^{+} and GAL-9^{-} cells showed no difference in expression but this could most likely be due to the very minimal CTLA-4 surface expression seen (**Fig. 22B**). Interestingly, LAG-3 expression on GAL-9^{+} cells was found to be quite unique. LAG-3 is the only tested inhibitory receptor whose pattern on GAL-9^{-} versus GAL-9^{+} cells substantially deviates between T cells and NK cells (**Fig. 22C**). Although on both T and NK cells, the majority of the

GAL-9^{+} cells have LAG-3 expression, both NK cell populations show one GAL-9^{+}LAG-3^{+} population. T cells however appear to have at least 5 different discernable populations. One GAL-9^{-}LAG-3^{-} population, and 4 populations with different combinations of GAL-9 and/or LAG-3 expression (**Fig. 23 A&B**).

I found that with TIGIT, on both the T and NK cell population, GAL-9^{+} cells had lower expression than GAL-9^{-} cells (**Fig. 22D**). Lastly, although in the T cell population there was no difference in TIM-3 expression on GAL-9^{+} cells compared to GAL-9^{-} cells, on NK cells, GAL-9^{+} cells had lower TIM-3 expression than GAL-9^{-} cells (**Fig. 22E**). Next, I investigated the expression of CD45RO on the bulk T cells and found no difference in expression on GAL-9^{+} cells compared to GAL-9^{-} cells (**Fig. 24A**). With regards to CCR7 expression, GAL-9^{+} cells had higher CCR7 expression than GAL-9^{-} cells (**Fig. 24B**). Lastly, I examined CD25 expression on GAL-9^{-} and GAL-9^{+} cells and found no difference in CD25 expression (**Fig. 24C**).

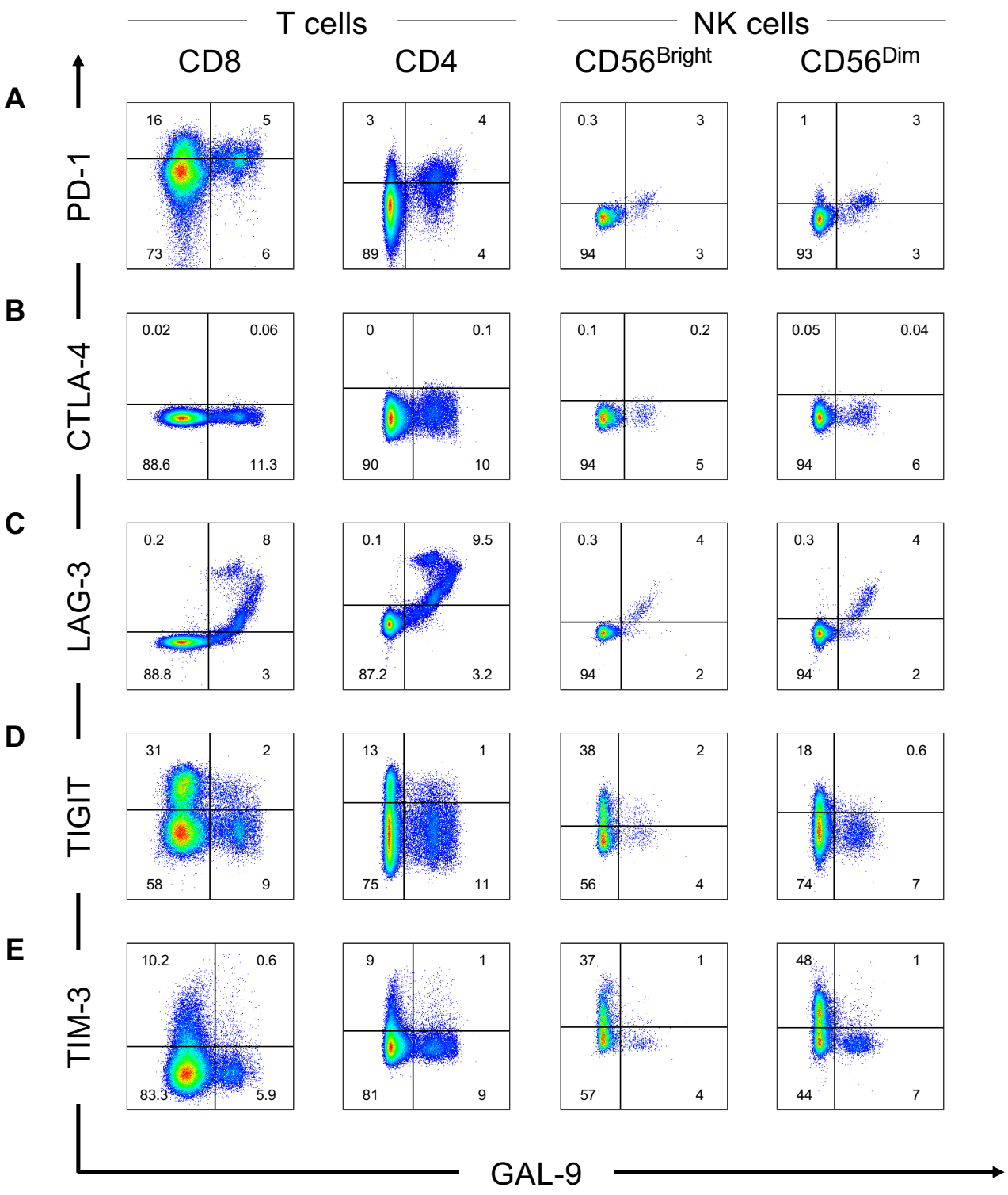

T cells
NK cells
CD8
CD4
CD56Bright
CD56Dim
A
PD-1
16
5
73
6
3
4
89
4
0.3
3
94
3
1
3
93
3
B
CTLA-4
0.02
0.06
88.6
11.3
0
0.1
90
10
0.1
0.2
94
5
0.05
0.04
94
6
C
LAG-3
0.2
8
88.8
3
0.1
9.5
87.2
3.2
0.3
4
94
2
0.3
4
94
2
D
TIGIT
31
2
58
9
13
1
75
11
38
2
56
4
18
0.6
74
7
E
TIM-3
10.2
0.6
83.3
5.9
9
1
81
9
37
1
57
4
48
1
44
7
GAL-9

Figure 22. Expression of inhibitory receptors on GAL-9^{+} and GAL-9^{-} cells. Representative plots showing the expression of (A) PD-1, (B) CTLA-4, (C) LAG-3, (D) TIGIT, and (E) TIM-3 on GAL-9^{+} and GAL-9^{-} cells in the bulk CD8 T cell, bulk CD4 T cell, CD56^{Bright} NK cell and CD56^{Dim} NK cell populations.

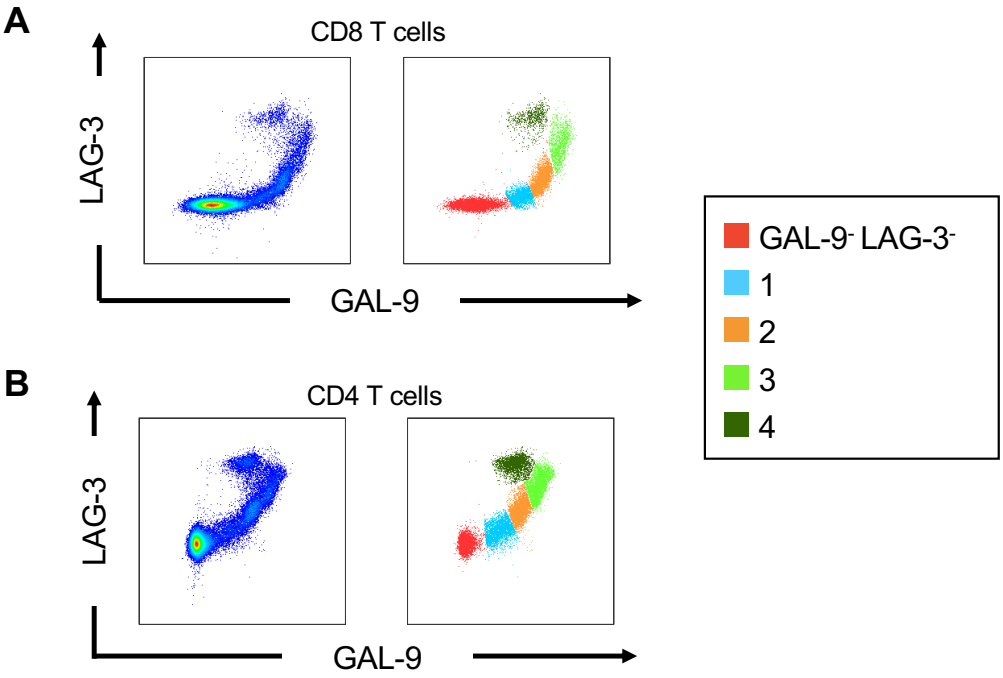

A
CD8 T cells
LAG-3
GAL-9
B
CD4 T cells
LAG-3
GAL-9
GAL-9- LAG-3-
1
2
3
4

Figure 23. 5 populations of cells in the LAG-3 vs GAL-9 plot. (A & B) In the bulk CD8 and CD4 T cell populations of chronic HCV patients, plotting LAG-3 against GAL-9 reveals 5 populations of cells. 1 with LAG-3$^-$GAL-9$^-$ cells and 4 other populations with different degrees of GAL-9 and LAG-3 expression.

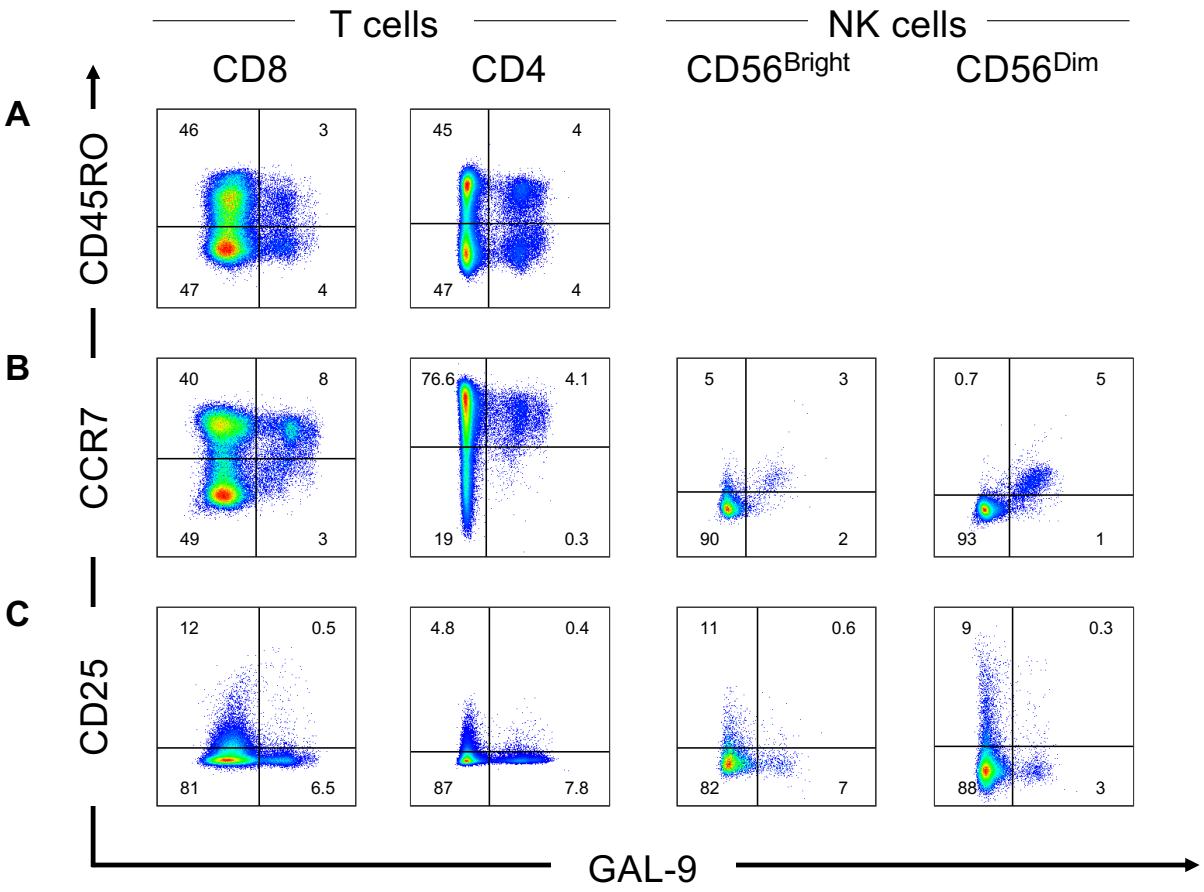
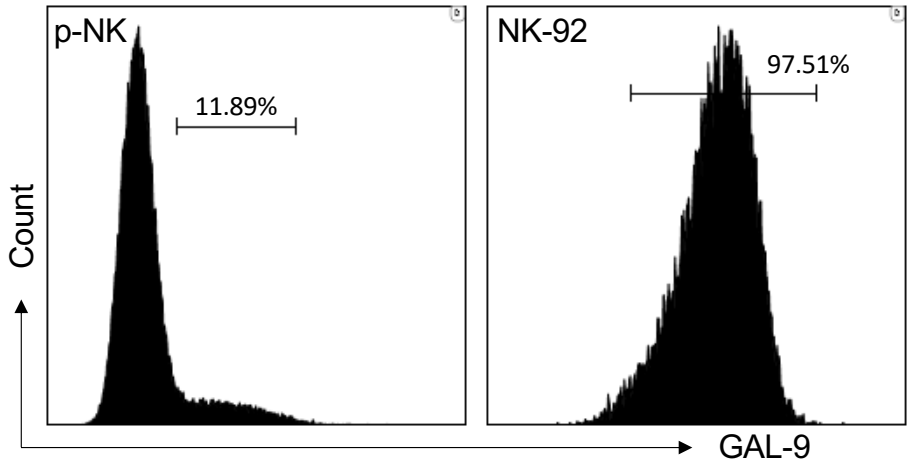

T cells
NK cells
CD8
CD4
CD56Bright
CD56Dim
A
CD45RO
B
CCR7
C
CD25
GAL-9
46
3
47
4
45
4
47
4
40
8
49
3
76.6
4.1
19
0.3
5
3
90
2
0.7
5
93
1
12
0.5
81
6.5
4.8
0.4
87
7.8
11
0.6
82
7
9
0.3
88
3

Figure 24. Expression of CD45RO, CCR7 and CD25 on GAL-9^{+} cells and GAL-9^{-} cells. Representative plots showing the expression of (A) CD45RO, (B) CCR7, and (C) CD25, on GAL-9^{+} and GAL-9^{-} cells in the bulk CD8 T cell, bulk CD4 T cell, CD56^{Bright} NK cell and CD56^{Dim} NK cell populations.

3.7 Primary-NK and NK-92 Cell GAL-9 Surface and Gene Expression.

To further investigate the role of GAL-9, I sought to identify an appropriate cell culture model. Using PBMC from chronic HCV patients with different degrees of fibrosis was essential to identify inhibitory receptor and GAL-9 mediated phenotypes that could play a role in patient immunosuppression. However, after identifying these phenotypes, a model is needed to fully understand the functional role of these phenotypes. Therefore, developing an NK and T cell line that can be expanded easily as well as manipulated to increase/decrease the expression of a specific protein is important. The two cells chosen as potential NK cell culture systems were a primary NK cell line (p-NK) derived from a healthy individual, and NK-92 cells, which are an immortalized NK cell line. I first determined baseline surface expression of GAL-9 in cultured p-NK and NK-92 cells using flow cytometry and found ~12% and ~98% of GAL-9^{+} cells, respectively (**Fig. 25A**). Next, I determined the expression of GAL-9 at the RNA level of these cells. RNA from p-NK and NK-92 cells were extracted, and reverse transcription polymerase chain reaction (RT-PCR) was used to covert the extracted RNA to complementary deoxyribonucleic acid (cDNA). Subsequently, primers targeting the cDNA of mRNA from the GAL-9 encoding gene, *LGALS9* was used to confirm GAL-9 gene expression. *LGALS9* expression in p-NK cells, NK-92 cells, and macrophages, which are known to express and secrete GAL-9, was measured. Hypoxanthine-guanine phosphoribosyl transferase 1 gene (*HPRT1*) was used as the housekeeping gene to access the quality of the PCR template. The PCR confirmed GAL-9 expression at the mRNA level in p-NK cells and macrophages, but gene expression could not be confirmed in NK-92 cells (**Fig. 25B**).

A


p-NK
11.89%
NK-92
97.51%
Count
GAL-9

B

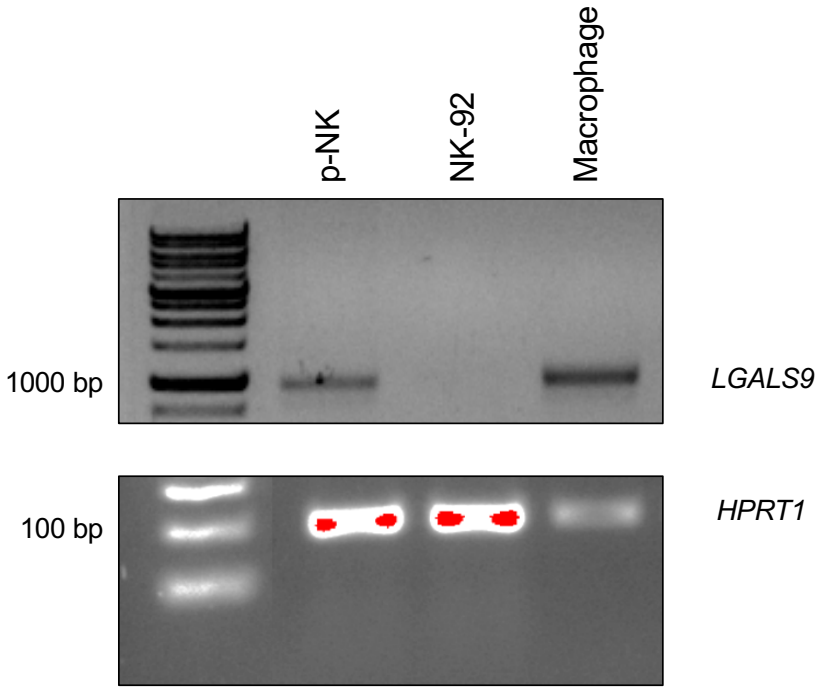

p-NK
NK-92
Macrophage
1000 bp
LGALS9
100 bp
HPRT1

Figure 25. GAL-9 protein and *LGALS9* gene expression in NK cells. (A) Histogram showing the frequency of p-NK and NK-92 cells positive for surface expression of GAL-9. (B) Agarose gel electrophoresis (2% agarose) of reverse transcription-PCR amplified products using human *LGALS9* and *HPRT1* primer sets. Lane 1 contains a DNA size marker, lane 2 contains p-NK cDNA, lane 3 contains NK-92 cDNA and lane 4 contains macrophage cDNA.

4. DISCUSSION

4.1 Study Cohort.

My study consisted of 30 chronically infected HCV patients with different degrees of liver fibrosis. The degree of patient's liver fibrosis ranged from no fibrosis (F0) to cirrhosis (F4) and patients were grouped into a no to moderate fibrosis group (F0-F2) and an advanced fibrosis group (F3-F4). Although the development of DAAs has significantly impacted the treatment and cure of chronic HCV, advanced liver fibrosis is still associated with a poor response to vaccination, recurrent infections and an increased risk of developing HCC[79-81]. This means that patients with advanced liver fibrosis can still remain immunosuppressed after being cured from chronic HCV. The goal of my study was to target and help these patients who, although no longer suffer from chronic HCV, still face a host of immunological issues. The majority of HCV patients' studies measuring inhibitory receptor expression compared the expression in patients with chronic HCV to healthy controls. With my study's patient populations, I have the unique ability to focus on the differences in inhibitory receptor expression phenotypes of patients all with chronic HCV infections, but different degrees of liver fibrosis. My study groups were comprised of an equal number of patients, n=15 (Table 2). The F0-F2 group represents less advanced stages of fibrosis, and although there are significant differences in fibrosis between F2 and F0 patients, I had only 2 patients in stage F2. Similarly, the F3-F4 group represents a more advanced stage of fibrosis, and although there is a significant difference in the extent of fibrosis between F3 and F4 patients, I had only 3 patients in stage F3. This means a majority of my F0-F2 group have no to minor degrees of fibrosis, and a majority of my F3-F4 group are cirrhotic.

4.2 T Cell Subsets During Chronic HCV Infections.

The majority of studies investigating T cells in chronic HCV patients focus on the HCV-specific T cells in order to draw conclusions. The information obtained from these studies remains important to understand the function and changes of HCV-specific T cells during chronic HCV to potentially explain the viral persistence seen in these individuals. However, with the new age of DAAs, HCV viral persistence in most chronic patients has become somewhat of a non-issue. What has persisted is the immunosuppression of patients with advanced liver fibrosis due to chronic HCV infections. Vranjkovic *et. al*., have shown that bulk $CD8^+$ T-cells of HCV-infected individuals with advanced liver disease were dysregulated and showed sustained activated status compared to those with minimal liver fibrosis, and this persisted post-DAA treatment[217]. This immunosuppression of patients with advanced liver fibrosis is implicated in not just susceptibility to infections, but also an increased risk of liver cancer. In order to elucidate the cause of this immunosuppression in these patients, the bulk T cell population, and not just the HCV-specific one, has to be investigated.

A change in T cell subsets during chronic HCV infections has been documented previously. Specifically, a decrease in the bulk naïve $CD4^+$ T cell frequency and an increase in the central memory population in the peripheral blood has been observed in chronic HCV patients when compared to healthy individuals[224]. Interestingly, although the absolute count of naïve $CD4^+$ T cells was also shown to decrease in chronic HCV patients, the count of the central memory population was not significantly different. This population shift was attributed to naïve $CD4^+$ T cells having a higher susceptibility to cell death after TCR activation and was theorized to contribute to the inadequate immune response seen in chronic HCV patients. Although I observed similar results in Fig. 6D with F3-F4 patients having lower frequencies of the naïve $CD4^+$ T cell

population and higher of the central memory population, it did not rise to the level of significance. Nevertheless, there is evidence to suggest a shift from the naïve population to the central memory one and this is most likely due to naïve $CD4^+$ T cell death following continuous TCR activation, which will have occurred more extensively in patients with advanced fibrosis.

Another well-established change in cell frequencies is the increase in bulk T_{regs} frequencies during chronic HCV infections, and this expansion is triggered by the HCV core protein[225-227]. Although I expected F3-F4 patients to have a higher frequency of bulk T_{regs} than F0-F2 patients, given their role in both immunosuppression, and their role in fibrosis development through the release of TGF-β, I found no significant difference in this subset (Fig. 21C). However, this does not exclude the possibility of intrahepatic enrichment of T_{regs} in patients with advanced fibrosis compared to those without.

4.3 Inhibitory Receptors with Higher Expression on T and NK Cells of Patients with Advanced Liver Fibrosis.

NK and T cells play a vital role in the anti-viral and anti-tumour responses. Chronic HCV patients with advanced stages of fibrosis have an increased susceptibility to infection and an increased risk of HCC post-viral clearance with DAAs[79-81, 217]. Thus, it is imperative to understand the immune dysfunction in these patients to improve their immune function after they are cured by DAAs. Previously, it has been shown that the expression of inhibitory receptors is a mechanism contributing to immune cell suppression of the NK cell and bulk T cell population[195, 204, 213, 228, 229]. Therefore, there is a need to understand the differences in immune dysregulation between patients with minimal liver fibrosis and those in the advanced stages to create targeted strategies to improve immune function.

No studies to my knowledge have reported a difference in PD-1 or LAG-3 expression on $CD56^{Dim}$ NK cells of chronic HCV patients even though the expression of these receptors on NK cells during other chronic infections translated into impaired degranulation and IFN-γ production capabilities[172, 182, 230, 231]. Here I show that within chronic HCV patients, those with advanced fibrosis have a higher expression of PD-1 and LAG-3 on these NK cells (Fig. 11B) and hypothesize that this contributes to the immune dysfunction observed in these patients. Similarly, TIGIT expression has been shown to play a role in suppressing effector $CD4^+$ T cell responses[232]. Furthermore, its expression has been shown to be increased on bulk $CD4^+$ T cells of HIV patients when compared to healthy controls[214]. HCV-specific CD4 T cells of chronic HCV patients also had a higher expression of TIGIT when compared to spontaneous clearers, and this again speaks to the role of this receptor during HCV infections[233]. I found that patients with advanced fibrosis had a higher expression of TIGIT on their bulk $CD4^+$ T cells (Fig. 9B), and this could again contribute to the immunosuppression seen in these individuals.

Lastly, I observed a higher expression of TIM-3 on the $CD56^{Bright}$ NK cells of chronic HCV patients with advanced liver fibrosis (Fig. 10B). On $CD56^{Dim}$ NK cells, the role of TIM-3 to mark dysfunctional cells during persistent infections seems to be clear[194, 195]. However, $CD56^{Bright}$ NK cells have poor cytotoxic capabilities and play a mainly cytokine production role. The impact of TIM-3 expression on this subset during chronic HCV infections still needs to be explored, but in HIV patients on anti-retroviral therapy, TIM-3 expression on these cells was associated with an increased IFN-γ production ability[213]. Thus, a similar phenomenon could be true for chronic HCV patients. Both T and NK cell subsets had unique inhibitory receptors, which were upregulated in chronic HCV patients with advanced liver fibrosis. This speaks to a likely unique role of each receptor on the different subsets during chronic HCV infection and the subsequent fibrosis.

4.4 Co-expression of Inhibitory Receptors on T and NK Cells of Patients with Advanced Liver Fibrosis.

Although the expression of only one inhibitory receptor does not suggest lymphocyte exhaustion, the expression of multiple inhibitory receptors is a clear indicator of extensively exhausted immune cells[204, 221]. On the other hand, immune cells not expressing any inhibitory receptors are known to exhibit superior effector function[204]. To begin to understand the extent of inhibitory receptor co-expression on the T and NK cells, I first applied Boolean gating and observed that F3-F4 patients had a higher frequency of NK cells, which expressed more than 3 (i.e., 4 or 5) inhibitory receptors, suggesting the extensive exhaustion of these cells (Fig. 12A). Furthermore, these patients showed a lower frequency of T and NK cells that did not express any of the measured inhibitory receptors (Fig. 12B). Therefore, my data demonstrates that patients with advanced liver fibrosis contain higher proportions of NK cells expressing multiple inhibitory receptors and lower proportions of T and NK cells free from inhibitory receptor expression. Interestingly, when I included GAL-9 expression in the Boolean gating analysis, I found that all T and NK cell subsets of F3-F4 patients had significantly higher expression of >3 inhibitory receptors or GAL-9 while all subsets had significantly lower expression of none of the inhibitory receptors and GAL-9 (Fig. 12 C & D). By including GAL-9 in the Boolean analysis, I was able to see significant differences between patients with advanced liver damage and those with minimal liver damage and this again speaks to potential importance of GAL-9 expression on the lymphocytes of these F3-F4 patients.

t-SNE analysis of chronic HCV patients showed that the T cells co-expressing LAG-3 and GAL-9 were PD-1^+ cells. It also showed that F3-F4 patients had a higher frequency of $CD4^+$ T cells with this expression pattern. Given the vital role of the TIM-3/GAL-9 interactions in

modulating T/NK cell function, the higher frequency of T and NK cells expressing GAL-9 in patients with advanced liver fibrosis when compared to F0-F2 patients is a phenotype potentially denoting immune dysregulation. I also observed a similar pattern on $CCR7^{+}$ $CD56^{Dim}$ NK cells of F3-F4 patients. Initially, I found a higher frequency of CCR7 expressing $CD56^{Dim}CD16^{+}$ NK cells in patients with advanced fibrosis than those with less fibrosis (Fig. 7E). CCR7 is a receptor vital for the migration of immune cells into secondary lymphoid organs such as the lymph nodes, and $CD56^{Dim}$ NK cells in the peripheral blood lacking CCR7 have been shown to be cytotoxic effector cells[234, 235]. The increase in $CD56^{Dim}$ $CCR7^{+}$ NK cells in F3-F4 patients suggests that the frequency of cytotoxic effector NK cells is reduced. The phenotype was accompanied by the close co-expression of LAG-3, GAL-9 as well as PD-1. Studies have previously shown that LAG-3 and PD-1 are co-expressed on both antigen-specific and bulk tumour-infiltrating lymphocytes (TILs) in various human tumours as well as antigen-specific CTLs in LCMV chronically infected mice, and LAG-3 blockade was not sufficient to reverse the CTL exhausted phenotype[204, 228, 236]. Notably, combined blockade of LAG-3 and PD-1 vastly improves CTL function to control tumour growth[228]. The combined blockade of LAG-3 and PD-L1 was also shown to improve viral control in LCMV chronically infected mice[204]. Thus, my findings reasons that combined blockade of LAG-3, GAL-9, and PD-1 could potentially improve the function of T/NK cells in patients with advanced liver fibrosis.

My t-SNE analysis also revealed a higher frequency of $TIGIT^{med\text{-}hi}$ $CD25^{+}$ $CD4^{+}$ T cells (T_{regs} & T_{effs}) in F3-F4 patients when compared to F0-F2 patients. T_{regs} begin to express IL-10 and FGL-2 after TIGIT engagement, and FGL-2 expression allows T_{regs} to selectively suppress Th1 and Th17 pro-inflammatory responses[237]. TIGIT expression on T_{regs} is also implicated in their lineage stability[238]. Altogether, my data revealed that patients with advanced liver fibrosis have an

increase in the frequency of $TIGIT^+$ $CD25^+$ CD4 T cells, which I found to be approximately 50% $TIGIT^+$ $T_{regs,}$ and evidence suggests $TIGIT^+$ T_{regs} could possess increased immunosuppressive capabilities and increased stability.

Lastly, CD25 is the IL-2Rα-chain, and its expression plays a crucial role in the response of NK cells in chronic HCV patients. Co-culturing of NK cells with HCV infected hepatocytes has been shown to increase their CD25 surface expression[239]. $CD56^{Dim}$ NK cells with increased expression of CD25 have enhanced responsiveness to low doses of IL-2, thus improving their production of IFN-γ and cytotoxicity in these conditions[240]. Here I showed that patients with advanced stages of fibrosis have a higher frequency of $CD25^+$ $CD56^{Dim}$ NK cells than patients with minimal fibrosis (Fig. 7C). The t-SNE analysis revealed that these $CD25^+$ cells also highly co-express TIM-3 and TIGIT. TIM-3 expression denotes functionally exhausted $CD56^{Dim}$ NK cells with impeded cytokine production and cytotoxicity[195]. Similarly, TIGIT engagement on NK cells directly inhibits their cytotoxicity and cytokine secretion by prematurely terminating the activity of NF-κB, PI3K and MAPK[185, 186]. Increased TIM-3 expression has also been observed on both HCV-specific and bulk $CD8^+$ and $CD4^+$ T cells of chronic HCV patients and was found to be particularly enriched in the intrahepatic T cells[203]. Knowing TIM-3 and TIGIT are co-expressed on NK cells, which are already primed for enhanced function ($CD25^+$), a combined immune blockade therapy can be employed to release these exhausted cells, thus conferring protection to the host against other infections and HCC. TILs of B16F10 melanoma in mice highly co-express TIM-3 and TIGIT, and their effector function is only restored in $TIGIT^{-/-}$ mice treated with anti-TIM-3 antibodies[241]. Blockade of TIM-3 or TIGIT has separately been shown to restore NK cell function[229, 242]. Thus, my data suggests that high co-expression of TIM-3 and TIGIT on $CD25^+$ NK cells hinders their function, and a combined blockade therapy using anti-TIGIT and anti-TIM-3

antibodies could provide a potential therapeutic benefit by improving NK cell function in chronic HCV patients with advanced liver fibrosis.

4.5 Expression of Inhibitory Receptors on GAL-9$^+$ T and NK Cells.

GAL-9 is one of the ligands of TIM-3, and the role of TIM-3/GAL-9 interactions on modulating immune cell functions has been well studied[212, 243, 244]. I found that CD8$^+$ and CD4$^+$ T cells as well as CD56^{Dim} and CD56^{Bright} NK cells in patients with advanced liver fibrosis had higher frequencies of GAL-9$^+$ cells when compared to F0-F2 patients (Fig. 8-11B). Notably, GAL-9 was the only tested protein which was consistently higher on all T and NK cell subsets in F3-F4 patients. This speaks to a potentially vital role played by this protein in chronic HCV and liver fibrosis. GAL-9$^+$ NK cells in HIV patients had reduced cytotoxicity with lower expression of perforin and granzyme B[213]. Similarly, GAL-9$^+$ bulk CD8$^+$ T cells in HIV patients showed hindered cytotoxic ability, while GAL-9$^+$ bulk CD8$^+$ and CD4$^+$ T cells showed decreased IFN-γ production ability[214]. Also, GAL-9$^+$ T cells showed a lower proliferative capacity compared to GAL-9$^-$ cells. Furthermore, GAL-9 binding to TIM-3 has been shown to trigger cell death in Th1 and IFN-γ producing CD8$^+$ T cells[191]. It reasons, therefore, that GAL-9 in chronic HCV infections marks dysfunctional T and NK cells.

The first interesting question about GAL-9, is how flow cytometric surface detection is possible as GAL-9 is a secreted protein lacking a cytoplasmic domain. GAL-9 is a protein, which is secreted by several immune cells, including B cells, T cells, macrophages and mast cells[245, 246]. Although GAL-9 secretion had been well established, the exact secretory pathway remained somewhat of a mystery due to it lacking a signal sequence necessary for secretion using the ER-

Golgi pathway[247, 248]. Furthermore, the questions of how GAL-9 is translocated across the lipid bilayer, or whether cells expressing GAL-9 at the RNA level are necessarily GAL-9 secreting cells remained unanswered. Oomizu *et. al*. hypothesized that GAL-9-secreting cells express GAL-9 on the cell surface during the secretory process, and this translocated intermediate can be detected using antibodies[249]. They found that indeed antibodies could detect surface expression of GAL-9 on $CD4^+$ T cells and also found that intracellular expression of GAL-9 did not necessarily correlate with its secretion[249]. Instead, the surface expression of GAL-9 was the best way to identify GAL-9 secreting cells. Therefore, the surface expression of GAL-9 I observe on the T and NK cells of chronic HCV patients is most likely a secretory intermediate of these cells.

The next question of interest is whether this GAL-9 expressed by cells modulates their function in a TIM-3 dependent manner. I found that, specifically on NK cells, the majority of GAL-9^+ cells were TIM-3^- (Fig. 22E). This lack of TIM-3 expression on GAL-9^+ cells could possibly be due to TIM-3 internalization after GAL-9 engagement. CTLA-4 internalization and intracellular storage after binding to CD80/CD86 has been identified to play a major role in its regulation of T cell activation[166, 250]. The internalized CTLA-4 is recycled and once again recruited to the surface during TCR signaling[251]. TIM-3 has been found to have similar intracellular storage compartments, which were identified to be recycling endosomes[252]. Furthermore, TIM-3 also contains an adaptor protein complex 2 binding domain which when dephosphorylated on CTLA-4, leads to internalization via clathrin-mediated endocytosis[251]. The role of this TIM-3 internalization remains in question. In CTLA-4, this internalization is used to remove the expression of its ligand (CD80/CD86) from the surface of APCs, thus inhibiting the activation of cells[253, 254]. However, this same function has not been verified with regards to TIM-3 internalization. Therefore, the subsequent internalization of TIM-3 after GAL-9 binding might

explain the lack of TIM-3 expression on GAL-9^{+} cells. A model for GAL-9 antibody detection and TIM-3 internalization is illustrated in **Fig. 26**.

Although I expected the higher frequency of GAL-9^{+} T and NK cells in F3-F4 patients to coincide with a lower frequency of TIM-3^{+} cells, this was not observed. In fact, although both T cell subsets and CD56^{Dim} NK cells showed no difference in TIM-3 expression between the 2 fibrosis groups, CD56^{Bright} cells of F3-F4 patients showed higher expression of TIM-3 (Fig. 10B). This pattern might provide some insight into the exact signaling mechanism of action of secreted GAL-9. Secreted ligands can act through either autocrine, paracrine or endocrine signaling. In autocrine signaling, the secreted ligand acts on a receptor expressed on the same cell. In paracrine, the ligand acts on a nearby cell, while in endocrine, the ligand will travel via the circulatory system to reach its intended target.

Given the downregulation of TIM-3 expression on GAL-9^{+} NK cells do not coincide with a global decrease of TIM-3 expression but, in some cases, an increase, this suggests GAL-9 regulation of cells is most likely autocrine and not endocrine or paracrine. If it was endocrine or paracrine, presumably the global TIM-3 expression would be decreased. Instead, what I see is a lack of TIM-3 expression on the NK cells highly expressing GAL-9. Thus, I postulate that GAL-9 secreted by NK cells of chronic HCV patients acts on TIM-3 in an autocrine manner. If true, this will not be the first documentation of a TIM-3/GAL-9 autocrine signaling loop. A TIM-3/GAL-9 autocrine loop has been found to be critical to maintain the self-renewal of leukemic stem cells during human acute myeloid leukemia[255]. Importantly, this proposed TIM-3/GAL-9 autocrine loop does not negate the possibility of GAL-9 produced by other cells in chronic HCV patients acting on T and NK cells. As previously stated, during chronic HCV infections, GAL-9 is increasingly secreted by macrophages and monocytes in the liver[212]. Thus, the GAL-9 secreted by these cells

could also be acting on T and NK cells in the peripheral blood in paracrine or endocrine manners. Finally, there is a chance that GAL-9 expressed by T and NK cells of chronic HCV patients regulate their activity in a TIM-3 independent manner. GAL-9 interacts with CD40 to inhibit the proliferation of $CD4^+$ T cells during autoimmunity as well as induces cell death[211]. GAL-9/CD40 interaction has been shown to modulate T cell function and could very well also be implicated in these chronic HCV patients.

Another interesting pattern observed with respect to the expression on GAL-9 cells, was the very high expression of PD-1 on $GAL\text{-}9^+$ cells (Fig. 22A). Notably, in HIV patients, the vast majority of $PD\text{-}1^+$ bulk $CD8^+$ and $CD4^+$ T cells were also $GAL\text{-}9^+$ [214]. Both $GAL\text{-}9^+$ cells and $GAL\text{-}9^+PD\text{-}1^+$ cells were found to be highly exhausted, with impaired IFN-γ production. The expression of PD-1, a very well-defined lymphocyte exhaustion marker, points again to the exhaustive nature of these $GAL\text{-}9^+$ T and NK cells. On the other hand, the majority $GAL\text{-}9^+$ cells are $TIGIT^-$ (Fig. 22D). This pattern is interesting, as GAL-9 and TIGIT were found to have dichotomous functions on the NK cells of HIV patients[213]. In these HIV patients, $GAL\text{-}9^+$ NK cells were found to have impaired cytotoxicity, but enhanced IFN-γ production, while $TIGIT^+$ cells had hindered IFN-γ production, but enhanced cytotoxicity. These diverging roles on NK cells could explain the TIGIT and GAL-9 expression pattern observed. This study however does not show the level of GAL-9 and TIGIT co-expression on cells, thus they assume GAL-9 and TIGIT are inherently expressed on different NK cell populations.

The last interesting expression I identified was the expression pattern of LAG-3 on $GAL\text{-}9^+$ T cells (Fig. 22C). $GAL\text{-}9^+$ NK cells have similar PD-1 and LAG-3 expression patterns, but in T cells, the LAG-3 pattern is very peculiar. There appears to exist multiple populations with varying degrees of GAL-9 and LAG-3 expression (Fig. 22C & Fig. 23). This pattern is similar to

the one observed with respect to the expression of GAL-9 vs CD160 on the bulk $CD4^+$ and $CD8^+$ T cells of HIV patients[214]. To understand if these populations have unique functions within these patients would require further research.

4.6 Limitations and Future Directions.

Although I utilize an extensive number of inhibitory receptors, there are other inhibitory receptors at play, which were not included in this study, but could be critical in inducing and maintaining immune dysregulation. Increasing the number of inhibitory receptors measured and potentially including the expression of activating receptors could provide a fuller picture of the nature of immune suppression in chronic HCV patients with advanced liver fibrosis. Due to the limited sample size, I acknowledge that statistically significant differences do not necessarily translate into biological significance, thus I am looking forward to my results being reproduced in other studies with larger sample sizes.

In addition, my data represents the status of circulating immune cells, and may not reflect the phenotypic and functional picture of their counterparts in the liver. Nonetheless, information regarding the immunological status of T and NK cells in the peripheral blood should be vital to understand the susceptibility to other infections in patients. Future studies could analyze the potential divergence in inhibitory receptor expression between the lymphocytes in the peripheral blood and those in the liver. This could further elucidate the role of inhibitory receptor expression in the development of HCC. It will also help to answer the question of if HCV replication in the liver applies a selective pressure on the nature of inhibitory receptor expression and co-expression.

Lastly, I did not include healthy controls in the study. Thus, my data itself might not be sufficient to distinguish phenotypes of immune dysregulation associated with advanced liver damage from those found in healthy controls. Nonetheless, there is ample literature that has compared the expression of inhibitory receptors in healthy controls to chronic HCV patients. A much more fitting question, is if DAA treatment impacts the expression and co-expression of inhibitory receptors on T and NK cells of patients with advanced liver fibrosis. Although patients with chronic HCV infections who have been cured by DAAs might experience a reversal of their liver fibrosis, HCV-specific $CD8^+$ T cells of F3-F4 patients remain dysfunctional post-DAA treatment[217]. This signals that DAA treatment might not resolve the increased and sustained expression of these receptors on T and NK cells of these patients. If true, this further highlights the need to identify targets for combinatorial therapy to hopefully improve T and NK cell function in these patients.

The next steps of this project will aim to further elucidate the role of GAL-9 surface expression on T and NK cell function. This will include the impact of knocking out and over expressing GAL-9 on cell proliferation and function. Currently, I have confirmed, using flow cytometry, that GAL-9 is expressed on the surface of p-NK and NK-92 cells (Fig. 25A). PCR then confirmed the expression of *LGALS9* gene encoding the GAL-9 protein in p-NK cells (Fig. 25B). The absence of a transcript in NK-92 cells might be due to the multiple isoforms and transcripts of GAL-9 that exist. Primers targeting other GAL-9 isoforms will be used to test GAL-9 expression at the RNA level of NK-92 cells. This expression at the gene and protein level will also be investigated in Jurkat cells, which will act as the T lymphocyte cell culture model. Knockout and over-expression cells are currently being generated and subsequently, CFSE proliferation assays,

ELISA tests to measure GAL-9 secretion and stimulation to measure cytotoxicity and cytokine production will be conducted to fully understand the role of GAL-9.

I hypothesize that cells over-expressing GAL-9 will be dysfunctional. Ultimately, these cells will have a hindered proliferative capability, lower expression of pro-inflammatory cytokines such as IFN-γ and TNF-α, and potentially higher expression of anti-inflammatory cytokines such as IL-10. Given the high expression of GAL-9 in the liver during HCV infections, I also expect that cells overexpressing GAL-9 will have high expression of TGF-β which could contribute to the development of fibrosis. Finally, I expect that T and NK cells with high GAL-9 expression will have decreased expression of perforin and granzyme B which will translate into impaired cytotoxicity.

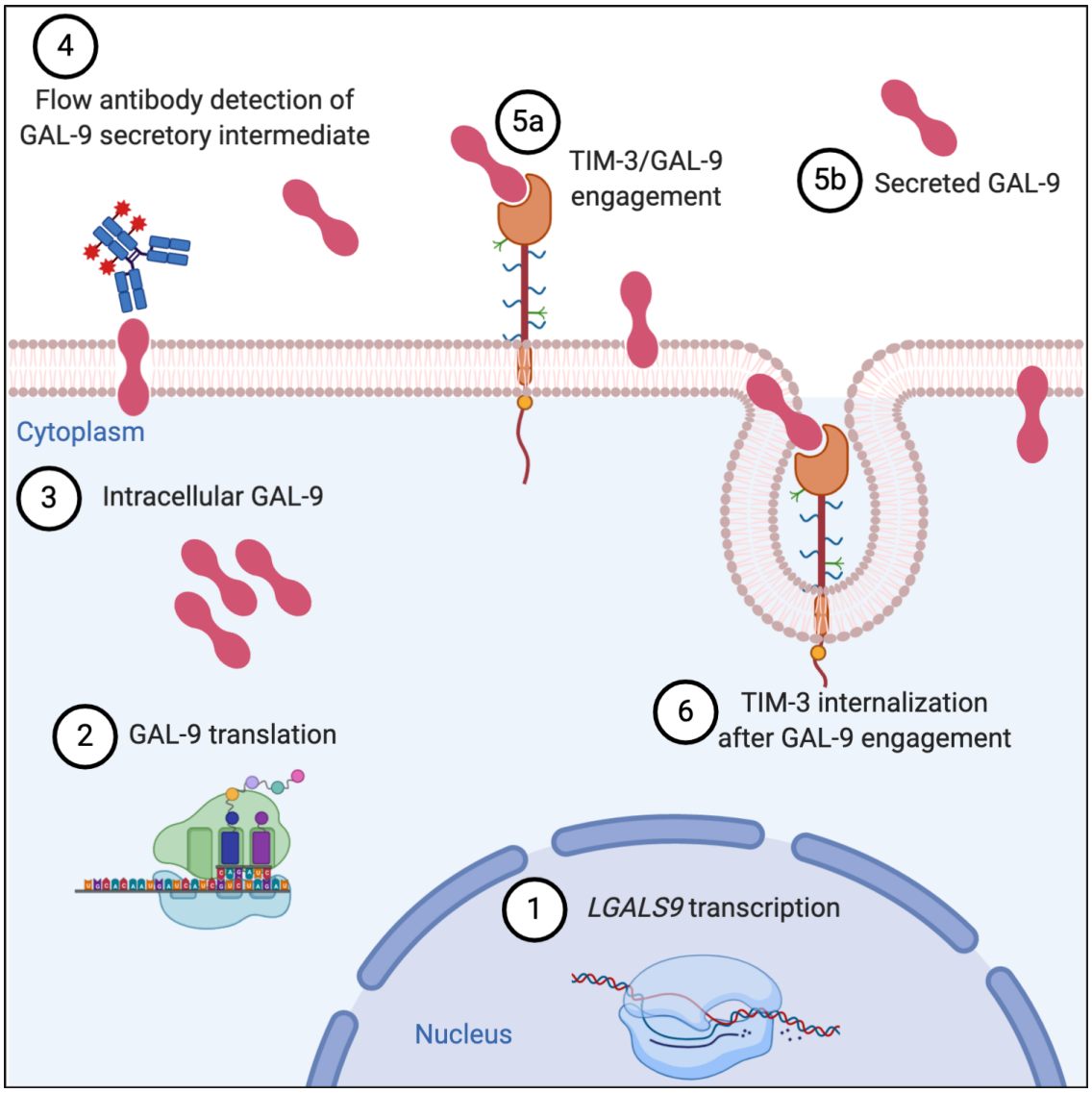

4
Flow antibody detection of
GAL-9 secretory intermediate
5a
TIM-3/GAL-9
engagement
5b
Secreted GAL-9
Cytoplasm
3
Intracellular GAL-9
2
GAL-9 translation
6
TIM-3 internalization
after GAL-9 engagement
1
LGALS9 transcription
Nucleus

Figure 26. Model of GAL-9 surface expression detection using flow cytometry. Transcription and translation of GAL-9 in cells leads to GAL-9 intracellular storage. During GAL-9 secretion, the secretory intermediate can be detected on the cell surface using fluorophore-labelled antibodies, and this identifies GAL-9^{+} cells. I propose that secreted GAL-9 can be engaged by TIM-3 receptors present on the same cell, and this leads to TIM-3 internalization. This figure was generated using Biorender.

5. CONCLUDING REMARKS

In conclusion, I investigated PBMCs from chronic HCV patients with minimal to no fibrosis, and chronic HCV patients with advanced fibrosis by analyzing the co-expression of five inhibitory receptors and GAL-9 on T/NK cells. A recent meta-analysis found that PD-1 inhibitor monotherapy and anti-CTLA-4 monotherapy had 18.6% efficacy when used for treating liver cancer in chronic HBV and HCV patients[256]. Ultimately, they concluded that immune checkpoint inhibitors are safe for these patients, but virus reactivation remains a possibility. My findings revealed multiparametric phenotypes that characterize immune suppression in chronic HCV patients with advanced liver fibrosis. Mechanisms underlying these phenotypes would provide a better understanding of immune dysregulation associated with advanced liver damage and increased susceptibility to infections and HCC and will lead to the development of targeted therapies to improve their immune function. Examples of potential combinatorial therapy approaches are illustrated in **Fig. 27**.

As previously stated, GAL-9 was the only protein that was consistently higher in expression on the T and NK cells of F3-F4 patients when compared to F0-F2. Additionally, F3-F4 patients had a higher frequency of cells in both the T and NK t-SNE clusters co-expressing GAL-9, LAG-3 as well as PD-1 and this cluster representation correlated with patients' LSM. Finally, GAL-9^{+} bulk T and NK cells from HIV patients were shown to have impaired cytotoxicity and cytokine production[213, 214]. Thus, my data suggests that GAL-9 mediated lymphocyte dysfunction could play a vital role in the immunosuppression seen in patients with advanced liver fibrosis. A summary of all my major findings are illustrated in **Fig. 28**.

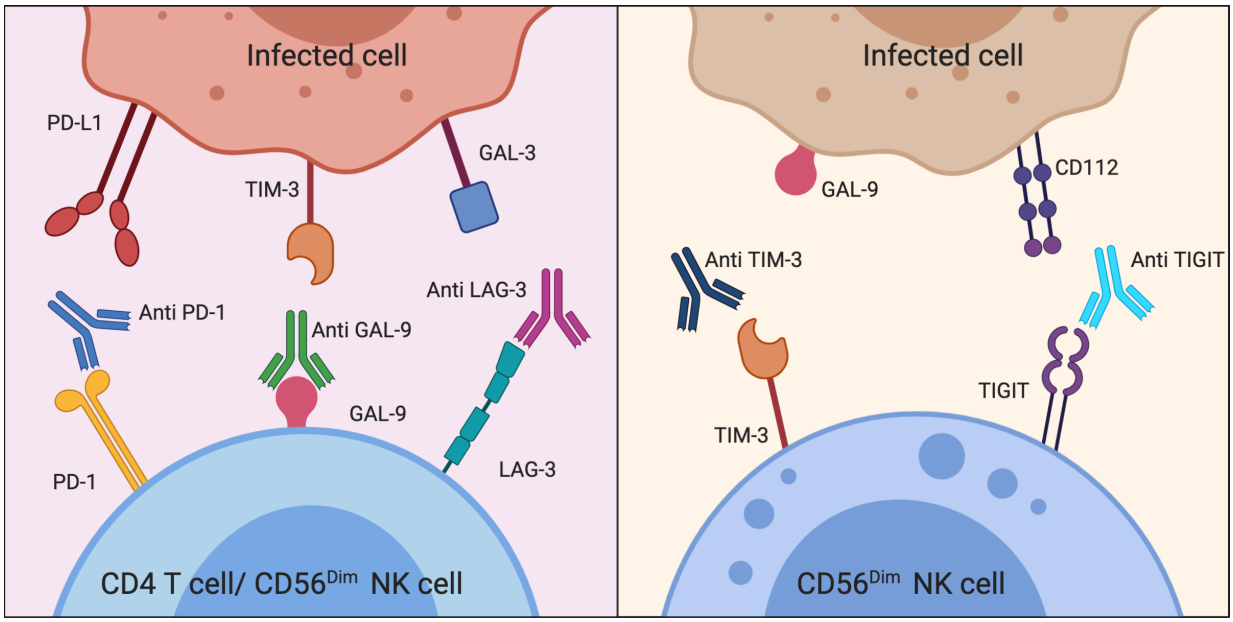

Infected cell
PD-L1
TIM-3
GAL-3
Anti PD-1
Anti GAL-9
Anti LAG-3
GAL-9
PD-1
LAG-3
CD4 T cell/ CD56Dim NK cell
Infected cell
GAL-9
CD112
Anti TIM-3
Anti TIGIT
TIGIT
TIM-3
CD56Dim NK cell

Figure 27. Potential combinatorial checkpoint blockade therapy using the identified t-SNE clusters with increased frequency in F3-F4 patients. CD4 T cell and $CD56^{Dim}$ NK cell clusters co-expressing PD-1, LAG-3 and GAL-9 were identified to have higher frequencies in F3-F4 patients. A $CD56^{Dim}$ NK cell cluster co-expressing TIGIT and TIM-3 was also identified to be increased in these patients. Combinatorial immune checkpoint blockade therapy targeting these clusters can be used to improve T and NK cell function in F3-F4 patients. This figure was generated using Biorender.

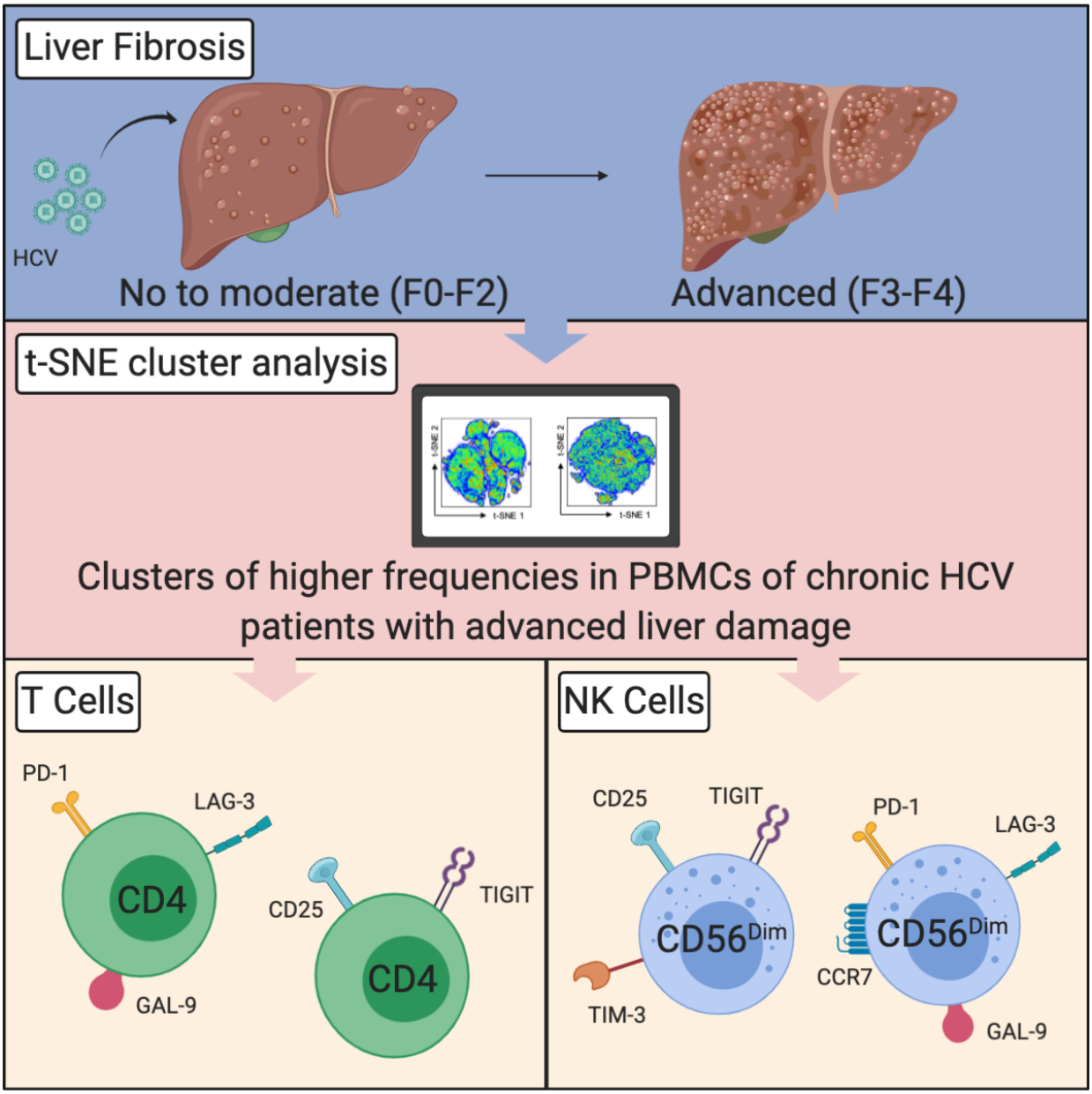

Liver Fibrosis
HCV
No to moderate (F0-F2)
Advanced (F3-F4)
t-SNE cluster analysis
t-SNE 2
t-SNE 1
Clusters of higher frequencies in PBMCs of chronic HCV patients with advanced liver damage
T Cells
PD-1
LAG-3
CD4
GAL-9
CD25
TIGIT
CD4
NK Cells
CD25
TIGIT
CD56Dim
TIM-3
PD-1
LAG-3
CD56Dim
CCR7
GAL-9

Figure 28. Summary figure showing the t-SNE clusters found to have higher frequencies in F3-F4 patients with chronic HCV infections. This study has identified 4 t-SNE clusters where F3-F4 chronic HCV patients have higher cell frequencies. In the T cell cluster; $CD25^{+}TIGIT^{med\text{-}hi}$ CD4 cells and $PD\text{-}1^{med}LAG\text{-}3^{med\text{-}hi}GAL\text{-}9^{med\text{-}hi}$ CD4 cells. In the NK cell cluster; $CD25^{+}TIGIT^{med\text{-}hi}TIM\text{-}3^{med\text{-}hi}$ $CD56^{Dim}$ NK cells and $CCR7^{+}$ $PD\text{-}1^{med}LAG\text{-}3^{med\text{-}hi}GAL\text{-}9^{med\text{-}hi}$ $CD56^{Dim}$ NK cells. This figure was generated using Biorender.

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7. CONTRIBUTION OF COLLABORATOR

Blood samples from chronically infected HCV^+ individuals and informed consent were collected in collaboration with Dr. Curtis Cooper at The Ottawa Hospital. He also provided information on patients' age, sex, ethnicity, ALT and AST levels, as well as LSM. Lastly, he assigned patient to their corresponding fibrosis groups.

8. APPENDIX

31/07/2020

Université d'Ottawa
Bureau d'éthique et d'intégrité de la recherche

University of Ottawa
Office of Research Ethics and Integrity

CERTIFICAT D'APPROBATION ÉTHIQUE | CERTIFICATE OF ETHICS APPROVAL

Numéro du dossier / Ethics File Number	H-07-20-5941
Titre du projet / Project Title	Immune and Metabolic Function During Viral Hepatitis Infection
Type de projet / Project Type	Recherche de professeur / Professor's research project
Statut du projet / Project Status	Approuvé / Approved
Date d'approbation (jj/mm/aaaa) / Approval Date (dd/mm/yyyy)	31/07/2020
Date d'expiration (jj/mm/aaaa) / Expiry Date (dd/mm/yyyy)	11/02/2021

Équipe de recherche / Research Team

Chercheur / Researcher	Affiliation	Role
Seung-Hwan LEE	Département de biochimie, microbiologie et immunologie / Department of Biochemistry, Microbiology and Immunology	Chercheur Principal / Principal Investigator
Curtis COOPER	Département de médecine / Department of Medicine	Chercheur principal - site d'examen primaire / Primary review site PI
Cynthia TSIEN	Ottawa Hospital	Co-chercheur / Co-investigator
Angela CRAWLEY		Co-chercheur / Co-investigator
John PEZACKI		Co-chercheur / Co-investigator
Mary-Anne DOYLE	Département de médecine / Department of Medicine	Co-chercheur / Co-investigator

Conditions spéciales ou commentaires / Special conditions or comments

OHSN REB 20100009-01H

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31/07/2020

Université d'Ottawa

Bureau d'éthique et d'intégrité de la recherche

University of Ottawa

Office of Research Ethics and Integrity

Le Comité d'éthique de la recherche (CÉR) de l'Université d'Ottawa, opérant conformément à l'*Énoncé de politique des Trois conseils* (2014) et toutes autres lois et tous règlements applicables, a examiné et approuvé la demande d'éthique du projet de recherche ci-nommé.

L'approbation est valide pour la durée indiquée plus haut et est sujette aux conditions énumérées dans la section intitulée "Conditions Spéciales ou Commentaires". Le formulaire « Renouvellement ou Fermeture de Projet » doit être complété quatre semaines avant la date d'échéance indiquée ci-haut afin de demander un renouvellement de cette approbation éthique ou afin de fermer le dossier.

Toutes modifications apportées au projet doivent être approuvées par le CÉR avant leur mise en place, sauf si le participant doit être retiré en raison d'un danger immédiat ou s'il s'agit d'un changement ayant trait à des éléments administratifs ou logistiques du projet. Les chercheurs doivent aviser le CÉR dans les plus brefs délais de tout changement pouvant augmenter le niveau de risque aux participants ou pouvant affecter considérablement le déroulement du projet, rapporter tout évènement imprévu ou indésirable et soumettre toute nouvelle information pouvant nuire à la conduite du projet ou à la sécurité des participants.

The University of Ottawa Research Ethics Board, which operates in accordance with the *Tri-Council Policy Statement* (2014) and other applicable laws and regulations, has examined and approved the ethics application for the above-named research project.

Ethics approval is valid for the period indicated above and is subject to the conditions listed in the section entitled "Special Conditions or Comments". The "Renewal/Project Closure" form must be completedfour weeks before the above-referenced expiry date to request a renewal of this ethics approval or closure of the file.

Any changes made to the project must be approved by the REB before being implemented, except when necessary to remove participants from immediate endangerment or when the modification(s) only pertain to administrative or logistical components of the project. Investigators must also promptly alert the REB of any changes that increase the risk to participant(s), any changes that considerably affect the conduct of the project, all unanticipated and harmful events that occur, and new information that may negatively affect the conduct of the project or the safety of the participant(s).

Catherine PAQUET
Directeur / Director
Pour/For **Daniel LAGAREC** Président(e) du/ Chair of the **Comité d'éthique de la recherche en sciences de la santé et sciences / Health Sciences and Sciences Research Ethics Board**

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Curriculum Vitae

EDUCATION

University of Ottawa, Ottawa, ON
Master of Science - Microbiology and Immunology (CGPA: 10)
Sep 2018 - Mar 2021

University of Ottawa, Ottawa, ON
Honours Bachelor of Science - Biomedical Science (CGPA: 9.59)
Sep 2014 - May 2018

EMPLOYMENT

University of Ottawa, Ottawa, ON
Laboratory technician
(May 2018 - Aug 2018)

The Children's Hospital of Eastern Ontario (CHEO), Ottawa, ON
Research assistant
(Jun 2017 - Apr 2018)

Self-employed Tutor, Ottawa, ON
Science and Math Tutor
(Dec 2015 - Dec 2019)

The Ottawa General Hospital, Ottawa, ON
Boutique Cashier
(Jun 2015 - Aug 2016)

PUBLICATIONS

- Okwor, C.I.A., Oh, J.S., Crawley, A.M., Cooper, C.L. & Lee, S.H. (2020). Expression of Inhibitory Receptors on T and NK Cells Defines Immunological Phenotypes of HCV Patients with Advanced Liver Fibrosis. *iScience*, 101513
- Okwor, C.I., Klaassen, R.J., Harrison, M.A., Tang, K., Laforest, I., Bowerman, B. & Simpson, E. (2018). Predictors of Hydroxyurea Use in Children with Sickle Cell Disease | *Blood* | American Society of Hematology. **132**(Suppl 1)

AWARDS

- CanHepC - *Master's Trainee Fellowship* ($20,000/year Jul 2019 - Jan 2021)
- University of Ottawa - *Excellence Scholarship* ($10,000/year Sep 2019 - Dec 2020)
- Faculty of Medicine Award in Education - *Excellence in Microbiology and Immunology Graduate Studies Program* (Dec 2020)
- Faculty of Medicine Research Day - *Best Oral Presentation* (Sep 2020)

- BMI Seminar and Poster Day - *Best Immunology Video* (May 2020)
- Canadian Liver Meeting - *Young Investigator Award* (Feb 2020)
- University of Ottawa - *International Student Entrance Scholarship* ($7,500/year Sep 2018 - Aug 2019)
- Cytokines in Inflammation, Ageing, Cancer and Obesity (CIACCO) 8th Symposium - *Poster Award* (May 2019)
- University of Ottawa - *Merit Scholarship* ($4,000, Sep 2016 - Apr 2018)
- CHEO Research Institute - *Summer Studentship Award* ($5,000 Jun 2017 - Aug 2017)
- The Ottawa Hospital - *Outstanding Service Award* (Apr 2017)
- Columbia International College - *Best in Calculus* (Jun 2014)
- Columbia International College - *Donnell Insurance Brokers Limited Residence Community Award* (Jun 2014)
- Duke of Edenborough Award - *Bronze Medal Recipient* (Sep 2013 - Mar 2014)

SCIENTIFIC MEETINGS

- CIACCO 8th Symposium - *Poster Presentation*
- Canadian Society for Immunology 32nd Annual Meeting - *Poster Presentation*
- Canadian Liver Meeting 2020 - *Poster Presentation*

EXTRACURRICULAR ACTIVITIES

- Biochemistry Microbiology and Immunology Graduate Student Association, BMIGSA -*Vice-president External* (May 2019 - Aug 2020)
- Graduate Student Association of the University of Ottawa, GSAED - *BMI Representative* (May 2019 - Aug 2020)
- Ultimate Frisbee - *Team Member* (Jun 2019 - Mar 2020)
- University of Ottawa Nigerian Student Association - *Executive Member* (Aug 2018 - Sep 2019)

COMMUNITY INVOLVEMENT

- Canadian Liver Foundation - *Volunteer* (Jun 2019 - ongoing)
- Ottawa General Hospital Volunteer Resources - *Office Helper and Team Lead* (May 2015 - ongoing)
- The Rehabilitation Centre - *Mobile Manicure Unit Team Lead* (Dec 2018 - ongoing)
- Let's Talk Science - *Volunteer* (Sep 2018 - ongoing)

- Ultimate Frisbee - *Team Member* (Jun 2019 - Mar 2020)
- University of Ottawa Buddy Program - *Mentor* (Sep 2019 – Dec 2019)
- Sickle cell awareness Group of Ontario - *Volunteer* (Jul 2017 – Dec 2018)
- St Joe's Library Assistant and Food Bank - *Volunteer* (Apr 2017 – Sep 2018)
- University of Ottawa Residence Move-in - *Volunteer* (Sep 2015 – Sep 2017)