Natural Killer Cell Function in Chronic HCV Infection

by

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Dedicate to my family and friends

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Abbreviations

Aboriginal	AB
Alanine aminotransferase	ALT
Alpha	α
Antibody-dependent cellular cytotoxicity	ADCC
Antigen presenting cell	APC
Aspartate aminotransferase	AST
Beta	β
Body mass index	BMI
Caucasian	CA
Culture medium	CM
Cytolytic T lymphocyte	CTL
Dendritic cells	DC
Direct-acting antiviral	DAA
Early virological response	EVR
Gamma	γ
Gamma glutamyl transpeptidase	GGT
Granulocyte macrophage colony-stimulating factor	GM-CSF
Hepatitis C virus	HCV
Hepatitis B virus	HBV
High viral load	HVL
Human immunodeficiency virus	HIV
Human leukocyte antigen	HLA
Immunoreceptor tyrosine-based inhibitory motifs	ITIM
Injection drug users	IDU
Interferon	IFN
Interferon regulatory factor	IRF
Interferon-stimulated genes	ISG
Interleukin	IL
Internal ribosomal entry site	IRES
Intracellular cytokine staining	ICCS
Killer cell immunoglobulin-like receptors	KIR
Low-density lipoprotein	LDL
Low viral load	LVL
Lymphocytic choriomeningitis virus	LMCV
Lysosomal-associated membrane protein 1	LAMP1
Myeloid dendritic cells	MDC
Natural killer	NK
Natural killer T cell	NKT

Non-responder	NR
Non-structural	NS
Pattern recognition receptor	PRR
Pegylated interferon	pegIFN
Peripheral blood mononuclear cell	PBMC
Plasmacytoid dendritic cells	PDC
Polymerase chain reaction	PCR
Programmed cell death protein 1	PD-1
Protease inhibitor	PI
Rapid virological response	RVR
Ribonucleic acid	RNA
Scavenger receptor class B	SR-B1
Signal transducer and activator of transcription	STAT
Single nucleotide polymorphism	SNP
Sustained virological response	SVR
T helper	Th
TNF-related apoptosis-inducing ligand	TRAIL
Tumor necrosis factor	TNF

Abstract

NK cells control viral replication through cytotoxicity and IFNγ production. These functions were assessed in chronic HCV infected patients undergoing treatment. Aboriginals have genetic polymorphisms that may enhance NK cell function suggesting more effective clearance of chronic HCV than Caucasians.

NK cell function was similar at baseline between ethnicities. At 3 months of treatment, Caucasian had higher NK killing potential compared to Aboriginal patients. This had no effect on treatment outcomes. NK cell cytotoxicity negatively correlated with viral loads while NK IFNγ production, particularly within the CD56bright subset, positively correlated with viral load suggesting that viral loads control NK cells function through an unknown mechanism. NK cell killing reflect fibrosis, but not liver damage measured by liver enzymes. IFNγ production,by NK cells does not reflect fibrosis nor liver enzymes levels. Lastly, NK cell function does not associate with therapeutic outcomes of chronic HCV infection suggesting that they do not directly play a role in therapeutic clearance of HCV.

Introduction

1.1 Hepatitis C virus

The Hepatitis C virus (HCV) was first discovered in 1989 when it became apparent that most cases of hepatitis resulting from blood transfusions were not due to hepatitis A or B viruses [1, 2]. HCV is now estimated to infect 200 million people worldwide [3]. People with acute HCV tend to remain asymptomatic and have few clinical manifestations. Acute HCV infections may eventually develop into chronic infections. Chronic HCV results in ~20% of infected people to progress to liver cirrhosis over the course of decades [4, 5]. Once this occurs, the only possible intervention is a liver transplant. Although treatment continues to evolve, current regimens require IFN and may be only moderately successful with multiple side effects.

1.2 HCV infection

1.2.1 HCV prevalence, incidence and natural history

The global prevalence rate of HCV is estimated to be 2-3% which accounts for 170-200 million people [6]. The prevalence rates are estimated to be 5.3% in Africa, 4.6% in the Eastern Mediterranean, 3.9% in the West Pacific, 2.15% in Southeast Asia, 1.7% in the Americas and 1.03% in Europe [7].

Within Canada, the prevalence rate is estimated to be 0.8% [8]. The prevalence of HCV in the Canadian Aboriginal population varies. However, higher incidences of acute HCV infection are generally reported [9, 10]. Nonetheless, within Canada, lower rates of chronicity have been observed compared to other populations [10-12].

1.2.2 HCV transmission

HCV transmission occurs primarily through direct percutaneous exposure to blood mainly through illicit injection drug use (IDU) [13]. Prior to the `1990's, transfusion or transplantation from infectious donors were also common forms of transmission. In countries that now routinely test blood donors, transfusion-associated HCV infection has nearly been eliminated [14]. Other risk factors for acquiring HCV infection include unsafe therapeutic injections and occupational exposure to blood primarily through accidental needle sticks. HCV is also less efficiently transmitted by mucosal exposures to blood or serum-derived fluids such as vertical transmission from mother to child or engaging in sexual intercourse with an infected partner [15, 16].

1.2.3 HCV life cycle

HCV is a member of the Flaviviridae family with a positive-strand RNA genome. The liver is the major site of HCV replication with HCV RNA being detectable in 5-50% of hepatocytes in chronically infected individuals [17, 18]. The HCV virus attaches to a cell via specific interactions between host cell receptors and viral proteins resulting in pH-dependent endocytosis which is believed to be a characteristic of the family Flaviviridae life cycle [19]. Although a detailed mechanism for HCV has yet to be determined, studies have suggested the importance of CD81, scavenger receptor B1 (SR-B1) and LDL receptor (LDLr) for viral entry [20-24]. C-lectins, including L-SIGN and DC-SIGN, along with a tight junction component, claudin-1, have also been suggested as potential co-receptors for virus attachment based on their affinity to HCV glycoprotein E2 [25-27].

Once viral entry has occurred, the plus strand RNA is translated to produce the NS proteins, including the viral RNA dependent RNA polymerase essential for generating the membranous

web and subsequent negative strand synthesis. This complementary negative-strand is then used to produce genomic positive-strand RNA. Multiple interactions between host cell membrane and viral nonstructural proteins control the assembly and activity of this process.

After viral replication, translation of the HCV RNA molecules occurs via a cap-independent IRES-mediated process with direct recruitment of each ribosome to the starting site of translation [28]. The main translation product from the HCV genome is a large precursor polyprotein that is processed by cellular and viral proteases to generate the structural and non-structural proteins.

Viral assembly involves oligomerization of the capsid proteins and encapsidation of viral genomic RNA. Once this nucleocapsid forms in the cytoplasm, it acquires an envelope as it buds through an intracellular membrane. It is then believed that HCV particles are released through the host cell secretory pathway as HCV structural proteins have been observed in the endoplasmic reticulum and the Golgi apparatus [29].

1.2.4 HCV genome and encoded proteins

The positive strand HCV RNA genome is about 9,600 nucleotides [30] with a single large open reading frame that encodes a 3,000 amino acid polyprotein flanked by short 5' and 3' untranslated RNA regions [1]. The polyprotein can be divided into two segments: the NH_2-terminal region containing the structural proteins core, E1 and E2 and the $COOH^-$ terminal region containing the nonstructural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. The nucleocapsid protein (core) is highly conserved among the different HCV genotypes. Core and the two envelops glycoproteins (E1 and E2) are the major structural components of the virion [1]. HCV proteins are multifunctional and have roles in viral propagation and immune modulation. Core is essential for viral replication, virion formation and pathogensis. E1 and E2 proteins are necessary for viral entry, but E2 in particular is also immune modulatory. The small

p7 protein assembles into a membrane-bound multimer which may function as an ion channel [31]. Its function is believed to be in particle assembly and release, and is not certain whether it is incorporated into virus particles. NS2 is thought to play a role in viral morphogenesis and release. NS3 in conjunction with NS4A forms the NS3-4A complex which is essential for viral polyprotein processing and RNA replication. NS4B is believed to play a critical role in assembly of the HCV replication process and also has GTPase activity which is important for RNA replication. NS5B is an RNA-dependent RNA polymerase. When considering that HCV has a high replication rate of between 10^{10} and 10^{12} virions per day, one can see the enormous potential to create a diverse viral population known as quasispecies. These viral quasispecies can escape immune surveillance resulting in viral persistence.

Similar to other RNA viruses, HCV shows astonishing genetic diversity due to its error prone RNA-dependent RNA polymerase. Six distinct HCV genotypes have been identified to date and a consensus has been reached on the nomenclature used to describe them along with the much larger number of HCV subtypes [32]. Regardless of the HCV genotype, HCV proteins interact with the immune system.

1.3 Immune system

1.3.1 Innate and adaptive immunity

The immune system can broadly be classified into two parts: the innate and the adaptive immune systems. Innate immunity is the body's first line of defense against an infectious disease. Components of innate immunity consist of physical barriers such as skin, mucosal surfaces that prevent pathogens from adhering to the epithelium. Innate immunity deals with most pathogens without requiring adaptive immunity. It is able to recognize different pathogens through different pattern recognition receptors (PRRs) that can distinguish components of

bacteria, viruses and fungi from healthy cells. Hallmarks of the innate immune system include rapid, non-specific responses and no classical memory responses. Adaptive immunity is critical when a pathogen overwhelms innate immunity. Antigen presenting cells such as dendritic cells and macrophages act as a mediator between innate and adaptive immunity. These cells present specific antigens from the pathogen to effector cells of the adaptive immune system. Effector cells include a repertoire of T and B cells, of which a select few will undergo clonal expansion to generate cell-mediated and humoral immunity. Hallmarks of the adaptive immune system include a delayed response, specificity and memory responses.

1.3.2 Cellular responses to HCV

1.3.2.1 Natural killer cells

Natural killer (NK) cells are $CD56^{+}CD3^{-}$ lymphocytes which are part of the innate immune system as they can induce death of allogeneic cells and autologous cells undergoing various forms of stress such as microbial infection or malignant transformation [33, 34]. They comprise 5-15% of lymphocytes within the periphery and may account for 40-60% of intrahepatic lymphocytes [35, 36]. Their principle function is to sense pathogen infected or transformed cells and, upon activation, eliminate these cells. They act through two main effector functions: target cell lysis, as well as cytokine and chemokine secretion [37-39]. NK cells are also involved in bidirectional cross-talk with dendritic cells (DCs). Through direct cell contact, activated NK cells enhance DC maturation and production of IL-12 while DCs enhance NK cell cytotoxicity [40, 41]. Please see Section 1.3.3 for NK cell functions.

1.3.2.2 Dendritic cells and monocytes

DCs are capable of taking up and processing viral antigens to present them to other immune cells [42]. They are a critical component that bridges innate and adaptive immunity. DCs detect the presence of pathogens, process antigenic material, and present it through major histocompatibility complex molecules to lymphocytes, including T cells [43]. There are two major subsets of DCs, myeloid dendritic cells (MDCs) and plasmacytoid dendritic cells (PDCs), based on their development from either myeloid or lymphoid precursors and distinct cytokine profiles: myeloid DCs produce IL-12 and IL-10 with little interferons while plasmacytoid DCs produce abundant type-1 interferons and little of anything else [44].

During chronic HCV infections, there is selective impairment in the maturation process of both MDCs and PDCs [45, 46]. Furthermore, the PDC compartment in patients with chronic HCV appears reduced relative to healthy donors. This may contribute to the defective type-1 interferon response [47, 48]. HCV core protein has been shown to interact with toll-like receptor (TLR)2 expressed by monocytes to increase IL-10 secretion by monocytes. This is believed to result in the inhibitory effect of HCV on DC differentiation [49]. Taken all together, this may contribute to the failure to maintain a sustained HCV-specific T cell response required for HCV eradication.

1.3.2.3 T cells

There are different subsets of T cells, each with their own specific functions. The main subsets are $CD4^+$ T cells, $CD8^+$ T cells and regulatory T cells. $CD4^+$ T cells provide the necessary help for both cellular and humoral immunity. They provide costimulatory signals to B cells, activate antigen presenting cells (APCs) and prime and sustain the $CD8^+$ T cell response. They are classified based on their function and the types of cytokines they produce. They can be divided into Th1 cells that produce cytokines such as IFN-γ and IL-2, Th2 cells that primarily

produce IL-4, IL-5 and IL-13, and Th17 cells that mainly produce IL-17 [50, 51]. $CD8^+$ T cells have two effector functions: i) to eliminate infected target cells and ii) secrete of cytokines. Secreted cytokines include IFN-γ and TNF-α which use different pathways to create an antiviral environment. Regulatory T cells are classically defined as $CD4^+CD25^+$, and are divided into two major subsets: thymus-derived natural Tregs and peripherally inducible Tregs. They function mainly in self-tolerance and to limit harmful immune responses [52, 53]. They do so directly through contact-dependent mechanisms and indirectly via TGFβ1 and IL-10 production [54, 55].

Numerous studies have indicated that individuals with initial, strong, and multi-specific T cell responses may clear virus spontaneously [56-58]. In contrast, during chronic HCV infections, $CD4^+$ and $CD8^+$ T cells lose their proliferative and effector functions in a process known as T-cell exhaustion [59]. HCV-specific $CD4^+$ T cells have been shown to have impaired IFN-γ and IL-2 secretion [60, 61]. HCV-specific $CD8^+$ T cell responses during chronic infections are weak and target only a narrow selection of viral epitops compared to people who clear the virus [62]. Regulatory T cells are elevated in the peripheral blood of individuals with chronic HCV infections and are believed to inhibit the Th1 $CD4^+$ T cell response [63, 64]. Th17 cells have been shown to be correlated with ALT levels, but not HCV RNA in individuals with chronic HCV. Thus they may not play a role in controlling disease progression during the infection as seen in other liver diseases [65-67].

It is believed that HCV develops its viral quasispecies during the time required to generate an adaptive immune response. Thus CTLs have not had time to expand and acquire effector functions [68, 69]. It is believed that these escape mutations within epitopes restricted by HLA class I alleles result in the development of chronic HCV infection [70, 71].

1.3.2.4 B cells

B cells are lymphocytes that are an essential component of the adaptive immune system and they play a role in the humoral immune response to pathogens. They are capable of serving as antigen-presenting cells, but their main function, once activated with T cell help and after differentiation into plasma B cells, is to make antibodies against antigens. Another major function of B cells is to differentiate after activation into memory B cells that are long-living cells that can respond much more quickly to secondary exposures to the same antigen.

As previously mentioned, chronic HCV is characterized by viral quasispecies. By the time antibodies are made specific to the HCV virus, it has already mutated making those neutralizing antibodies obsolete. Clonal expansion of peripheral $CD5^+$ B cells has been reported [72, 73]. B cell regulatory dysfunction is also reported as important regulatory B cell receptors such as Fas, TNF receptors I and II, and B-lymphocyte stimulator have been shown to be elevated which may induce B cell clones and autoantibody production [74, 75]. The end result of HCV-related B cell clonal expansion and dysfunction is up to 40% of infected individuals have extrahepatic symptoms including mixed cryoglobulinemia, glomerulonephritis, systemic vasculitis, Sjögren's syndrome, and B cell non-Hodgkin's lymphoma [76].

1.3.3 Natural killer cell functions

As previously mentioned, NK cells have two main functions. They can kill target cells directly through cytolysis or indirectly through cytokine production. There are three main mechanisms of NK cell cytolysis. The first involves the delivery of secretory lysosomes or granules containing perforin and granzyme B into the immunological synapse between the NK cell and its target [77, 78]. These cytotoxic granules are formed during development and NK cells constitutively express perforin and granzymes B [79]. Lining these cytolytic granules is the

lysosomal-associated membrane protein (LAMP)-1, otherwise known as CD107a, which can readily be detected via flow cytometry [80]. Once inside the synapse, it facilitates entry of granzymes into the target cell. Once inside the target cells, granzymes, in particular granzyme B, promotes apoptosis through proteolysis of a few different substrates. It can directly cleave and activate caspases [81-83]. Granzyme B may also promote caspase-independent apoptosis by triggering mitochondrial permeabilization [84, 85]. A second mechanism of NK cell killing is known as antibody-dependent cell-mediated cytotoxicity (ADCC). This process occurs through the activating receptor CD16 found on the surface of NK cells. CD16 is a low-affinity activating Fc receptor (FcγRIIIA) that allows NK cells to bind to and eliminate antibody-coated cells [86], [87]. The final mechanism of NK cell killing is through the binding of TNF-related apoptosis-inducing ligand (TRAIL) on the surface of NK cells to its death receptor DR4 (TRAIL-R1) and DR5 (TRAIL-RII) on the surface of target cells [88].

The other main function of NK cells is to produce cytokines with antiviral functions including IFNγ and TNFα. The importance of NK cell-produced IFNγ is well documented with detectable protein levels observed during some but not all viral infections [89-92]. The production of these cytokines is also important in the development of antiviral T cell responses.

HCV E2 has been shown to inhibit protein kinase receptor activity via crosslinking with CD81 to inhibit NK cell functions including cytotoxicity and IFN production [93, 94]. Inhibiting NK cells early in infection may allow HCV to establish a replication advantage prior to the induction of specific immune responses from which the host may never recover. NK cells have a number of different regulatory receptors which participate in the activation/inhibition of cytotoxicity and cytokine production.

1.3.4 Natural killer cell activation

Unlike other lymphocytes, NK cells do not express antigen-specific receptors, but are still able to selectively eliminate virus-infected and tumor cells. NK cell activation is controlled by a delicate balance between activating and inhibitory signals. Two models of NK cell activation have been proposed and are called the 'missing self' and 'induced self' models [95, 96]. The 'missing-self hypothesis' suggests that NK cells eliminate target cells that show reduced or abnormal MHC or HLA class I molecules. In other words, they attack target cells that are missing expression of self-molecules normally found on healthy cells. The 'induced self' model suggests that stressed cells upregulate expression of "inducible self" ligands which NK cells are able to recognize without the need for altered MHC or HLA class I molecules. More activation tips the balance in favour of NK cell activation.

Three groups of NK cell receptors have been distinguished: the immunoglobulin superfamily, the C-type lectin superfamily and the natural cytotoxicity receptors. The immunoglobulin superfamily killer-cell immunoglobulin-like receptor (KIRs) and lectin-like receptors can be activating or inhibitory, while natural cytotoxicity receptors have only activating functions. For the immunoglobulin and C-type lectin superfamilies, inhibitory and activating elements can be found in the cytoplasmic tails of these receptors. Inhibitory receptors signal through intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs) located in the cytoplasmic tail. In contrast, activating receptors signal through immunereceptor tyrosine-based activating motifs (ITAMs) which are not contained in the receptor's cytoplasmic tails but rather in associated molecules. It is believed that a balance of inhibitory and activating signals is responsible for NK cell activation.

KIR receptors recognize classical MHC classI-like molecules including HLA-A, -B and –C. They are able to recognize polymorphisms within these molecules. Each individual has their own KIR repertoire based on their genes. The KIR cluster contains 15 genes and 2 pseudogenes [97]. Due to different allelic variants and levels of expression, different ethnic populations of NK cells can express a variety of KIRs [98, 99].

The C-type lectin superfamily recognizes non-classical MHC class I or class I-like molecules. Three C-type lectin receptors include NKG2D, a receptor for the stress-inductible MHC class I-like molecule MICA, MICB and ULBP1, NKG2C [100, 101], a receptor for the non-classical MHC class I molecule HLA-E [102], and NKRP-1, a putative NK cell receptor for still undefined carbohydrates, glycolipids and/or glycoproteins [103].

Natural cytotoxicity receptors are immunoglobulin-like receptors including NKp30, NKp44 and NKp46. Although several viral ligands of natural cytotoxicity receptors have been identified, little is known about their cellular targets. One study shows that they bind to heparin sulfate on cancer cells [104]. These receptors are specific to NK cells and once crosslink, participate in cytotoxicity and cytokine release [105]. The activation status of NK cells correlates with liver inflammation. Increased expression of NKG2A, CD69 and CD107a, a marker of NK cell degranulation, on peripheral NK cells have been associated with disease activity [106-109].

1.3.5 NK cell subsets

As mentioned, NK cells kill target cells directly through cytolysis or indirectly through cytokine production. NK cells can be divided into two subsets based on their expression of CD56 and CD16. Approximately 90% of peripheral blood NK cells are CD56dimCD16^{+} whereas the remaining 10% are CD56brightCD16+/-dim [110]. Although early studies show that resting CD56dim NK cells were naturally more cytotoxic compared to CD56bright NK cells [111], it has

subsequently been observed that these subsets have similar levels of cytotoxicity after activation with IL-2 or IL-12 in vitro [112, 113]. CD56dim NK cells, having greater expression of CD16, therefore it is believed that they participate more in ADCC compared to the CD56bright NK cell subset. The CD56bright subset are the main producers of immunoregulatory cytokines or chemokines including interferon IFN-γ, tumor necrosis factor (TNF)-α, TNF-β, granulocyte macrophage-colony stimulating factor (GM-CSF), IL-10, and IL-13 [114, 115]. The CD56dim subset, under the same conditions, produces negligible amount of immunoregulatory cytokines [114].

1.3.6 NK cells and the liver

The liver is enriched with NK cells known as intrahepatic NK cells. Under normal conditions, intrahepatic NK cells account for about 30% of resident lymphoid cells but may reach up to 90% in liver diseases [35]. Intrahepatic NK cells are embedded in the endothelial lining of the liver sinusoids and was originally described as 'pit' cells [116]. Intrahepatic NK cells can be sub-classified based on the density and size of their granuoles (low density/small granuoles vs. high density/large granuoles). High density/large granuoles most resemble peripheral NK cells. It has been shown, in rats, that peripheral blood high-density–large granular cells migrate to the liver and differentiate into liver-specific low-density–small granular NK cells [117].

There is limited data on intrahepatic NK cells. Liver biopsies from healthy people are rare. Intrahepatic NK cells may behave differently than peripheral NK cells due to the tolerogenic environment of the liver. This hyporesponsive state of intrahepatic NK cells has been described in the early stages of hepatitis B virus infection in humans and may contribute to the establishment of chronic viral infection [118]. Murine intrahepatic NK cells are less cytotoxic

and have an alterered cytokine profile producing less IFNγ and greater levels of immunoregulatory cytokines such as IL-10 compared to peripheral and splenic NK cells [119]. Most evaluations of intrahepatic NK cells are compared to peripheral NK cells or to intrahepatic cells from other liver diseases. In general, however, a larger portion of intrahepatic NK cells express activation molecules and TRAIL receptors compared to the peripheral blood compartment [109, 120].

1.3.7 NK cells in acute HCV infections

People with acute HCV infections rarely come to the attention of clinicians as most infections are asymptomatic. Therefore, limited data exists during this phase of infection with regard to NK cells. The majority of individuals (55-85%) exposed to HCV develop chronic infections [121]. Those individuals who do not develop a chronic infection clear the virus spontaneously. Differences in viral genotype and host age, gender and ethnicity affect spontaneous clearance. African Americans have been shown to have reduced rates of spontaneous clearance, while Canadian Aboriginals have increased rates of spontaneous clearance when compared to Caucasians [10-12, 122, 123]. A possible explanation for ethnic differences in spontaneous clearance could be the prevalence of different KIR profiles among different ethnicities. The presence of different KIR receptors has been shown to influence the outcome of HCV infections [124, 125]. African Americans have unique KIR gene profiles which may account for their increased rate of chronicity [126]. Previously, the Rempel lab has shown that relative to Caucasians, Canadian Aboriginals possess KIR clusters that display a greater immune activating phenotype associated with genes of the KIR B haplotype [127]. Similarities exist between the KIR gene profiles seen in the Manitoban Aboriginal population and KIR gene profiles reported to be associated with spontaneous clearance of HCV [125, 128, 129].

Single nucleotide polymorphisms (SNPs) within the IL-10 gene promoter may also in part explain the ethnic disparity in spontaneous clearance of HCV. HCV protein induction of IL-10 may impair IFNγ production and proliferation of HCV-specific T cells in acute HCV infection [49, 130]. SNPs can be classified as high, intermediate or low IL-10 producers based on IL-10 production from PBMC [131]. SNPs defined as low IL-10 producers have been associated with HCV clearance [132, 133]. The Rempel lab has previously shown that Aboriginals have a genetic tendency to produce less IL-10 compared to Caucasians and that this may contribute towards higher spontaneous clearance rates seen in this population [134].

Upon any viral infection, peripheral blood NK cells become more prevalent and activated. One study reported an increase in the number CD56bright NK cells and a reduction in the number of CD56dim NK cells in chronic HCV infections [135]. Despite this change in NK cell subset composition, both cytotoxicity and IFNγ production of NK cells are greater in acute HCV infections compared to healthy controls [135]. This same study has found that expression of the activating receptor NKG2D was also increased in the acute phase of HCV infection. Another study reported an increase in NK cell activity as measured by a degranulation assay [136]. They found lower IFNγ production however this may be attributed to opioid use by the subjects. NK cell activity correlated with the magnitude of HCV virus-specific T cell responses during spontaneous clearance which implies a coordinated innate and adaptive immune response during acute HCV infection.

1.3.8 NK cells in chronic HCV infections

In contrast to acute HCV infections, more studies have been done examining NK cell in the context of chronic HCV infections. Within the peripheral blood compartment of individuals with chronic HCV, NK cell frequencies in both absolute and percentage of total lymphocytes are

reduced when compared to healthy individuals [107, 137, 138]. A number of studies have documented a relative increase in peripheral CD56bright, but not CD56dim NK cells [107, 137, 139, 140]. When examining effector functions of NK cells, there is more conflicting information. Some groups report an increase [108, 141], one reports no difference [137], and yet others report a decrease [142-144] in NK cytotoxic activity between individuals with chronic HCV infections compared to healthy individuals. IFNγ production from NK cells, however, generally appear decreased in chronic HCV infections supporting the T cell exhaustion hypothesis [108, 109, 141]. Changes in NK cell phenotype include an increase in activating receptor expression including NKG2C, NKp44, NKp46 and NKp30 [109, 140, 145]. Conflicting information with regard to NKG2D expression exists with reports suggesting upregulation, downregulation or no change [140, 145-147].

Few studies have examined the role of intrahepatic NK cells in chronic HCV infections. TRAIL was found to be down-regulated on intrahepatic NK cells from HCV infected patients compared to intrahepatic NK cells from healthy controls [148]. The intrahepatic NK cell compartment is also significantly reduced in patients with chronic HCV compared to healthy people [107, 148, 149]. Accompanying a reduced intrahepatic NK cell compartment, there is reduced cytotoxicity and similar IFNγ production from intrahepatic NK cells when compared to intrahepatic NK cells from otherwise healthy subjects who agreed to donate a liver tissue fragment during laparoscopic cholecystectomy [148]. Another study suggests reduced cytotoxicity and IFNγ production from intrahepatic NK cells in cirrhotic patients with chronic HCV compared to healthy donors [150]. Needless to say, more research is needed to address the role of intrahepatic NK cells during chronic HCV infections.

1.4 HCV treatment

1.4.1 Treatment for chronic HCV infections

Current standard of care antiviral therapy includes pegylated interferons (pegIFN) and ribavirin, although this is rapidly changing. PegIFN is a product of pegylation, the attachment of inert polyethylene glycol polymers, to IFN. This results in a larger molecule with a longer half-life due to reduced clearance while maintaining its biological activity. Interferon monotherapy used before the development of pegIFN, was successful in 15-20% of cases [151]. Using the current standard of care treatment regime, successful treatment occurs in approximately 50% of genotype 1 infections and up to 80% for genotype 2 or 3 [152, 153]. This is an improvement over interferon monotherapy which was successful in 15-20% of cases [151]. Treatment success, or sustained virological response, is defined as having undetectable HCV RNA six months after the end of treatment. More than 97% of patients who achieve a sustained virological response (SVR) remain HCV RNA free as detected by PCR for the following 5-14 years [154, 155].

Although the mechanism of action of interferon is currently unknown in the context of HCV infection, it is believed that induction of interferon-stimulated genes (ISGs) is important for favourable outcomes. It has been shown that HCV viral loads begin to decline approximately 8 hours after a single dose of interferon [156]. This rapid initial decline in viral loads during the first one to 2 days of treatment is followed by a gradual second phase of viral elimination over the course of several months [157].

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is an oral purine nucleoside analogue with broad activity against viral pathogens [158]. Although the exact antiviral mechanism of ribavirin is unclear, the current theory is that it acts as a mutagen to increase the probability of lethal mutations being incorporated into the HCV genome. Ribavirin exposure has

been linked to an increase in HCV mutation frequency in HCV replicon cell lines, as well as in patients treated with ribavirin monotherapy [159]. In contrast, for those receiving combination pegIFN and ribavirin, the mutation frequency fell on treatment between 20 and 47 days [159]. Another study also identified an increase in HCV mutation frequency in humans treated with ribavirin monotherapy at 4 weeks but not at 24 weeks of treatment [160]. However, ribavirin monotherapy followed by combination interferon/ribavirin treatment did not support an increase in HCV mutations [161].

In addition, ribavirin may modulate the innate immune response. Tissue expression profiling of liver biopsies from patients treated with a single dose of peginterferon with or without ribavirin indicated that ribavirin increased expression levels of several ISGs and also decreased several negative regulators of interferon signaling such as PP2A and SOCS1 [162]. Evidence also suggests that ribavirin, at clinically achievable plasma levels, suppresses IL-10 production by PBMC from HCV RNA positive patients exposed to HCV core protein [163].

IFNα treatment is associated with a range of adverse effects including leucopenia, thrombocytopenia, autoimmunity, neutropenia, depression, fatigue, and "flu-like" symptoms which can become serious. Maintenance of pro-inflammatory cytokine expression during the first four weeks of pegIFN and ribavirin treatment has been associated with treatment induced depression [164]. Ribavirin is also associated with serious side effects including hemolytic anemia. Side effects from HCV treatment are sometimes dose limiting and may lead to discontinuation of treatment in approximately 20% of patients [153, 165].

New therapeutics for treating chronic HCV infections were introduced during 2011. These therapeutics are known as direct-acting antivirals (DAA) as they target specific viral elements pathways that interfere with HCV infection and replication. The best known DAAs are the first-

generation NS3/4A protease inhibitors (PI) that were added in combination with pegIFN and ribavirin to treat genotype 1 infections. These PIs are known under their trade names of boceprevir and telaprevir and increase the SVR rates in naïve patients upwards to 75% [166, 167]. More importantly, these benefits are even more important in raising SVR rates in treatment-experienced patients [168, 169].

Other potential targets of DAAs include both structural and nonstructural proteins [170, 171]. Structural proteins include the nucleocapsid core protein, the envelope glycoproteins E1 and E2. Nonstructural proteins targeted include the NS2/NS3 region, the NS3 serine protease RNA helicase, the NS4A peptide cofactor of NS3, the NS5A protein and the NS5B RNA-dependent RNA polymerase. The research behind these other DAAs is still in its infancy. As our understanding of HCV molecular biology improves, so will the development of drugs to improve treatment response rates.

1.4.2 Factors influencing treatment outcomes

1.4.2.1 Viral factors

Viral factors can influence treatment outcomes. These include viral genotypes and viral load. Viral genotype is perhaps the most important viral factor that influences treatment outcomes. SVR rates differ drastically among viral genotypes with SVR rates of approximately 50% for genotypes 1 and 4 compared to up to 80% for genotypes 2 and 3 [152, 153, 172]. High baseline viral loads (measurement of HCV RNA levels in the blood prior to commencement of treatment), at >800,000 IU/ml, have been associated with worse therapeutic outcomes in particular for genotype 1 infections [173-175].

Viral kinetics during the first few weeks of treatment are strong indicators of subsequent treatment outcomes. HCV RNA levels generally fall in a biphasic manner during treatment [156]. The first drop in HCV RNA occurs in the first few hours of treatment. It is due to an inhibition of viral replication by the direct, non-specific action of IFN. The second drop of HCV RNA occurs gradually leading to seroclearance. It is believed to be related to the elimination of infected cells. The presence of a rapid virological response (RVR), defined by undetectable HCV RNA after 4 weeks of treatment is the single best predictor of an SVR to current antiviral therapy for patients with genotype 1 infections (89% and 89% respectively) [176, 177], or patients with genotype 2 or 3 infections (89% and 81% respectively) [178, 179]. The presence of an early virological response (EVR), defined as at least a 2 log drop in HCV RNA from baseline levels at 12 weeks of treatment, is also predictive of successful treatment outcomes with 72% of patients subsequently developing an SVR [155]. EVRs can further be broken down into complete EVR (cEVR, a lack of HCV RNA at 12 weeks) and partial EVR (pEVR, a greater than 2 log drop in HCV RNA at 12 weeks). Achieving either a cEVR or a pEVR may be used to optimize treatment duration in previously non-RVR patients [180].

1.4.2.2 Host factors

Host factors that influence treatment outcomes include gender, age, ethnicity and co-morbidities. A gender effect on treatment response has been reported. Females had higher SVR rates in studies using conventional IFN-based therapy [181], but in studies using pegIFN-ribavirin, no gender differences were observed [153, 182]. Younger patients under 40 years of age also had higher SVRs on pegIFN and ribavirin therapy [152, 153, 172]. Ethnicity also impacts therapeutic success. Please see section 1.4.2.3 for details.

In addition, concurrent morbidities impair the ability to achieve a SVR. Chronic HCV patients with body mass indexes (BMIs) greater than 30 kg/m^2 are more likely to be insulin-resistant and have been shown to be a risk factor for nonresponse to antiviral therapy independent of viral genotype [183]. They are also more likely to have hepatic steatosis or steatohepatitis, which are also negative predictors of SVR rates [184, 185]. Excessive alcohol use further reduces the likelihood of a response to treatment [186]. Other virus co-infections, most notably HIV and HBV, have been associated with lower SVR rates [187]. It has been estimated that 3-6% of HCV carriers are co-infected with HBV with a higher prevalence rate in high-risk groups and in geographical regions are both viruses are endemic [188]. Up to 50% of HIV-infected patients are co-infected with HCV with prevalence being higher in injection drug addicts compared to other risk categories [189]. The presence of fibrosis and cirrhosis has been shown in several studies to be an unfavourable predictor of treatment outcomes [190-192].

1.4.2.3 Ethnicity

African-American patients, when compared to Caucasian patients, have been shown to have lower rates of SVR with genotype 1 infections [193, 194]. However, when looking at genotype 2 and 3 infections, there is no difference between these ethnicities [193, 195]. Patients of Asian descent, on the other hand, have been shown to have enhanced rates of SVR compared to Caucasian patients [196, 197]. Not a lot is known of Latino/Hispanic patients, however it has been suggested that they tend to have lower rates of SVR compared to Caucasians [196, 198]. Lastly, within the Canadian Aboriginal population, it has been suggested that they have similar rates of SVR compared to Caucasians [199]. However, this study does not take into account treatment-naïve individuals. When considering that 0 of the previously 14 treated Aboriginal patients achieved an SVR compared to 260 of the 854 previously Caucasian patients, this

information suggests that the rate of SVR within treatment-naïve Aboriginal patients is slightly higher than their Caucasian counterparts. Ethnic differences in treating chronic HCV infections may be due to different genetics between ethnic populations.

1.5 HCV treatment and immunity

1.5.1 Genetics

Recently, using genome-wide association studies, the importance of single nucleotide polymorphisms near a gene encoding a type III interferon, IL-28B, has been identified as a strong indicator of the ability to achieve a SVR with treatment [200, 201]. Different major and minor IL-28B alleles have been associated with SVR or non responders. More interestingly, the nonresponse allele frequency was much higher in African Americans compared to Hispanics or European Americans, while the effect of response genotype on SVR was essentially identical regardless of race [201]. In a independent, multiethnic population, the highest frequency of the major allele was seen in East Asians. Thus differences in allele frequency may help to explain the racial difference in SVR rates.

Another polymorphism which may impact IFN-based therapies for chronic HCV lies within the IL-10 gene. IL-10 can modulate immune responses and is a classical anti-inflammatory cytokine. When examining IL-10 polymorphisms, several groups reported distribution associations with treatment outcomes [133, 202]. It has been shown that serum IL-10 levels are lower in individuals who achieve a SVR compared to those who do not achieve a SVR [203, 204]. In contrast, no associations with treatment outcomes have been found [205-207]. With such conflicting information, it is difficult to correlate IL-10 SNPs with treatment outcomes.

Other genetic studies have indicated that certain KIR and HLA-C genes are associated with favourable responses to IFNα-based treatment regimes [208, 209]. It is interesting to note that these KIR genes are among those that have been beneficial in spontaneously resolving HCV infections.

1.5.2 Impact of treatment on immune system

As mentioned, current standard of care antiviral therapy includes pegIFNα and ribavirin. Exogenously administered IFN activates endogenous pathways of type I IFN signaling. Firstly, throughout the body, IFN binds to IFN-α/β receptors (IFNAR) and activates the Jak-Stat signal transduction pathway. IFNAR exists as a heterodimer of IFNAR-1 and IFNAR-2 [210], and can be found on nearly all cell types. Once activated, it recruits and phophorylates the tyrosine kinases Jak1 and Tyk2, which in turn phosphorylates STAT1 and STAT2. Phosphorylated STAT1 and STAT2 combine with IRF9 to form the heterotrimeric transcription factor known as ISGF3. ISGF3 binds to the interferon-stimulated response element upstream of interferon-stimulated genes (ISGs) to promote their transcription. One of these ISGs is IRF-7, which amplifies interferon-α and -β transcription, thus generating a positive feedback loop for the amplification of type I interferon signaling [211].

Type I IFNs have broad antiviral activity. Several hundred genes can be induced, yet others can be negatively regulated [212-214]. A large number of these genes have unknown functions, but taken altogether they establish an “antiviral state” in the cell [215]. This “antiviral state” protects the cell from viral infection by directly targetting many different stages of viral replication including viral entry, envelope uncoating, genome replication, protein assembly, and release of viral progeny to limit infection [216, 217]. Type I IFNs also have indirect immunomodulatory activity to control viral infections. They can target innate immunity by promoting neutrophil

survival [216, 218] and the activation of macrophages [219], natural killer cells [220] and DCs [221]. In addition to its crucial role in regulating innate antiviral immunity, type I IFNs can also enhances the adaptive immune response. Type I IFNs have been shown to have an important role in the differentiation of both $CD4^+$ and $CD8^+$ T cells [222, 223]. Furthermore, type I IFNs stimulate the proliferation of memory T cells [224], enhances dendritic cell differentiation [225, 226], and upregulates class I MHC expression on hepatocytes [227].

1.5.3 Impact of treatment of NK cells

IFNα is the foundation of current anti-viral therapy for chronic HCV infections because it is a potent stimulator of NK cells. It has been shown to increase NK cell cytolytic activitity [228, 229]. Two studies have shown that antiviral therapy shifts the balance of peripheral NK cell subsets by increasing the proportion of CD56+bright NK cells, while slightly declining the CD56+dim subset [230, 231]. These authors argue that this is an advantageous strategy as increased secretion of IFNγ by CD56+bright NK cells has an antiviral effect, whereas the decrease in the CD56+dim subset may limit damage to hepatocytes. A different study has shown that the reduction of NK frequency and suppression of IFNγ synthesis due to chronic HCV were reversed after antiviral therapy [138].

In addition, during IFNα therapy, there is an increase in NK cell cytotoxicity which may be due to enhanced degranulation or the capacity to induce apoptosis [232]. The exposure to IFNα *in vitro* induces TRAIL expression on NK cells, TRAIL expression inversely correlated with viral load, and that patients who responded to antiviral therapy had higher levels of TRAIL on CD56+dim cells than those who did not respond to therapy [106]. Increased frequency and total number of intrahepatic NK cells on treatment may also associate with SVR [149].

1.6 Project summary

Based on the ethnic differences in spontaneous and therapeutic HCV clearance which involve the KIR genes, we were interested in evaluating peripheral NK cell function between Caucasian and Aboriginal patients preparing to undergo treatment for chronic HCV infection. NK cell function was examined before treatment and after 12 weeks of treatment. The two functions of NK cells we were interested include NK cell cytotoxicity and cytokine production (IFNγ and TNFα). Three separate mechanisms of NK cell cytotoxicity were examined.

The main objectives of this study were:

1. To examine if NK cell function differed between Caucasian and Aboriginal patients with chronic HCV infections.
2. To examine if NK cell function in chronic HCV associated with viral control and/or cellular injury
3. To examine if NK cell function associated with therapeutic outcomes in chronic HCV infections

The hypothesis for objective 1 was that Caucasian patients would have inferior NK cell function when compared to Aboriginal patients. We hypothesized for objective 2 that NK cell function would negatively associate with viral loads, but positively associate with liver damage. Lastly, our hypothesis for objective 3 was that enhanced NK cell function would associate with favourable treatment outcomes.

2 Methods

2.1 Patient recruitment

2.1.1 Ethics

This study was approved by the University of Manitoba Research Ethics Board and was conducted in accordance with discussions with thre Health Research Information Committee, Assembly of Manitoba Chiefs. Participation was voluntary and written consent forms were signed by all study participants.

2.1.2 Recruitment

Caucasian and Aboriginal patients preparing to undergo treatment for chronic HCV infections were recruited through the Viral Hepatitis Investigation Unit, Health Sciences Centre, Winnipeg, MB, Canada. Standard of care inclusion and exclusion criteria for pegylated-IFN and ribavirin treatment applied. The majority of participants had HCV genotype 1 infections. Participants were HIV and HBV antibody negative. Hepatic inflammation was monitored by examination of serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) and bilirubin. Fibrosis and inflammation were graded according to the METAVIR scoring system. Viral loads were measured by quantitative PCR at Cadham Provincial Laboratory, MB, Canada. Early virological responses (EVR), defined as at least a 2 log reduction in viral titer in the serum at 3 months relative to pre-treatment levels, were recorded. The absence (SVR+) or presence (SVR-) of detectable HCV-RNA 6 months after the end of treatment was determined. In addition, seven Aboriginal patients with chronic HCV infections who did not start treatment were recruited from there Mount Carmel Clinic, Winnipeg, MB, Canada.

2.2 Sample preparation

2.2.1 Blood collection and PBMC isolation

Blood samples (~40ml) were drawn and collected in sterile 10 ml EDTA tubes (BD, Franklin Lakes, NJ). Isolation of PBMC from whole blood was performed in a biosafety cabinet under sterile conditions. Two parts blood was diluted with one part sterile 0.85% saline before being carefully layered on top of Histopaque® (Sigma, St. Louis MO) and spun at 600 x g for 30 minutes at room temperature. PBMC were collected using a 2ml pipette, washed twice with sterile 0.85% saline and spun at 300 x g for 10 minutes at room temperature. PBMC were then resuspended in 4ml of PBMC culture medium and counted using a hemocytometer. PBMC culture medium consisted of RPMI 1640 (Hyclone, Logan, UT) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen Life Technologies, Grand Island, NY) and 2-mercaptoethanol (Sigma). PBMC viability was determined using trypan blue exclusion with a 1 in 10 dilution of PBMC in culture medium in trypan blue (Sigma). Aliquots of fresh PBMC were used to evaluate NK cell cytotoxicity in chromium release assays and for *in vitro* cultures. PBMC were also frozen for flow cytometry analysis performed at a later date.

2.2.2 Plasma

Plasma samples (diluted 1:2) were acquired following density centrifugation of PBMC using a 2ml pipette. Samples were aliquoted into 2ml eppendorf tubes (Eppendorf, Hamburg, Germany) and stored at –80^{o}C until analysis for cytokine levels.

2.3 NK cell functional assays

2.3.1 Target Cell culture maintenance

K562 (ATCC® CCL-243™) and HepG2 (ATCC® HB-8065™) cell lines were used as targets for chromium release assay and functionality assessed by flow cytometry. NK-92 (ATCC® CRL-2407™) control cell line was used as a control for the chromium release assay. These cell lines were purchased from American Type Culture Collection (Manassas, VA). K562 cells were maintained in RPMI 1640 (Hyclone) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen Life Technologies). Medium was changed every 2 to 3 days. New cultures were started with 1.0×10^5 and were subcultured at 1.0×10^6 in a T25 flask (Corning, Tewksbury, MA). Adherent HepG2 cells were maintained in DMEM (Hyclone) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen Life Technologies). Medium was changed every 3 days. Subculturing occurred when plate confluency reached >90%. Cell monolayer was washed with sterile 0.85% saline and digested with TrypLE (Invitrogen) before subculturing 1 in 6 in a T75 flask (Corning). NK-92 cells were maintained in αMEM supplemented with 12.5% (v/v) fetal calf serum (Invitrogen Life Technologies), 12.5% (v/v) horse serum (Lonza, Basel, Switzerland) 0.2 mM inositol (Sigma), 0.1 mM 2-mercaptoethanol (Sigma), 0.02 mM folic acid (Sigma) and 100U/ml recombinant human IL-2 (R&D, Minneapolis, MN). Medium was changed every 2 to 3 days to start a new culture at 2.0×10^5 cells/ml into a new T25 flask (Corning).

2.3.2 NK cell cytotoxicity

To measure NK cell cytotoxicity, fresh PBMC were used in a standard 4 hour chromium release assay. This assay does not work on previously frozen cells. Isolated patient PBMC were cultured overnight with or without 100 IU/ml recombinant human IFNα-2b (PBL

InterferonSource, Piscataway, NJ) in 15ml sterile round bottom tubes (Corning Incorporated, Corning, NY) at a concentration of 5.0 x 10^6 PBMC/ml. The following day, the PBMC were washed and resuspended in PBMC culture medium at concentration of 2.5 x 10^6 PBMC/ml. Serial dilutions were made to get additional concentrations of 1.25 x 10^6 and 0.63 x 10^6 PBMC/ml. 100µl of each PBMC dilution was added to a 96 well v-bottom plate (Corning). 1.5 x 10^6 K562 or HepG2 target cells were put in sterile 50ml conical tubes (Corning) and spun at down at 300 x g. The supernatants were discarded and the target cells were then labeled with 500µCi Na_2CrO_4 (PerkinElmer, Waltham, MA) for 1 hour at 37°C, 5% CO_2. After the incubation, target cells were washed once with 1x Hank's Balanced Salt Solution (Invitrogen) and again with appropriate cell culture medium before being resuspended at 1.0 x 10^4 cells/ml. 100µl of each target cell suspension was added to each PBMC dilution along with wells with 100µl of appropriate cell culture medium to measure the spontaneous release or 5% Triton X-100 (Sigma) to measure the maximal release. After the 4 hour effector:target (E:T) incubation at 37°C, 5% CO_2, the 96 well v-bottom plate was spun and 100µl from each well was collected into a non-sterile 5ml round bottom tube (BD Biosciences) to measure counts per minute (cpm) on a Wallac Wizard 1470 Gamma Counter (PerkinElmer). All analyses was done in triplicate. NK cell lysis was defined as the amount of Cr-51 released into the medium upon target cell lysis. Specific lysis was calculated by the following formula:

$$\text{specific lysis (\%)} = \frac{\text{cpm test} - \text{cpm spontaneous release}}{\text{cpm max release} - \text{cpm spontaneous release}} \times 100$$

To compare NK cell cytotoxicity results from different patients, NK-92 cells were run in parallel to patient samples at E:T ratios of 10:1, 5:1, 2.5:1 1.25:1 and 0.6:1 to normalize patient data as described in the Results.

2.3.3 Flow analysis of activation markers

To examine activation markers on the surface of NK cells and their association to NK cell function, surface stains of NK cells were performed. Patient PBMC, previously frozen in liquid nitrogen, were thawed, washed twice in PBMC culture medium and aliquoted (2.5×10^5 cells) into 5 ml round bottom tubes (BD Biosciences). All tubes were washed with 1x PBS and resuspended in 1x PBS. Fixable Viability Dye eFluor® 506 was added to each tube for 20 minutes on ice. Tubes were then washed and resuspended in FACS buffer (0.5% BSA, 0.05% NaN_3 in 1x PBS). Fluorochrome conjugated antibodies against NK cell surface markers were added for 30 minutes on ice as per Table 1. PBMC were also used to examine mechanisms of lysis and intracellular cytokine staining.

PBMC were washed with FACS buffer and fixed with 2% paraformaldehyde (from a 16% stock, Canemco Inc., Lakefield, Quebec) for 20 minutes on ice. After washing in 1x PBS, PBMC were resuspended in FACS buffer until acquisition on a FACS Canto II (BD Biosciences) the following day. Acquisition was performed using FACSDiva software. Analysis was done using FlowJo (Tree Star Inc., Ashland, OR).

Flow cytometry was used to examine the appearance of protein involved in different NK cell lytic pathways on NK cells in conjunction with NK cell-specific cytokine production. Patient PBMC were washed twice in PBMC culture medium and PBMC (2.0×10^6 cells) were resuspended at a concentration of 1.0×10^6 PBMC/ml with either 4.0×10^5 K562 or HepG2 target cells at an E:T ratio of 5:1 in a sterile 5ml snap cap round bottom tube (BD Biosciences).

In addition, PBMC (2.0×10^6) were cultured with PMA/ionymycin (Sigma) as a positive control, or alone as a negative control. PBMC were stained with a mouse anti-human CD107a:PE-Cy5 (BD Biosciences) or with mouse IgG1:PE-Cy5 isotype control (BD Biosciences) for 1 hour at 37°C, 5% CO_2 before addition of 6μg/ml monensine (Sigma) and 10μg/ml brefeldin A (Sigma). Cells were incubated for five hours at 37°C, 5% CO_2, except for the PMA/ionomycin tube which was placed at 4°C after 3 hours to prevent excessive cell death. After five hours, all tubes were centrifuged, washed with 1x PBS and resuspended in 1x PBS. Fixable Viability Dye eFluor® 506 was added to each tube for 20 minutes on ice. Tubes were then washed and resuspended in FACS buffer. For each PBMC tube, two 2.5×10^5 PBMC aliquots were washed a second time. Staining was performed on 2.5×10^5 PBMC to reduce antibody usage in analysis of NK cell surface markers and Panel 2 (Table 1). Antibodies for surface staining were added and incubated for 30 minutes on ice. PBMC were washed with FACS Buffer and fixed with 2% paraformaldehyde (Canemco Inc.) for 20 minutes on ice. PBMC were washed with 1x PBS and permeabilized in FACS buffer with 0.1% saponin for 20 minutes on ice. After another wash with FACS buffer containing 0.01% saponin, PBMC were stained intracellularly for IFNγ and TNF-α for 30 minutes on ice. PBMC were washed with FACS buffer containing 0.01% saponin and resuspended in FACS buffer and kept at 4°C in the dark until acquisition. Samples were acquired the following day on a FACS Canto II flow cytometer (BD Biosciences). All data was analyzed using FlowJo software (TreeStar).

Table 1: Antibodies used for flow cytometry

Antibody	Clone	Conjugated fluorochrome	Compensation antibody
Viability Dye	-	eFluor® 506 (AmCyan)	-
NK surface markers			
CD3	SK7	PE-Cy7	CD3:PE-Cy7
CD56	B159	Alexa488	CD3:Alex488
CD16	3G8	APC-H7	CD16:APC-H7
CD69	FN50	PE	CD8:PE
NKG2D	1D11	PE	CD8:PE
Lytic mechanisms			
CD107a	H4A3	PE-Cy5	CD4:PE-Cy5
TRAIL	RIK-2	PE	CD8:PE
PD-1	MIH4	PE	CD8:PE
Intracellular staining			
TNF-α	MAb11	APC	CD3:APC
IFN-γ	B27	v450	CD3:v450

2.3.4 Data Analyses

All statistical analyses and figure generation were done using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA). All correlations shown were determined using the non-parametric Spearman's rank test. All differences between study cohorts were evaluated using the non-parametric Mann-Whitney U test. Within cohort significant differences were determined by paired t test. $p<0.05$ (two-tailed) was considered significant.

3 Results

3.1 Study demographics

Forty-five patients were enrolled in this study with eight patients not qualifying to start treatment. Viral loads (n=37) were assessed at baseline and at 3 months into treatment to determine EVR, and six months after treatment to determine SVR. Baseline liver enzymes (n=39) were assessed as a surrogate of liver inflammation or damage. Liver biopsies were also performed for sixteen patients to determine fibrosis and inflammation. Fibrosis stage was assessed by the METAVIR five point scale ranging from 0 (no fibrosis) to 4 (cirrhosis). Grade of inflammation was determined on a four point scale ranging from 0 (no inflammation) to 3 (severe inflammation).

3.2 NK-92 control cell line used for patient normalization

NK-92 cells were run in parallel to patient samples at E:T ratios of 10:1, 5:1, 2.5:1 1.25:1 and 0.6:1 to normalize percent lysis values between different patients to account for inter-assay variation. We believe that NK-92 cells are an adequate control as percent lysis values titrate down with decreasing E:T ratios (Fig. 1). In addition, each individual experiment yielded percent lysis results that were consistently all above or all below the mean values. If the NK-92 cells ran in parallel to patient PBMC had higher percent lysis values for that day compared to the mean value from all experiments, then a method to lower patient percent lysis values is required. For each patient at every E:T ratio, the percent lysis value was compared to the mean percent lysis value from all experiments to calculate a ratio. This ratio was calculated for every E:T ratio. The average of the five ratios was determined and this value was used to multiply the patient percent lysis values. For example, if an individual experiment yields NK-92 percent lysis values that are higher compared to the mean NK-92 percent lysis values, then a number is calculated (in this

case, greater than 1) to divide (and lower) the patient percent lysis values. On the other hand, if an individual experiment had low NK-92 percent lysis values, then the ratio (in this case, less than 1), would increase the patient percent lysis values. These normalized patient percent lysis values were used in subsequent analyses.

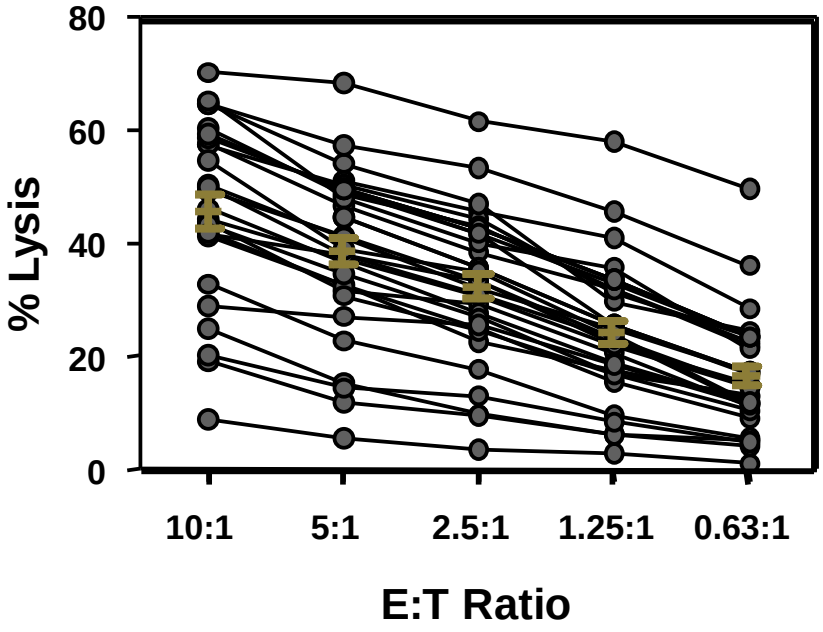

Figure 1: NK-92 control cell line used for patient normalization. NK-92 cells were run in parallel to patient PBMC in a four hour ^{51}Cr release assay against K562 target cells at E:T ratios of 10:1. 5:1, 2.5:1, 1.25:1 and 0.63:1. Grey dots and black lines indicate an individual experiment. Mean and standard error from all experiments are shown in red.

3.3 Caucasian versus Aboriginal patients with chronic HCV infections

3.3.1 Demographics based on Caucasian and Aboriginal ethnicity

Ethnicity, as observed between Caucasians, African Americans and South-East Asians, can influence therapeutic clearance of chronic HCV infections. Based on our labs' previous findings with KIR gene profiles as detailed in the Section 1.5.1, we hypothesized that Caucasian patients, when compared to Aboriginal patients, would display a worse therapeutic clearance of chronic HCV infections that would be reflected by decreased NK cell function [127],

Ethnicity had limited influence on baseline subject demographics and disease characteristics (Table 2). The Caucasian cohort had a slightly lower percentage of males and a higher median age, but these differences were not significant. The distribution of genotype 1 infections was also comparable (Caucasian 75% versus Aboriginal 90%). Baseline viral loads, liver enzymes including ALT, AST, GGT and bilirubin, fibrosis scores and grades of inflammation also did not differ between ethnicity. Caucasian and Aboriginal patients also demonstrated similar rates of treatment discontinuation (Caucasian, 3/20, 15% vs. Aboriginal, 3/9, 33%) and SVR for individuals completing treatment (Caucasian 47% vs. Aboriginal 44%).

Table 2: Baseline parameters between Caucasian and Aboriginal patients

Parameters	Caucasian (n=20)	Aboriginal (n=17)
Patient factors		
Male (%)	40%	53%
Age (median, range)	52.5 (23-65)	45 (31-65)
Viral factors		
Genotype 1 (%)	75%	90%
Viral Load (log, IU/ml, median, range)	6.11 (3.21-7.33)	5.79 (1.74-6.91)
ALT (median, range)	79 (27-330)	67 (22-248)
AST (median, range)	74 (22-221)	63 (20-142)
GGT (median, range)	59.5 (10-361)	54 (22-328)
Bilirubin (median, range)	10.0 (4.0-25.0)	9(2.4-21.6)
Fibrosis Scores (median, range)	2 (1-4)	2 (1-3)
Grade of Inflammation (median, range)	2 (1-3)	2 (1-3)
Treatment factors		
% EVR Genotype 1	60%	55%
% SVR Genotype 1	47%	44%
% Treatment Discontinuation	20%	33%

3.3.2 NK cell cytotoxicity between Caucasian and Aboriginal patients

We evaluated NK cell cytotoxicity to determine if ethnic differences exist at baseline and at three months of treatment corresponding to EVR assessment. At these time points, patients donated blood samples. PBMC, freshly isolated from blood, were incubated overnight in culture medium (CM) or culture medium supplemented with IFNα (1000 IU/ml). PBMC incubated overnight in CM yields spontaneous NK cell cytotoxicity while PBMC cultured overnight in the presence of IFNα yield maximal NK cell killing. The following day, PBMC were used in a standard four hour chromium (^{51}Cr) release assay against K562 target cells to assess NK cell cytotoxicity at E:T ratios of 25:1, 12.5:1 and 6.3:1. The ^{51}Cr release assay is the gold standard for evaluating NK killing potential. For the analysis, an E:T ratio of 12.5:1 was used since as the intermediate value it consistently fell within the linear portion of the NK cell killing curve.

This analysis revealed that at baseline overnight incubation with IFNα enhanced PBMC killing of targets relative to CM alone in Caucasian (Fig. 2, n=16, 1.42 fold, p=0.001) and Aboriginal (n=14, 2.34 fold, p=0.001) cohorts. However, there were limited differences between ethnic cohorts with response to spontaneous NK cell cytotoxicity (p=0.467) or IFNα-induced cytotoxicity (p=0.983).

In contrast, at 3 months of treatment, relative to CM, overnight incubation with IFNα did not increase median percent lysis values for either Caucasian (Fig. 3a, p=0.636) or Aboriginal patients (p=0.980). However, NK cells from the CA cohort (n=15) demonstrated greater spontaneous NK cell cytotoxicity (p=0.042) and IFNα-induced NK cell cytotoxicity (p=0.0219) compared to the Aboriginal cohort (Fig 3a, n=8).

To address the potential bias of the reduction in samples size from baseline to 3 months of treatment, a paired evaluation of baseline and 3 month samples from the same donor was performed. At baseline, similar increases in IFNα-induced NK cytotoxicity relative to spontaneous cytotoxicity were observed for Caucasian (Fig. 3b, n=12, 1.42 fold, p=0.0151) and Aboriginal (n=5, 1.77 fold, p=0.0465) patients. In addition, between ethnic cohorts, there were limited differences in spontaneous (p=0.958) and IFNα-induced (p=0.635) NK cell cytotoxicity.

As was shown in Fig. 3a, at 3 months of treatment, NK cytotoxicity did not increase upon IFNα stimulation. Additionally, spontaneous NK cell cytotoxicity from Caucasian patients was increased 3.93 fold when compared to Aboriginal patients, but this was not significant (p=0.0651). In contrast, a similar increase in IFNα-induced NK cell cytotoxicity was observed in Caucasian patients compared to Aboriginal patients (p=0.0234). Thus paired samples reflected the whole cohort analyses.

To further evaluate the impact of treatment on NK cell killing, the fold difference between cytotixicity at 3 months relative to baseline was determined for paired samples (Fig. 3c). The fold change in spontaneous NK cell killing was similar between Caucasian and Aboriginal cohorts (p=0.102). However, the fold change in IFNα-induced killing was significantly higher in Caucasian patients compared to Aboriginal patients was retained (p=0.0398).

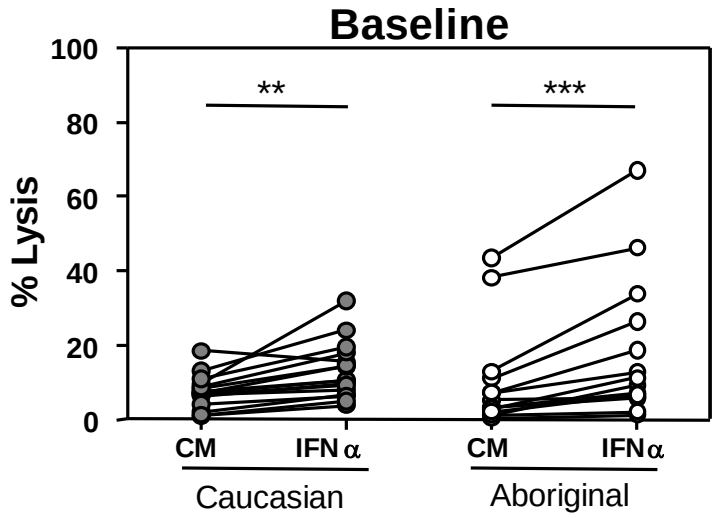

Figure 2: NK cell cytotoxicity is similar between Caucasian and Aboriginal patients with chronic HCV infections. PBMC were incubated overnight in culture medium (CM) and IFNα. NK cell cytotoxicity was measured by Cr-51 release assay. At E:T ratio of 12.5:1, similar NK cell cytotoxicity was observed between Caucasian and Aboriginal patients at baseline. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and IFNα treatments were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as **$p<0.01$, ***$p<0.001$.

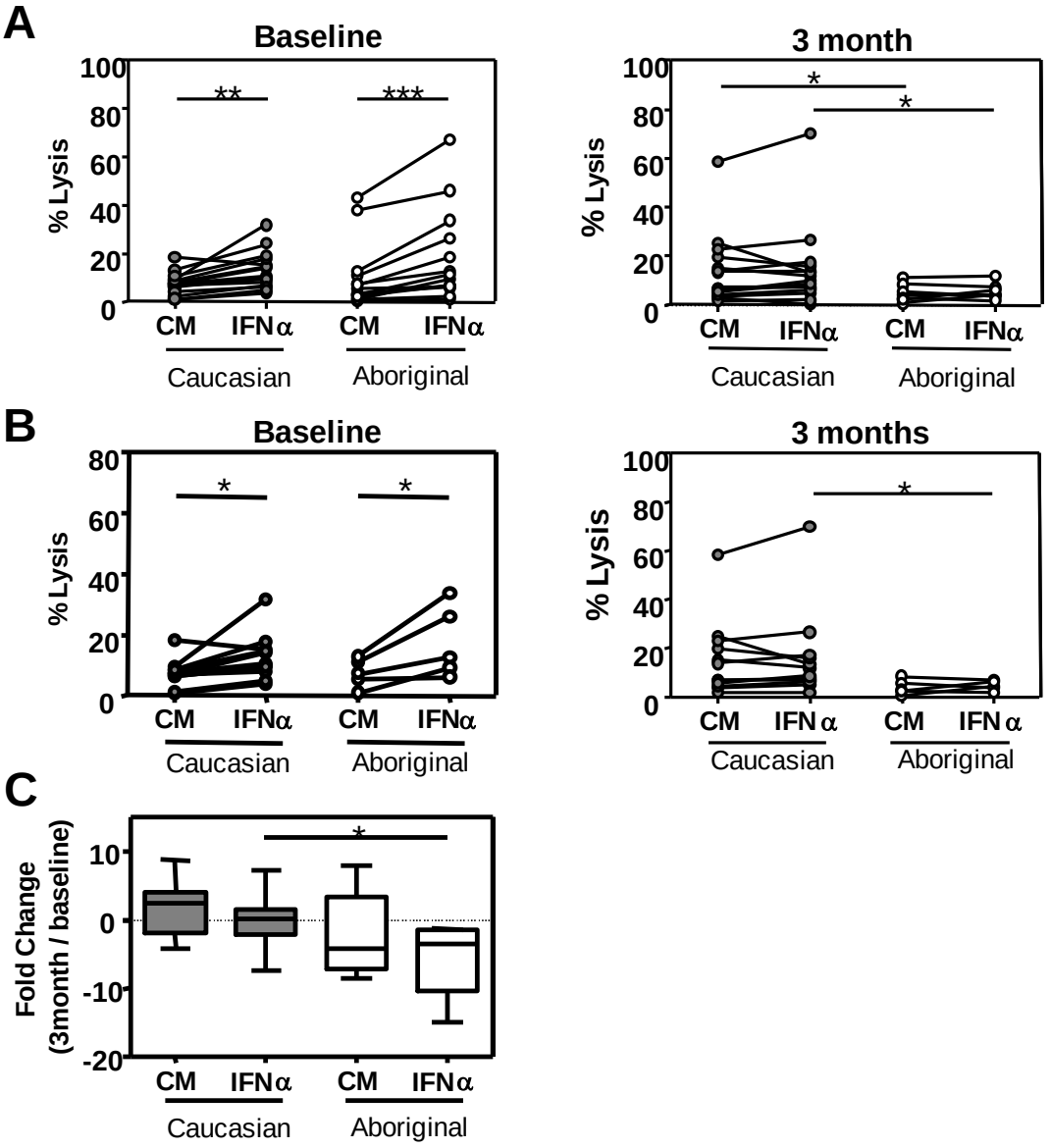

Figure 3: Decreased IFNα-induced NK cell cytotoxicity within Aboriginal patients at 3 months of treatment relative to baseline. PBMC were incubated overnight in culture medium (CM) and IFNα. NK cell cytotoxicity was measured by Cr-51 release assay. **A.** At E:T ratio of 12.5:1, similar NK cell cytotoxicity was observed between Caucasian and Aboriginal patients at baseline and at 3 months of treatment. **B.** For patients with samples available at baseline and 3 months, a paired analysis was performed. **C.** Fold change in NK cell cytotoxicity from baseline to 3 months for each condition indicated. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and IFNα were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.3.3 Degranulation marker CD107a between Caucasian and Aboriginal patients

An alternative method of assessing NK cell activation is lysosomal-associated membrane protein (LAMP)-1, also known as CD107a, expression on NK cells. CD107a is significantly upregulated on the surface of NK cells following stimulation with target cells such as K562 which lack MHC rendering them sensitive to NK cell killing [233]. Thus, the appearance of CD107a on the surface of NK cells is considered an alternate measure of killing potential.

For flow cytometric assessment of NK cell proteins, any remaining PBMC not required for the ^{51}Cr release assay were frozen in Cell Culture Freezing Medium (Gibco, Carlsbad, CA) (n=22). Due to treatment induced lymphopenia at 3 months, there was not sufficient cell numbers for subsequent analysis. Therefore, only baseline events were examined. To evaluate NK cell protein expression, PBMC were thawed and incubated with culture medium or K562 target cells for six hours. PBMC were then stained with antibodies specific for protein expression on NK cells (CD3-/CD56+) as per our ICCS protocol. Flow cytometric data was assessed as total NK cell population, as well as CD56dim and CD56bright populations. Refer to Figure 4 for our ICCS gating strategy and to Table 1 for the staining panel.

Relative to CM alone, K562 stimulation upregulated CD107a expression from Caucasian (Fig. 5a, n=10, 1.46 fold, p=0.0163) and Aboriginal (n=12, 1.75 fold, p=0.0022) NK cells. This was also observed for the CD56dim (Fig. 5b, Caucasian 1.28 fold, p=0.0132; Aboriginal 1.53 fold, p=0.0029) and CD56bright (Fig 5c, Caucasian 1.43 fold, p=0.0273; Aboriginal 2.67 fold, p=0.0001) subsets. At baseline, spontaneous (Fig. 5a, p=0.804) and K562-induced (p=0.156) CD107a expression within the total NK population did not differ between Caucasian and Aboriginal patients. The same observation was noted within the CD56dim and CD56bright subsets.

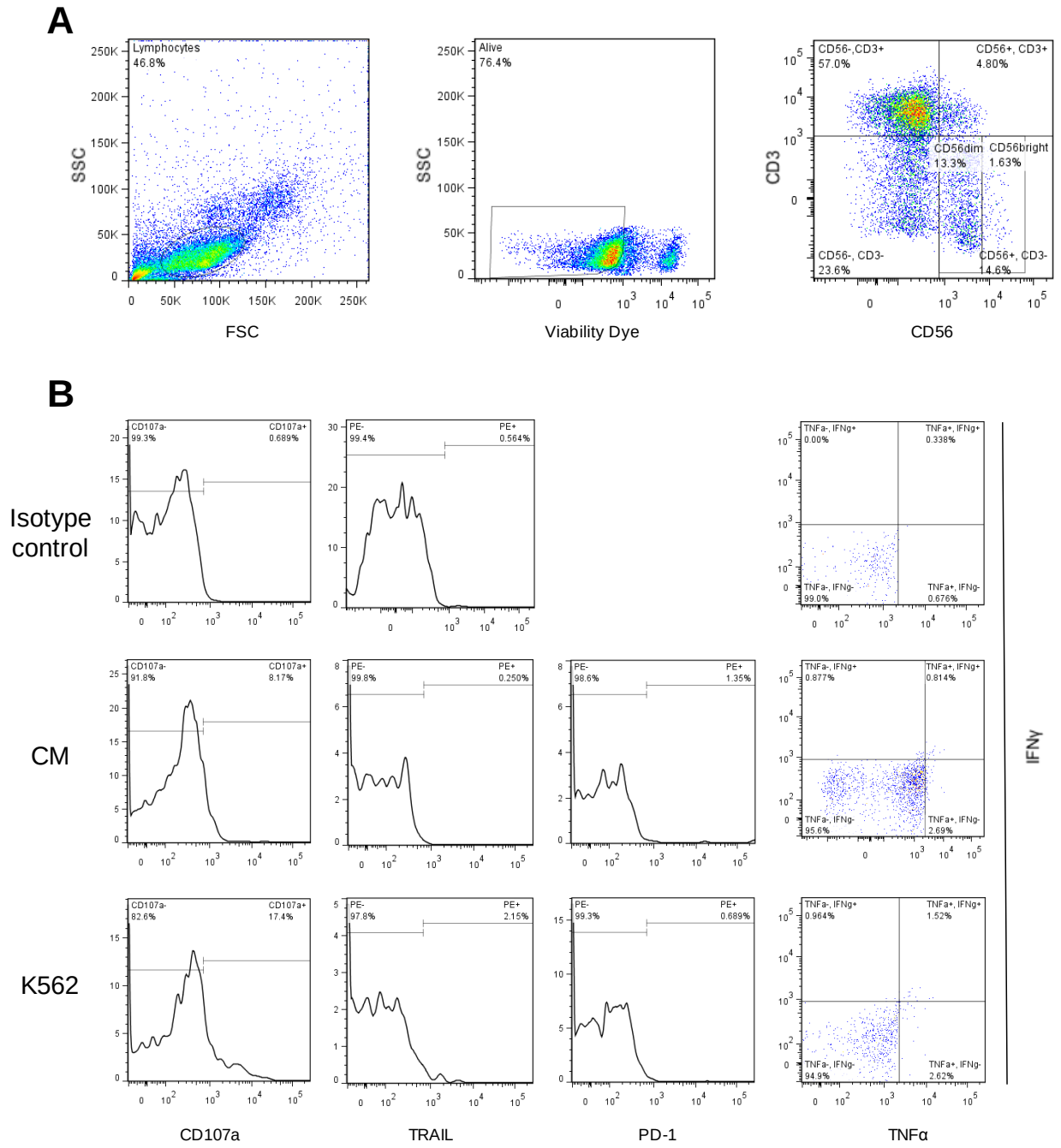

Figure 4: Representative flow cytometry data to illustrate gating strategy for our intracellular cytokine staining protocol. A. PBMC were first gated on forward scatter/side scatter (FSC/SSC) to visualize the lymphocyte population. Lymphocytes were then sorted into alive/dead populations based on our viability dye. Live cells were further sorted into the total NK, CD56dim and CD56bright populations based on CD3 and CD56 expression. **B.** Within the total NK, CD56dim and CD56bright populations, CD107a, TRAIL and PD-1 expression along with cell specific TNFα and IFNγ production were assessed from PBMC incubated with culture medium (CM) or K562 target cells against an isotype control.

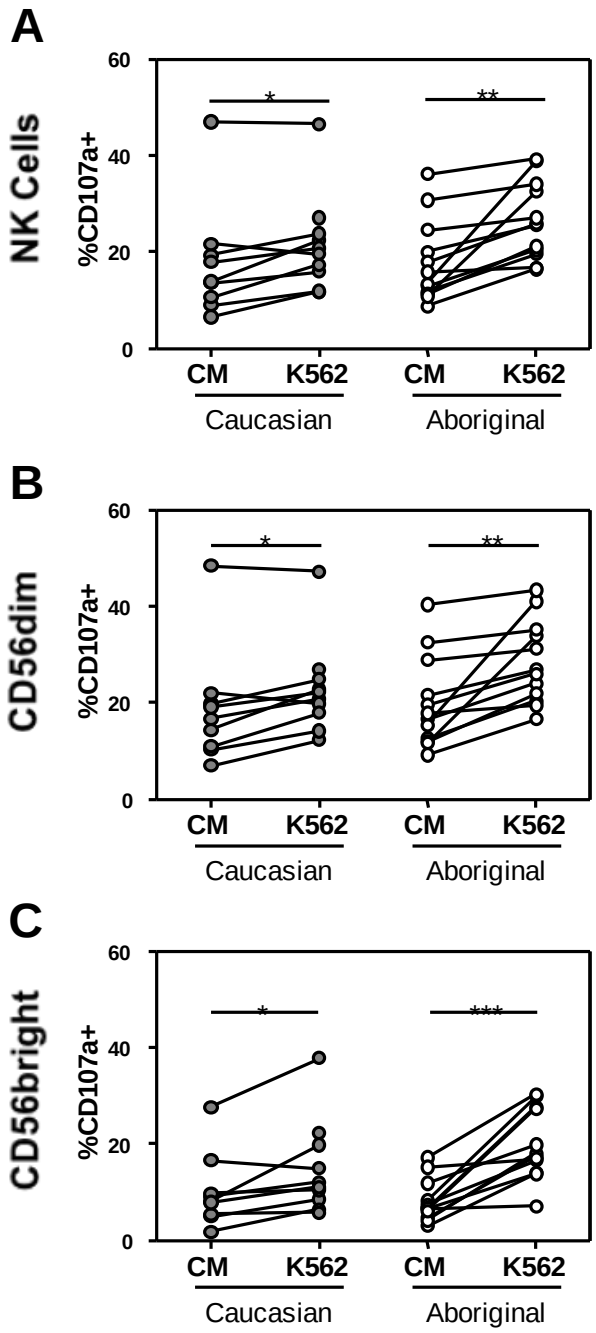

Figure 5: CD107a expression on NK cell subtypes is similar between Caucasian and Aboriginal patients. Frozen PBMC from patient samples prior to treatment (baseline) were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. CD107a expression was assessed in Caucasian and Aboriginal patients by flow cytometry for **A.** all NK cells, **B.** CD56dim and **C.** CD56bright populations. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and K562 were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.3.4 TRAIL expression between Caucasian and Aboriginal patients

Another process by which NK cells induce apoptosis of target cells is the binding of TRAIL (TNF-related apoptosis-inducing ligand) to its death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-RII) on the surface of target cells. Similar to examining the degranulation marker CD107a, TRAIL expression was examined on the surface of NK cells in our ICCS protocol.

In contrast to CD107a expression, relative to CM alone, stimulation of NK cells with K562 target cells did not induce expression of TRAIL within either Caucasian (Fig. 6a, n=10, p=0.999) or Aboriginal (n=12, p=0.284) patients in the total NK cell population or the CD56dim and CD56bright subsets (Figs. 6b and 6c). Between ethnic cohorts, no differences were observed in TRAIL expression from the total NK cell, CD56dim and CD56bright populations with CM alone or with K562 target cells.

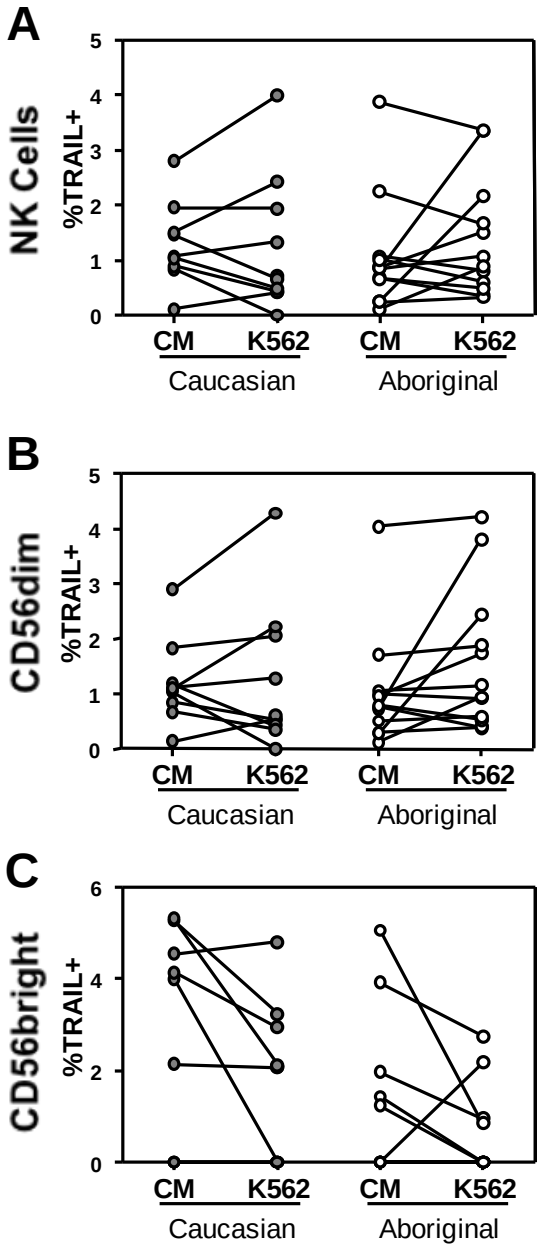

Figure 6: TRAIL expression on all NK cell subtypes is similar between Caucasian and Aboriginal patients. Frozen PBMC from patient samples prior to treatment (baseline) were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. TRAIL expression was assessed in Caucasian and Aboriginal patients by flow cytometry for **A.** all NK cells, **B.** CD56dim and **C.** CD56bright populations. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and K562 were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test.

3.3.5 Exhaustion marker PD-1 expression between Caucasian and Aboriginal patients

Programmed death-1 (PD-1) is an important marker of T cell exhaustion. It was previously implicated in the negative regulation of T cell function during chronic viral infections [234]. Recently, PD-1 expression was found to be elevated on NK cells in patients with persistent HCV infections when compared to healthy controls suggesting that PD-1 may play a role in suppressing NK function during chronic HCV infection [235]. Expression of PD-1 was examined in conjunction with other cell surface markers in our ICCS protocol.

Similar to TRAIL expression, relative to CM alone, stimulation of NK cells or the CD56dim and CD56bright subsets with K562 targets did not upregulate PD-1 expression within Caucasian or Aboriginal patients (Fig. 7a). Between ethnicities, when comparing spontaneous ($p=0.594$) and K562-stimulated ($p=0.692$) PD-1 expression on the surface of NK cells and the CD56dim and CD56bright subsets, no differences were found (Fig. 7).

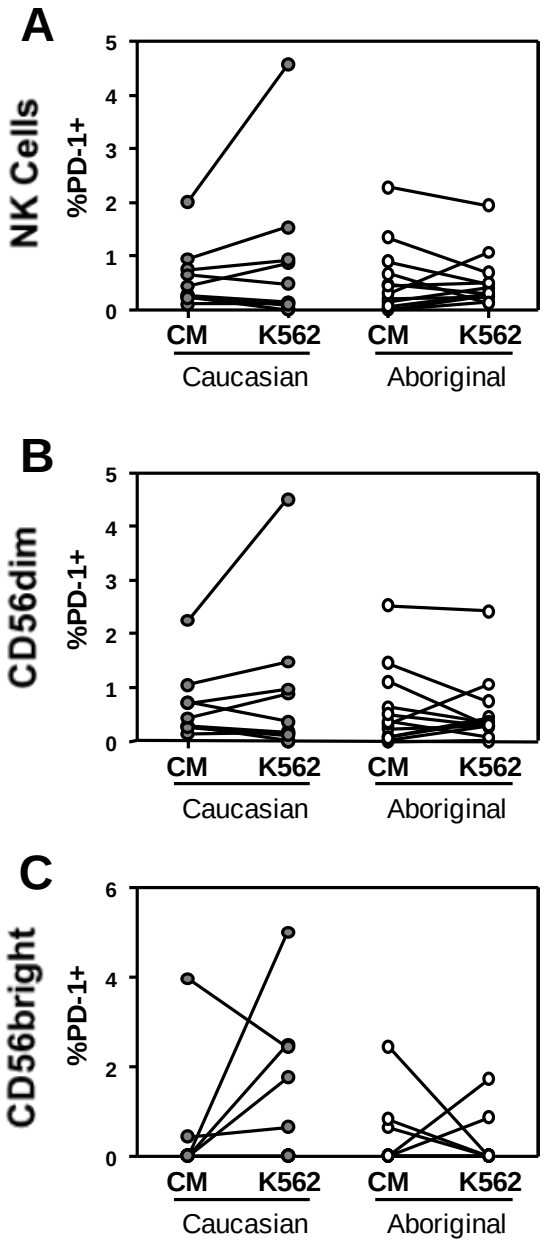

Figure 7: PD-1 expression on all NK cell subtypes is similar between Caucasian and Aboriginal patients. Frozen PBMC from patient samples prior to treatment (baseline) were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. PD-1 expression was assessed in Caucasian and Aboriginal patients by flow cytometry for **A.** all NK cells, **B.** CD56dim and **C.** CD56bright populations. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and K562 were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test.

3.3.6 Cell-specific IFNγ between Caucasian and Aboriginal patients

Aside from directly killing target cells through cytotoxicity, NK cells can also control viral replication indirectly through IFNγ production. NK cell-specific IFNγ production was determined by ICCS in parallel to CD107a quantification.

Stimulation with K562 cells did not enhance NK cell IFNγ production in the Caucasian cohort (Fig. 8a, n=10, p=0.142). In contrast, K562 stimulation by NK cells from Aboriginal patients (n=12, p=0.0011) upregulated IFNγ production 3.68 fold relative to CM alone. The same trends are observed within the CD56dim subset (Fig. 8b, Caucasian p=0.1229, Aboriginal p=0.0012), but not within the CD56bright subset (Fig. 8c, Caucasian p=0.2588, Aboriginal p=0.1865) suggesting that only CD56dim NK cells can upregulate IFNγ production upon stimulation with MHC lacking target cells. Between ethnicities, a greater percentage of NK cells from Caucasian patients produce IFNγ spontaneously (Fig. 8a, p=0.0360) and from K562 stimulation (p=0.0161) when compared to Aboriginal patients. Similar observations were seen within the CD56dim subset. A greater percentage of CD56dim cells from Caucasian patients produce IFNγ spontaneously (p=0.0428) and from K562 stimulation (p=0.021) when compared to Aboriginal patients (Fig. 8b). Within the CD56bright subset, no difference in percentage of NK cells that produce IFNγ spontaneously was observed between ethnicity (Fig. 8c, p=0.082). However, similar to all NK cells and the CD56dim subset, a greater percentage of CD56bright NK cells from Caucasian patients produced IFNγ upon K562 stimulation when compared to Aboriginal patients (p=0.0481)

In addition to examining NK cells, NKT and T cell-specific IFNγ production was also determined. Relative to CM alone, neither NKT (Fig. 8d, Caucasian p=0.767, Aboriginal p=0.393) or T cells (Fig. 8e, Caucasian p=0.368, Aboriginal p=0.806) significantly upregulated

IFNγ production upon K562 stimulation from either ethnicity. Within the NKT cell compartment, NKT cells from Caucasian patients produced significantly more spontaneous IFNγ when compared to Aboriginal patients ($p=0.0428$). IFNγ production from K562 stimulated NKT cells did not differ between ethnicity ($p=0.0927$). Within the T cell compartment, both spontaneous ($p=0.0209$) and K562 stimulated ($p=0.0076$) T cells from Caucasian patients produce significantly more IFNγ compared to Aboriginal patients (Fig. 8e).

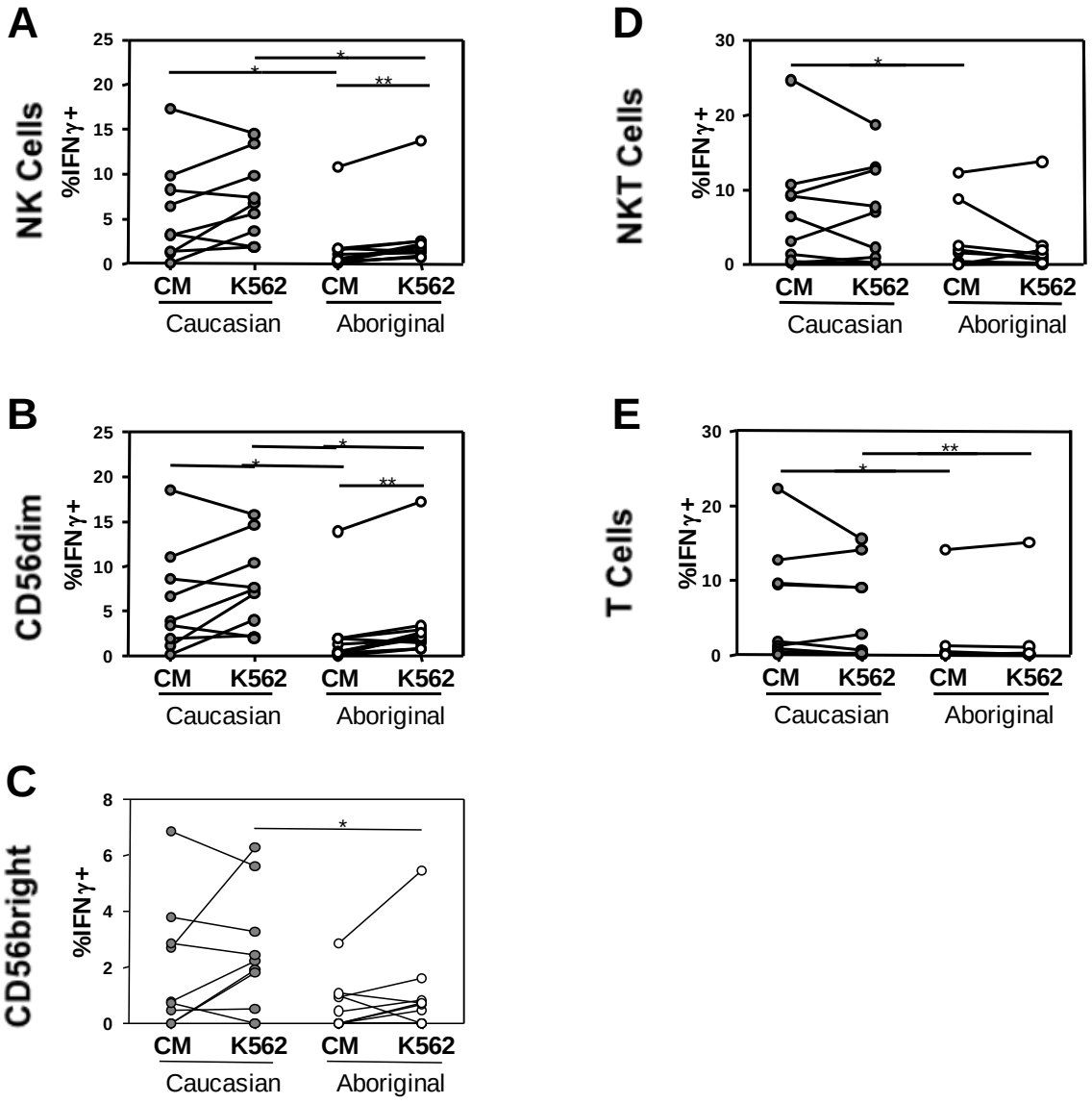

Figure 8: A greater percent of NK cell subsets from Caucasian patients produce IFNγ compared to Aboriginal patients. Frozen PBMC from patient samples prior to treatment (baseline) were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. Cell-specific IFNγ prodution was assessed in Caucasian and Aboriginal patients by flow cytometry for **A.** all NK cells, **B.** CD56dim, **C.** CD56bright, **D.** NKT cells and **E.** T cells. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and K562 were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as *$p<0.05$, **$p<0.01$.

3.3.7 Cell-specific TNFα production between Caucasian and Aboriginal patients

Strong Th1 cytokine profiles have been associated with increased therapeutic clearance of chronic HCV infections [236, 237]. TNFα is a pro-inflammatory cytokine produced by NK cells which contributes to this anti-viral state. We were interested in seeing if there was a difference in cell-specific TNFα production between Caucasian and Aboriginal patients with chronic HCV infections. Cell-specific TNFα production was assessed by flow cytometry.

Relative to culture medium, in the presence of K562 targets, a significantly greater portion of NK cells from Caucasian patients produced TNFα (Fig. 9a, 1.85 fold, p=0.0212). A similar increase was seen in Aboriginal patients, but this was not significant (1.60 fold, p=0.0598). Within the CD56dim compartment, relative to CM alone, a significantly greater percentage of CD56dim cells incubated with K562 targets produced TNFα in Caucasian (p=0.0184) and Aboriginal (p=0.0450) patients. Within the CD56bright compartment, relative to CM alone, incubation with K562 targets did not increase the percentage of TNFα producing CD56bright NK cell between Caucasian (p=0.235) or Aboriginal (p=0.272) patients. Between ethnicities, no differences were observed in the percentage of NK cells producing TNFα spontaneously (p=0.972) or in the presence of K562 targets (p=0.717). The same results were obtained within the CD56dim and CD56bright subsets.

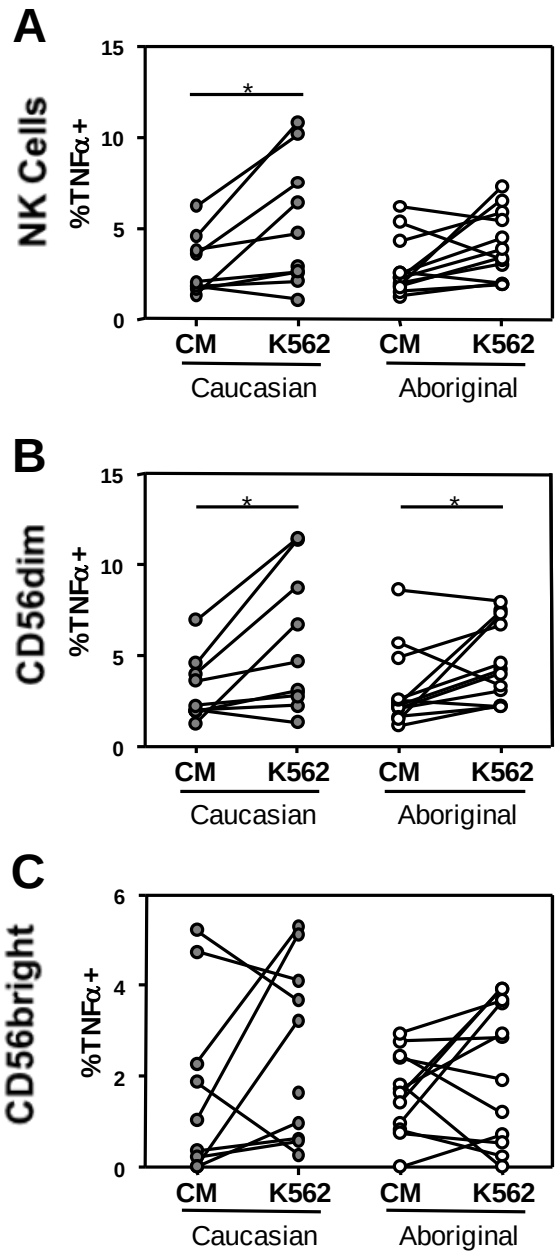

Figure 9: TNFα production within all NK cell subsets is similar between Caucasian and Aboriginal patients. Frozen PBMC from patient samples prior to treatment (baseline) were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. Cell-specific TNFα prodution was assessed in Caucasian and Aboriginal patients by flow cytometry for **A.** all NK cells, **B.** CD56dim and **C.** CD56bright populations. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and K562 were determined by paired U test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as *$p<0.05$.

3.3.8 NK cell functions between Caucasian and Aboriginal patients

As another method to assess NK cell activity, the number of functions displayed by NK cells within these cohorts was evaluated. Specifically, we included the appearance of degranulation marker CD107a, and NK cell-specific IFNγ and TNFα production. NK cells were divided according to the number of functions they displayed.

The number of NK cell functions was evaluated between Caucasian and Aboriginal patients prior to the start of treatment (Fig. 10). Relative to CM alone, the number of NK cell functions did not increase upon incubation with K562 targets. In addition, between ethnicities, there was no difference in the number of NK cell functions either cultured with CM or in the presence of K562 targets.

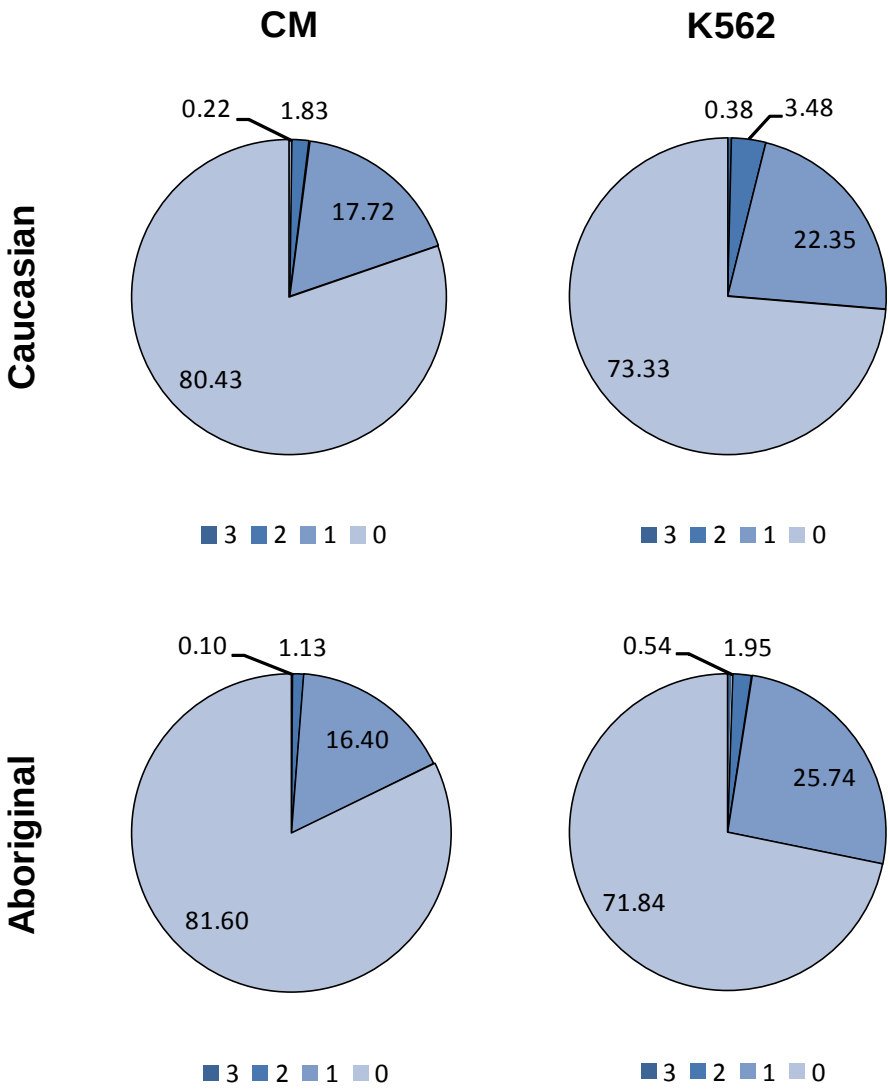

Figure 10: Number of NK cell functions are similar between Caucasian and Aboriginal patients. Frozen PBMC were thawed and incubated with culture medium for 6 hours to assess NK cell functions. NK cell functions examined include degranulation marker CD107a, NK cell-specific IFNγ and TNFα production. Pre-treatment spontaneous and K562-stimulated NK cell functions were evaluated in Caucasian and Aboriginal patients by flow cytometry. The percentage of total NK cells with 0, 1, 2 or 3 functions are indicated as the median values for each cohort condition. Statistical significance of number of functions between cohorts was determined using the Mann-Whitney U test.

3.4 Viral Loads

High baseline viral loads (defined in the literature as >800,000 IU/ml), have previously been associated with worse therapeutic outcomes in particular for genotype 1 infections [173-175]. NK cells are principle effectors against viral infections particularly early in infection. HCV is reported to inhibit NK cell function [93, 94]. Thus, we were interested in evaluating NK cell function in our patient cohort with regard to HCV loads.

3.4.1 Baseline parameters between high and low viral loads

Due to the association of baseline viral loads with treatment outcomes, we examined NK activity according to high and low viral loads. High viral loads (HVL) are defined as >800,000 IU/ml whereas low viral loads (LVL) are defined as ≤800,000 IU/ml [173-175]. Subject demographics and disease characteristics were similar between high and low viral loads (Table 3) including the distribution of genotype 1 infections (HVL, 92% versus LVL, 80%) and markers of liver disease and inflammation. Unexpectedly, HVL patients had surprisingly higher EVR ($p=0.170$) and SVR ($p=0.07$) rates when compared to LVL patients. We would expect the opposite trend as high viral loads have previously been associated with worse therapeutic outcomes in particular for genotype 1 infections [173-175].

Table 3: Baseline parameters between high and low viral loads

Parameters	HVL (n=13)	LVL (n=10)
Viral Load (log, IU/ml, median, range)***	6.67 (5.90-7.33)	5.44 (1.74-5.84)
Patient factors		
Male (%)	46	30
Caucasian (%)	77%	60%
Age (median, range)	54 (23-62)	50 (40-65)
Viral factors		
Genotype 1 (%)	92%	80%
ALT (median, range)	67 (27-330)	57 (38-181)
AST (median, range)	63 (22-221)	81 (32-168)
GGT (median, range)	54 (10-361)	65 (19-297)
Bilirubin (median, range)	7 (4-20)	9 (5-25)
Fibrosis Scores (median, range)	2 .5 (1-4)	3 (2-4)
Grade of Inflammation (median, range)	2 (1-3)	2.5 (2-3)
Treatment factors		
% EVR Genotype 1	75	38
% SVR Genotype 1	58	13
% Treatment Discontinuation	17	38

3.4.2 NK cell cytotoxicity and baseline viral loads

In chronic HCV infections, some studies suggest NK cytolytic functions can be unaffected [108, 137] or impaired [93, 142]. To further explore this debate, we evaluated the association between baseline viral loads and NK cell cytotoxicity. NK cell cytotoxicity was assessed by ^{51}Cr release assay as previously described. Correlations were used to evaluate the association between baseline viral loads and NK cell killing in the total study population.

At baseline (n=23) using our ^{51}Cr release assay, spontaneous NK cell cytotoxicity at E:T ratio of 12.5:1 negatively correlated with viral loads (Fig. 11a, r=-0.437, p=0.037). A similar trend was observed with IFNα-induced NK cell killing at E:T ratio of 12.5:1 (n=23), but this correlation was not significant (Fig. 11b, r=-0.372, p=0.081).

When distinguishing between high and low viral loads, our analysis revealed that overnight incubation with IFNα enhanced PBMC killing of targets relative to CM alone from both high (n=11, 1.42 fold, p=0.002) and low (n=12, 1.78 fold, p=0.003) viral loads. In addition, PBMC from high viral loads patients had significantly reduced spontaneous (p=0.021) and IFNα-induced (p=0.0151) NK cell killing at E:T ratio of 12.5:1 compared to PBMC from low viral load patients (Fig. 11c). This observation suggests patients with high viral loads may have difficulty in controlling viral replication due to reduced NK cytotoxicity.

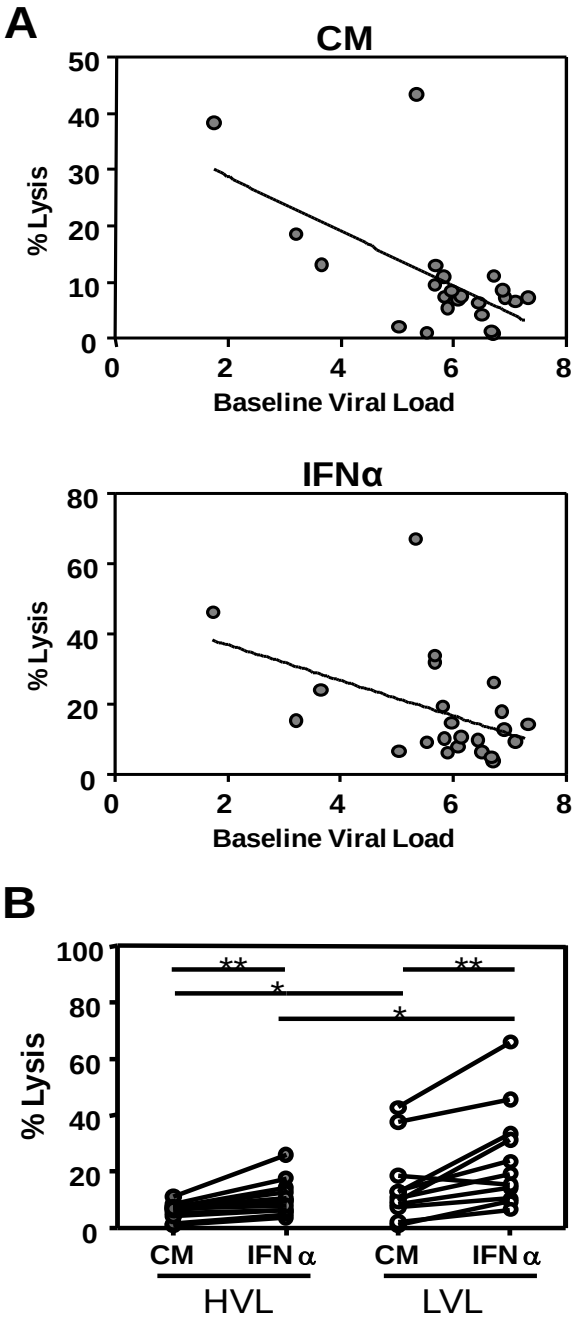

Figure 11: NK cell cytotoxicity negatively correlates with viral loads. PBMC were incubated overnight in culture medium (CM) and IFNα. NK cell cytotoxicity was measured by Cr-51 release assay. Baseline viral loads were plotted against NK cell cytotoxicity at 12.5E:1T ratio with **A.** CM (n=22) or **B**. IFNα stimulation (n=23). Spearman rank correlation, r, is shown. **C.** Baseline NK cell cytotoxicity was evaluated between high viral load (HVL) and low viral load (LVL) patients at 125E:1T ratio. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and IFNα were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as *$p<0.05$, **$p<0.01$.

3.4.3 Degranulation marker CD107a between high and low viral load patients

To evaluate how viral loads might influence mechanisms of NK cell killing, CD107a expression was examined with respect to viral loads. CD107a expression was evaluated on the total NK cell, CD56dim and CD56bright populations after stimulation with culture medium alone or with K562 target cells.

In contrast to NK cell cytotoxicity measured by our ^{51}Cr release assay, in the presence of CM alone, NK cell killing measured by CD107a expression positively correlated with viral loads (Fig. 12a, n=14, r=0.578, p=0.030). Similar observations were seen within the CD56dim population which associated with cytotoxicity (Fig. 12b, r=0.552, p=0.041) and CD56bright population which associated with cytokine production (Fig. 12c, r=0.78, p=0.001). However, in the presence of K562 targets, CD107a expression did not correlate with baseline viral loads for the total NK cell, CD56dim and CD56bright populations.

Relative to CM alone, K562 stimulation increased CD107a expression 1.12 fold within the total NK cell population from high viral loads, but this was not significant (Fig. 13a, p=0.099). The same results were seen within the CD56dim and CD56bright subsets. Similarly, relative to CM alone, K562 stimulation increased CD107a expression 1.41 fold within the total NK cell population from low viral loads, but again this was not significant (p=0.068). However, both the CD56dim (Fig. 13b, p=0.023) and CD56bright (Fig. 13c, p=0.027) subsets could induce CD107a expression upon K562 stimulation relative to CM alone.

Further analysis between high and low viral loads revealed limited differences in spontaneous (p=0.282) and K562 stimulated (p=0.388) percentages of CD107a expression on the total NK cell and CD56dim populations. In contrast, the CD56bright population from high viral loads

when compared to low viral loads showed increased spontaneous CD107a expression ($p=0.029$). However, similar to the total NK cell and CD56dim populations, the CD56bright population did not differ between high and low viral loads in its CD107a expression upon K562 stimulation ($p=0.371$).

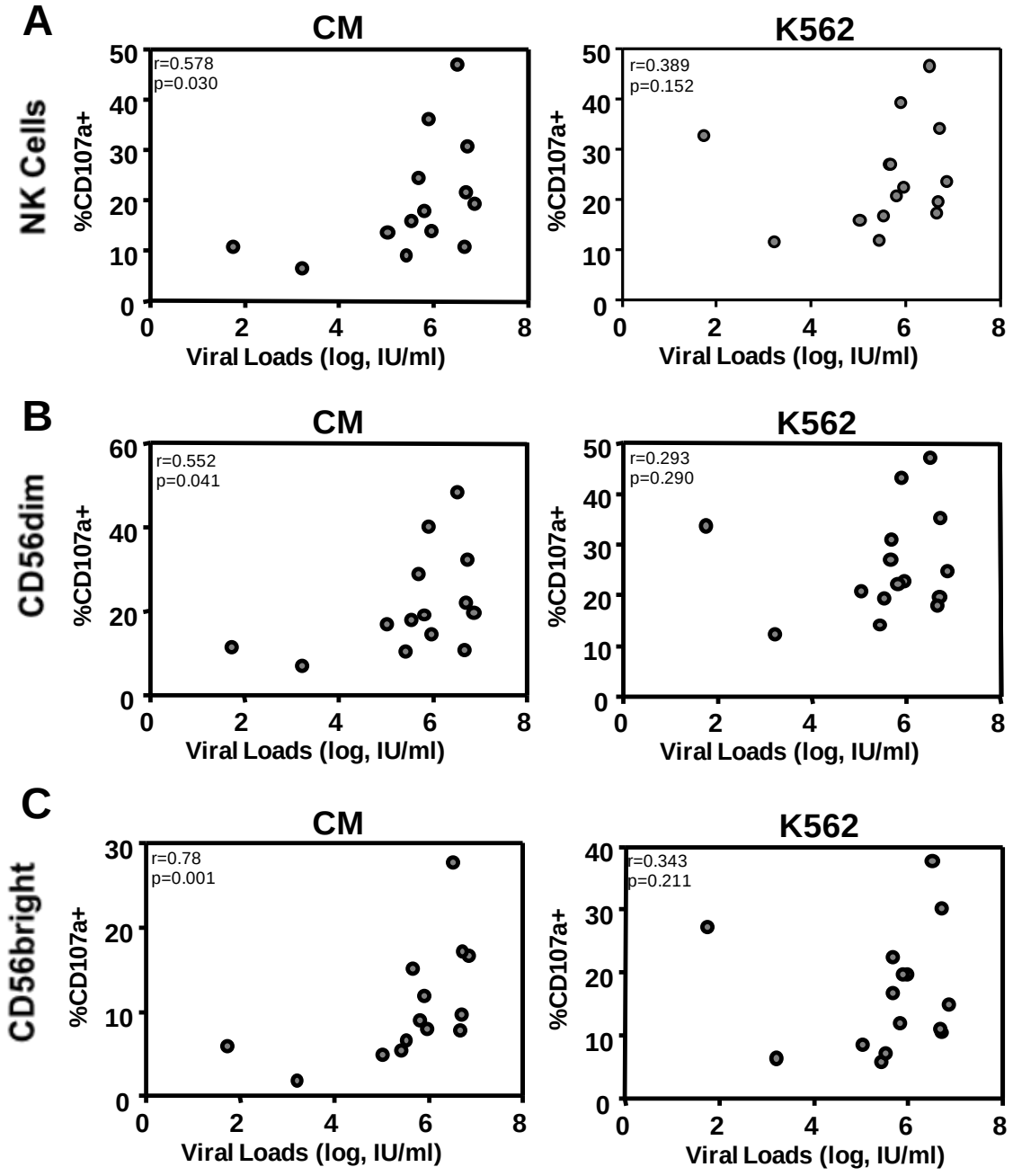

Figure 12: Spontaneous expression of CD107a in all NK cell subsets correlate with baseline viral loads. Frozen PBMC were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. Pre-treatment CD107a expression was assessed by flow cytometry and plotted against baseline viral for **A.** all NK cells, **B.** CD56dim and **C.** CD56bright populations. Spearman rank correlation, r, is shown. $p<0.05$ is considered significant.

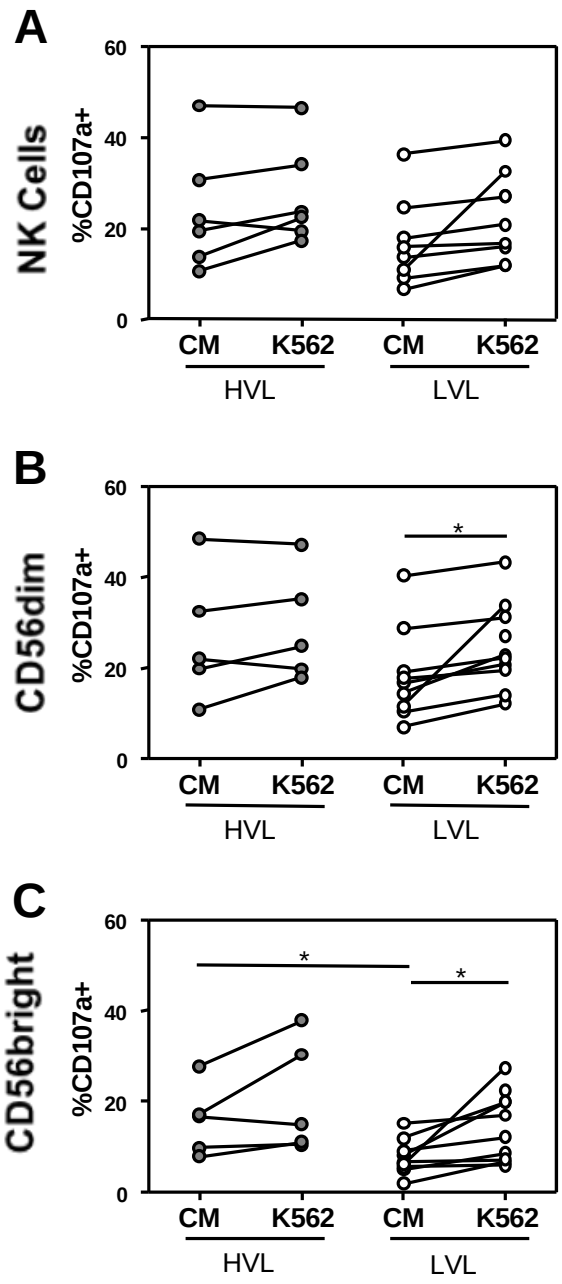

Figure 13: Increased CD107a expression on CD56bright NK cells in patients with highviral loads compared to patients with low viral loads at baseline. Frozen PBMC were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. Pre-treatment CD107a expression was evaluated by flow cytometry between high viral load (HVL) and low viral load (LVL) patients for **A.** all NK cells, **B.** CD56dim and **C.** CD56bright populations. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and K562 were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as *$p<0.05$.

3.4.4 Exhaustion marker PD-1 expression between high and low viral loads

PD-1 expression was found to be significantly elevated on the surface of NK cells in individuals with chronic HCV infections compared to healthy individuals [235]. We were interested to see if PD-1 expression on NK cells correlated with viral loads.

Spontaneous expression of PD-1 from the total NK and CD56bright populations did not correlate with baseline viral load (Fig. 14). Further evaluation spontaneous PD-1 expression within the CD56dim (Fig. 14b, r=0.512, p=0.061) revealed a potential correlation with baseline viral loads, but this was not significant. In contrast, K562-stimulated PD-1 expression positively correlated with baseline viral loads for the total NK (n=15, r=0.649, p=0.009), CD56dim (r=0.652, p=0.008) and CD56bright (r=0.763, p=0.0009) populations.

Relative to CM alone, incubation of the total NK, CD56dim and CD56bright populations with K562 target cells did not increase PD-1 expression within the high viral load cohort (Fig. 15). Likewise, incubation of the total NK, CD56dim and CD56bright populations with K562 targets, relative to CM alone, did not increase PD-1 expression within the low viral load cohort.

Subsequent analysis between high and low viral loads revealed no differences in spontaneous expression of PD-1 on the total NK (p=0.142) and CD56 bright (p=0.867) populations. In contrast, spontaneous PD-1 expression was increased on CD56dim cells (p=0.019) from the high viral load cohort compared to low viral load cohort. Upon incubation with K562 target cells, once again no differences were prevalent within the total NK population between the two viral load cohorts (Fig. Xa, p=0.114). However, both the CD56dim (p=0.040) and CD56bright (p=0.0007) populations from the high viral load cohort had increased PD-1 expression compared to the low viral load cohort.

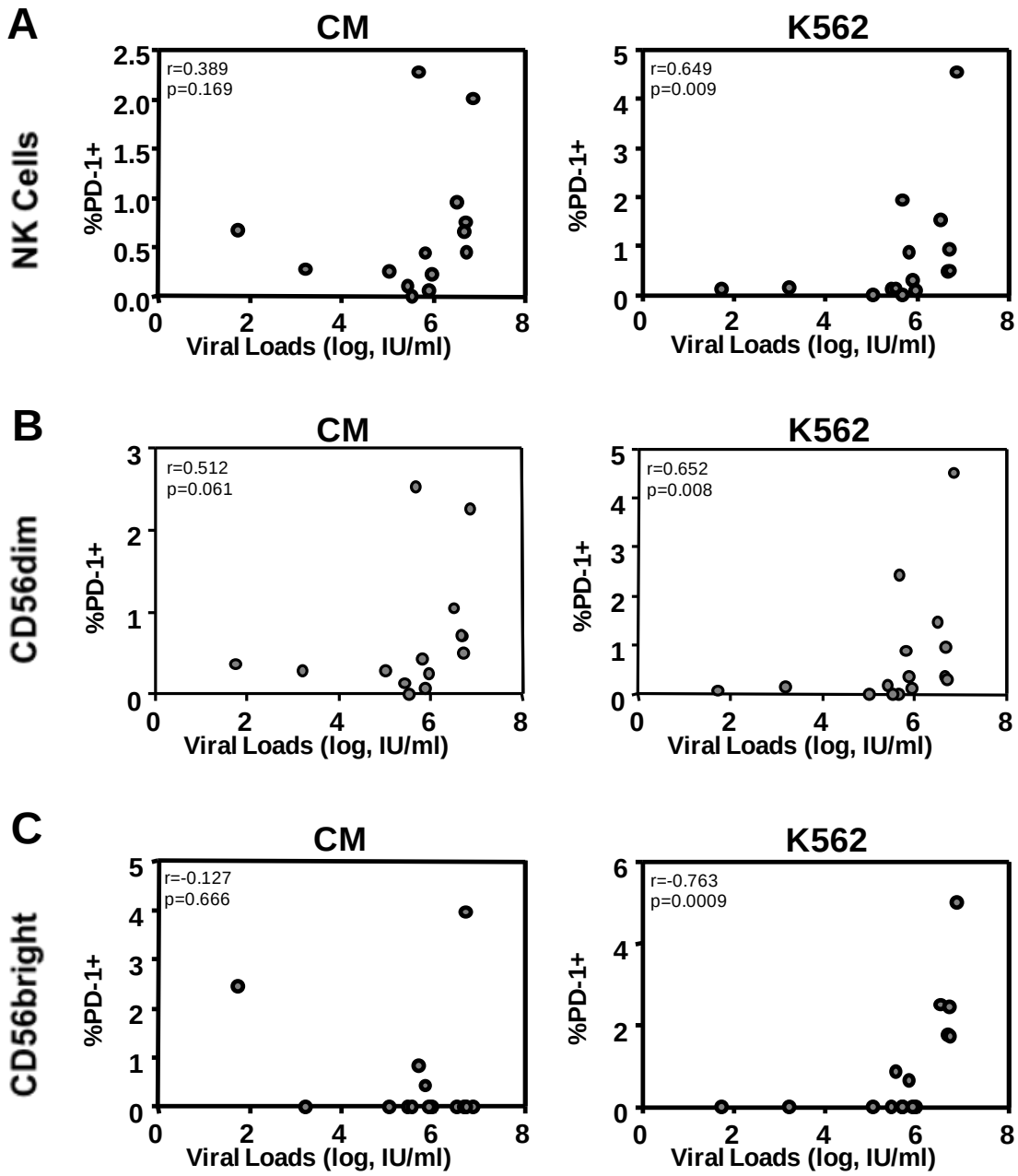

Figure 14: Expression of PD-1 in all NK cell subsets upon K562 stimulation correlate with baseline viral loads. Frozen PBMC were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. Pre-treatment PD-1 was assessed by flow cytometry and plotted against baseline viral loads for **A.** all NK cells, **B.** CD56dim and **C.** CD56bright populations. Spearman rank correlation, r, is shown. $p<0.05$ is considered significant.

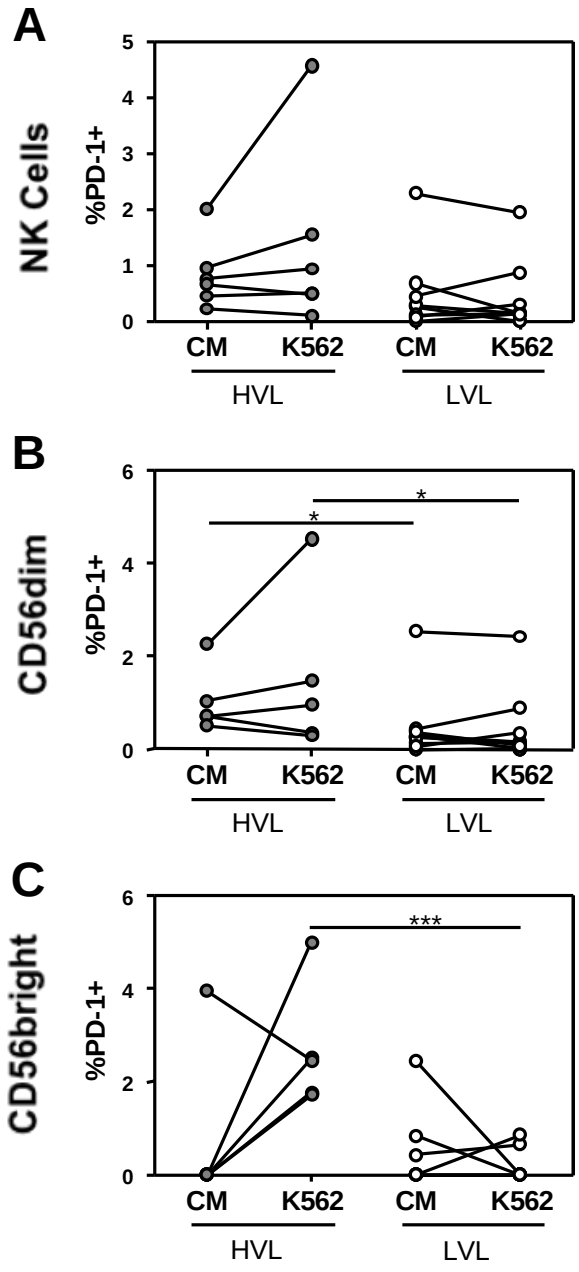

Figure 15: Increased expression of PD-1 on NK cells in patients with high baseline viral loads. Frozen PBMC were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. Pre-treatment PD-1 expression was evaluated by flow cytometry between high viral load (HVL) and low viral load (LVL) patients for **A.** all NK cells, **B.** CD56dim and **C.** CD56bright populations. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and K562 were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as *$p<0.05$, ***$p<0.001$

3.4.5 Cell-specific IFNγ between high and low viral loads

NK cell-specific IFNγ production is considered another mechanism by which NK cells can control viral replication. We examined whether correlations existed between NK cell-specific IFNγ production and baseline viral loads. NK cell-specific IFNγ production was determined by ICCS.

Spontaneous cell-specific IFNγ production from the total NK (Fig. 16a, n=14, r=0.334, p=0.240) and CD56dim populations (data not shown) did not correlate with baseline viral loads. In contrast, we found a positive correlation between spontaneous cell-specific IFNγ production and baseline viral loads within the CD56bright population (Fig. 16b, n=14, r=0.581, p=0.030). No correlations were observed between cell-specific IFNγ production with K562 stimulation from the total NK (Fig. 16a, n=15, r=0.293, p=0.290), CD56dim (data not shown) and CD56bright (Fig. 16b, n=15, r=0.216, p=0.439) populations.

Further analysis examined differences in cell-specific IFNγ production between viral load cohorts. Relative to CM alone, stimulation of the total NK (Fig. 17a, p=0.603) and CD56dim (data not shown) populations with K562 target cells did not upregulate IFNγ production within the high viral load cohort. In contrast, the CD56bright population did upregulate IFNγ production (Fig. 17b, p=0.0174). In addition, relative to CM alone, stimulation of the total NK (p=0.0082) and CD56bright (p=0.0049) populations with K562 targets did upregulate IFNγ production whereas the CD56dim (data not shown) population did not within the low viral load cohort.

Between viral load cohorts, limited differences were present in spontaneous (p=0.573) and K562-stimulated (p=0.388) IFNγ production from the total NK and CD56dim (data not shown)

populations. In contrast, the high viral load cohort had increased spontaneous IFNγ production within the CD56bright population ($p=0.0290$) compared to the low viral load cohort. Limited differences were observed in K562 stimulated IFNγ production from CD56bright cells between high and low viral loads ($p=0.7131$).

CD107a has been shown to correlate not only with target cell lysis, but also with cytokine secretion [233]. Thus we evaluated the relationship between CD107a expression and cell-specific IFNγ production. No relationship was apparent between CD107a expression and cell-specific IFNγ production when incubated in culture medium within the total NK (Fig. 18a, $r=0.227$, $p=0.322$) or CD56dim populations (Fig. 18b, $r=0.235$, $p=0.305$). However, a significant positive correlation was observed within the CD56bright population (Fig. 18c, $r=0.516$, $p=0.017$) suggesting that IFNγ is responsible for the appearance of CD107a within this cell population.

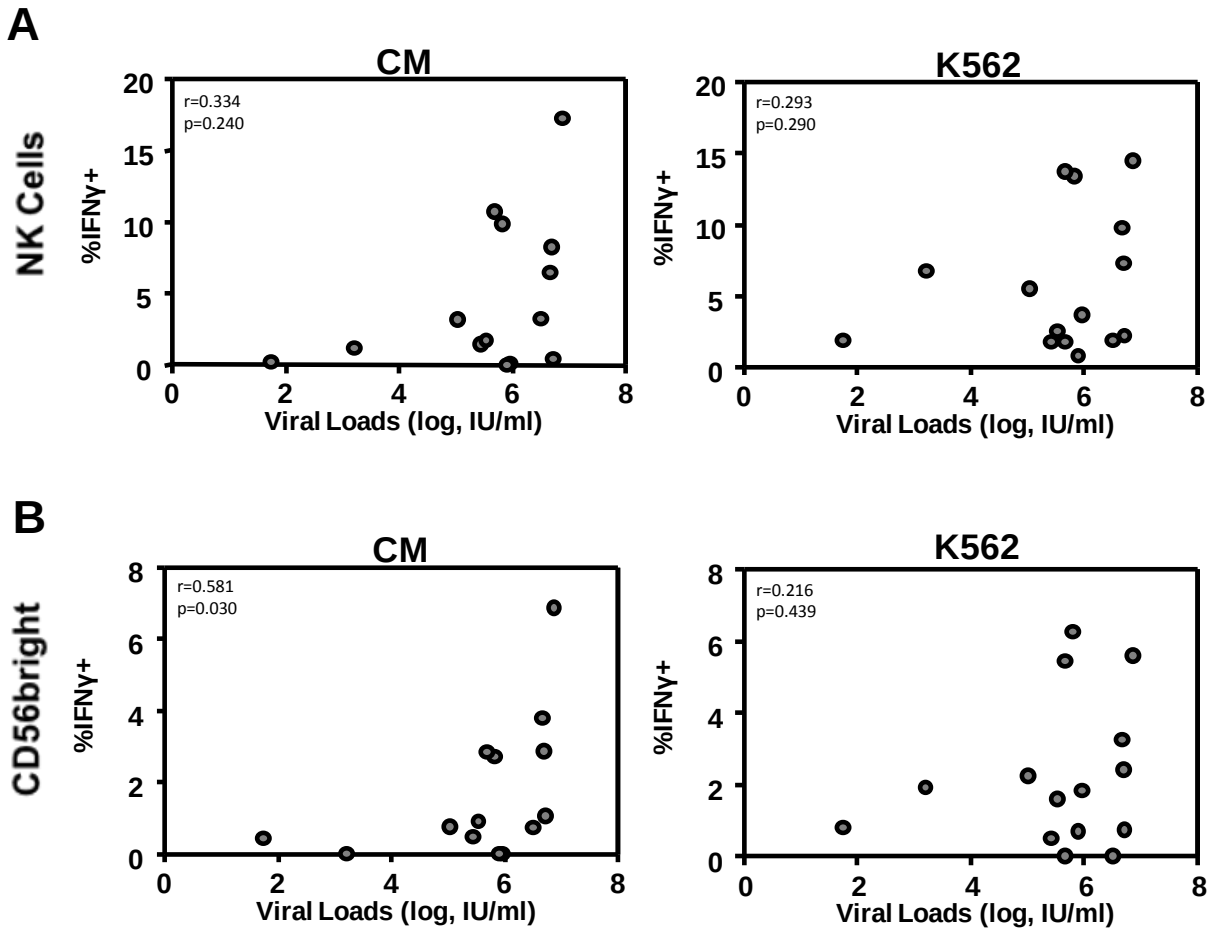

Figure 16: The portion of IFNγ producing CD56bright NK cells correlate with baseline viral loads. Frozen PBMC were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. Cell-specific IFNγ production was assessed by flow cytometry and plotted against baseline viral loads for **A.** all NK cells and the **B.** CD56bright population. Spearman rank correlation, r, is shown. $p<0.05$ is considered significant.

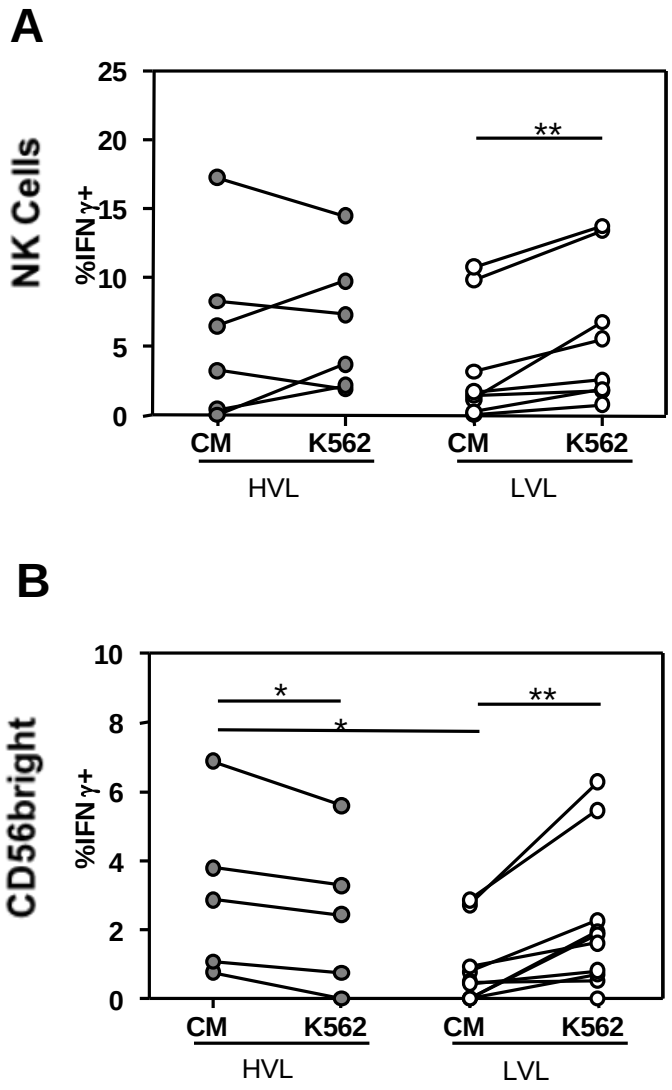

Figure 17: Increased portion of IFNγ producing CD56bright NK cells in patients with high baseline viral loads. Frozen PBMC were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. Pre-treatment cell-specific IFNγ production was evaluated by flow cytometry between high viral load (HVL) and low viral load (LVL) patients for **A.** all NK cells and the **B.** CD56bright population. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and K562 were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as *$p<0.05$, **$p<0.01$

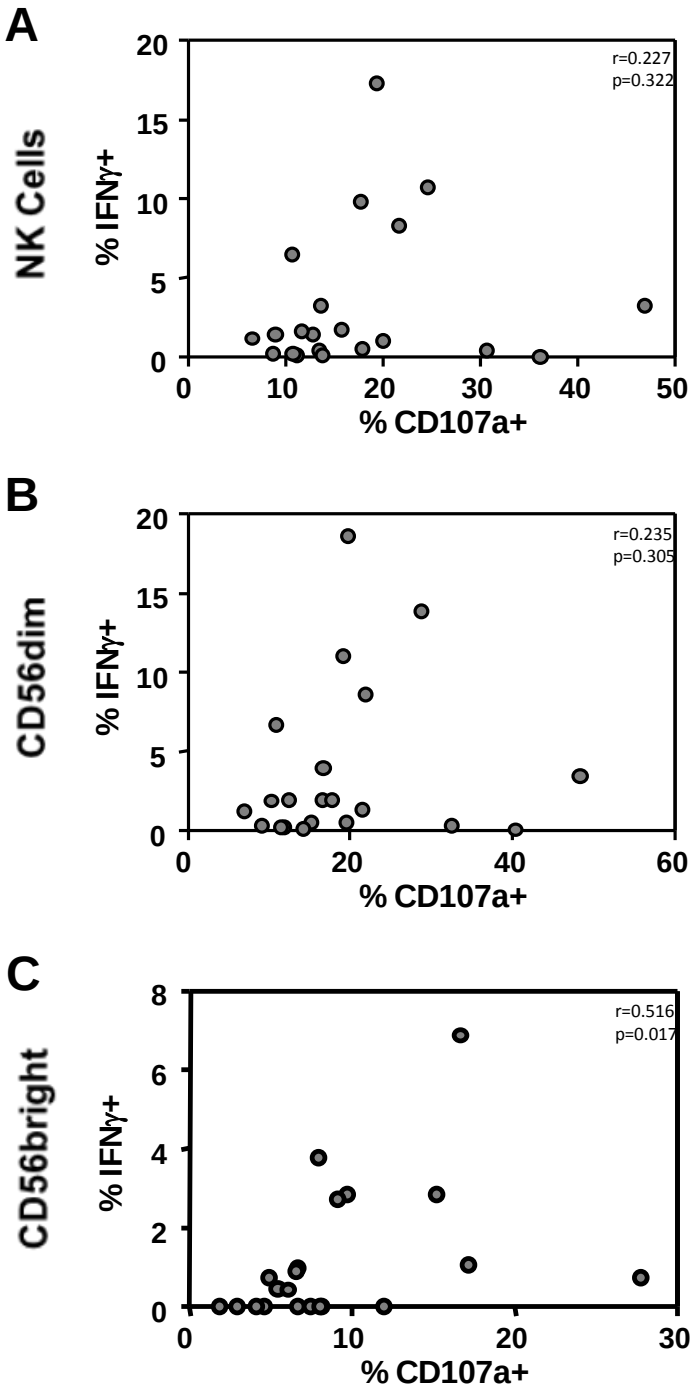

Figure 18: CD107a expression correlates with IFNγ production within the CD56bright population. Frozen PBMC were thawed and incubated with culture medium for 6 hours. Pre-treatment CD107a expression and cell-specific IFNγ production was assessed by flow cytometry. CD107a expression was plotted against IFNγ production for **A.** all NK cells, the **B.** CD56dim and **C.** CD56bright populations. Spearman rank correlation, r, is shown. $p<0.05$ is considered significant.

3.4.6 NK cell functions between high and low viral loads

The HCV E2 protein has been shown to inhibit protein kinase receptor activity via crosslinking with CD81 to inhibit NK cell functions including cytotoxicity and IFN production [93, 94]. In addition, NK cell activity had no relationship with declining viral loads in patients who cleared their acute HCV infection [238]. We evaluated the potential relationship between NK cell activity and viral loads from individuals with chronic HCV infections. The NK cell functions measured by ICCS to assess NK cell activity include CD107a and cell-specific IFNγ and TNFα production. NK cells were divided according to the number of functions they displayed.

Spontaneous numbers of NK cell functions were assessed against baseline viral loads. There was a trend towards a correlation between the percentage of NK cells with three (Fig. 19a, r=0.451, p=0.097) spontaneous functions, but this was not significant. Two spontaneous functions (Fig. 19b, r=0.398, p=0.159) did not associate with baseline viral loads. In contrast, one (Fig. 19c, r=0.635, p=0.015) spontaneous function positively correlated with baseline viral load while zero (Fig. 19d, r=-0.565, p=0.035) spontaneous functions negatively correlated with baseline viral loads.

Patient PBMC were also incubated with K562 target cells to assess K562-stimulated NK cell functions against baseline viral loads. Analysis of K562-stimulated NK cell functions revealed no correlation between the percentages of NK cells with three (Fig. 20a, r=-0.143, p=0.612), two (Fig. 20b, r=0.229, p=0.413), one (Fig. 20c, r=0.318, p=0.248) or zero (Fig. 20d, r=-0.364, p=0.182) functions against baseline viral loads.

Spontaneous and K562-stimulated percentage of NK cell functions were also evaluated between high and low viral loads prior to the start of treatment (Fig. 21). Relative to CM alone, the number of NK cell functions did not increase upon incubation with K562 targets within both viral load cohorts. In addition, in between viral load cohorts, there were no differences in spontaneous or K562-stimulated NK cell functions.

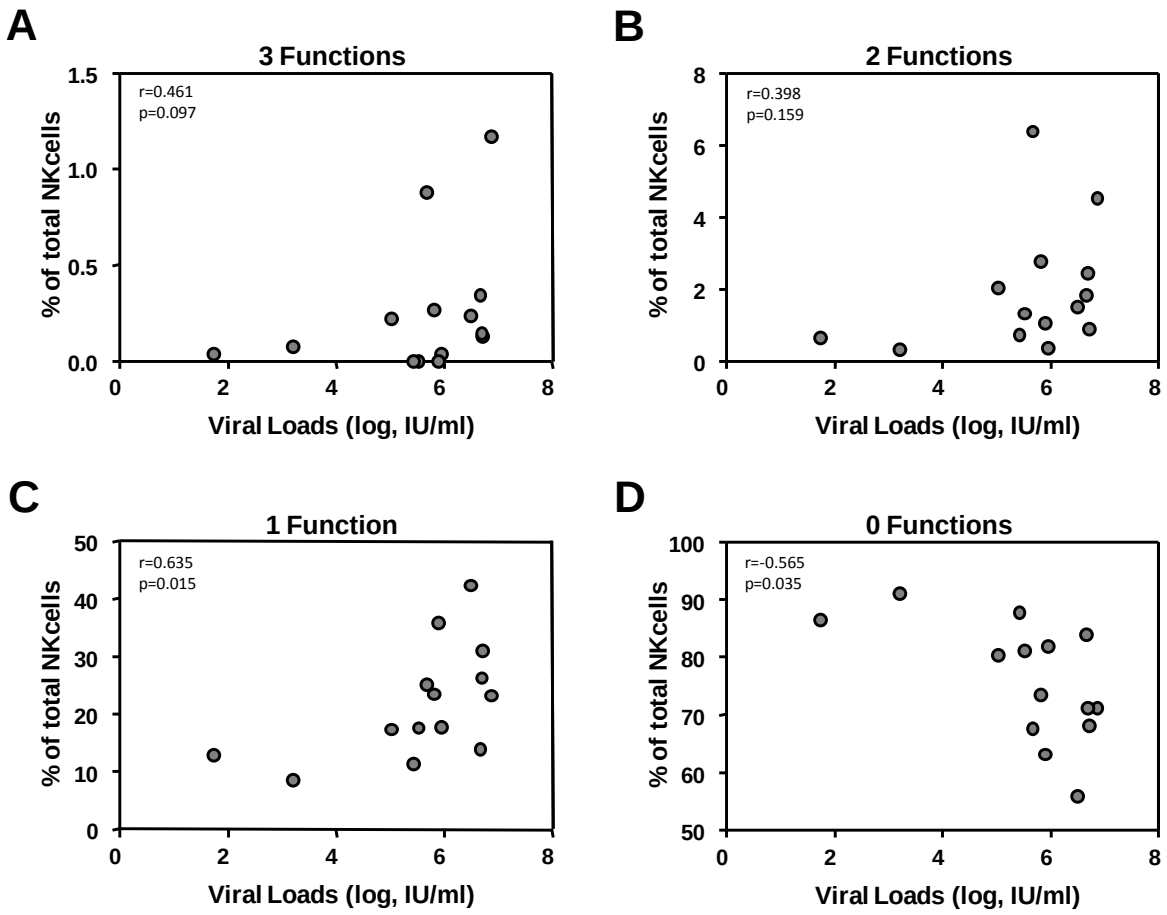

Figure 19: Spontaneous NK cell functions associate with viral loads. Frozen PBMC were thawed and incubated with culture medium for 6 hours to assess NK cell functions. NK cell functions examined include degranulation marker CD107a, NK cell-specific IFNγ and TNFα production The spontaneous percentage of totals NK cells with **A.** three, **B.** two, **C.** one or **D.** zero functions were plotted against baseline viral loads. Spearman rank correlation, r, is shown. $p<0.05$ is considered significant.

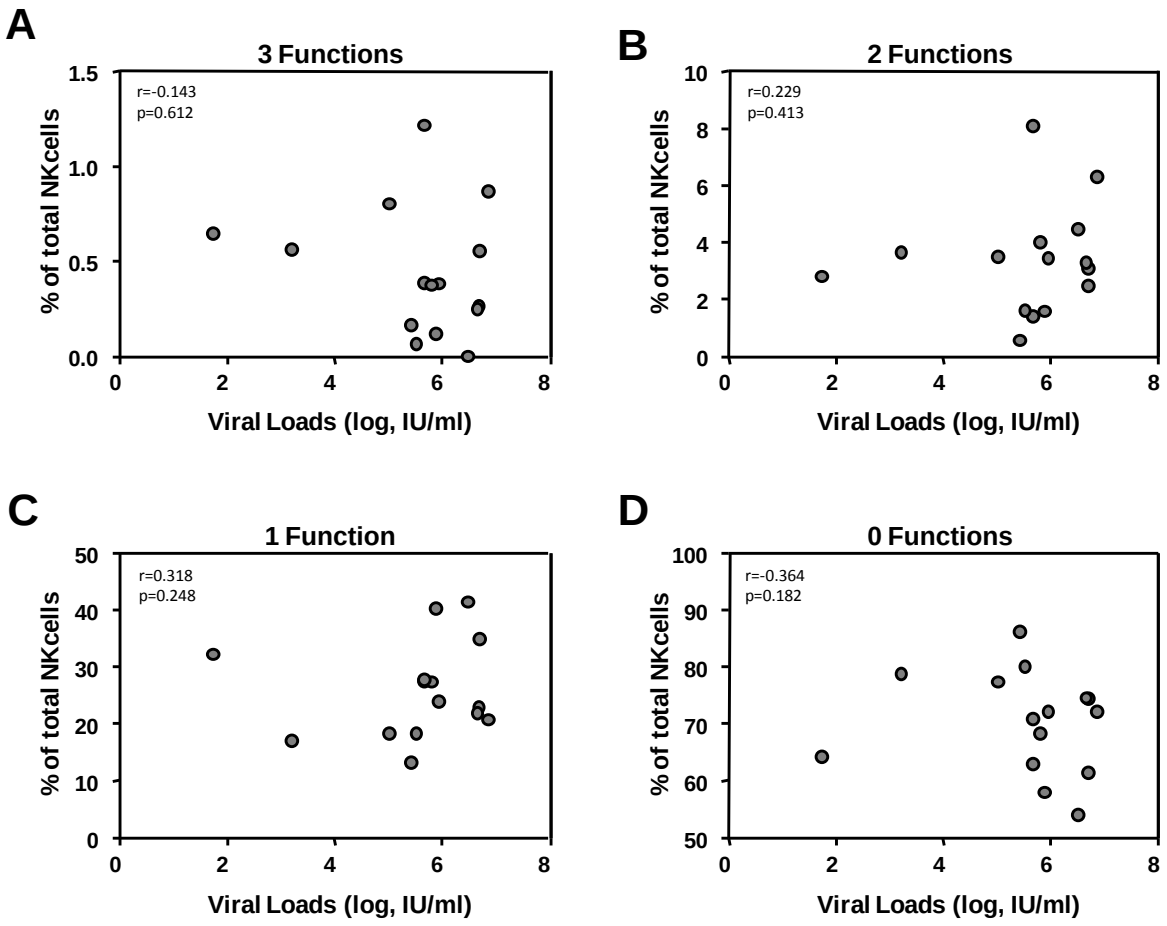

Figure 20: K562 stimulated NK cell functions do not associate with viral loads. Frozen PBMC were thawed and incubated with K562 target cells for 6 hours to assess NK cell functions. NK cell functions examined include degranulation marker CD107a, NK cell-specific IFNγ and TNFα production The spontaneous percentage of totals NK cells with **A.** three, **B.** two, **C.** one or **D.** zero functions were plotted against baseline viral loads. Spearman rank correlation, r, is shown. $p<0.05$ is considered significant.

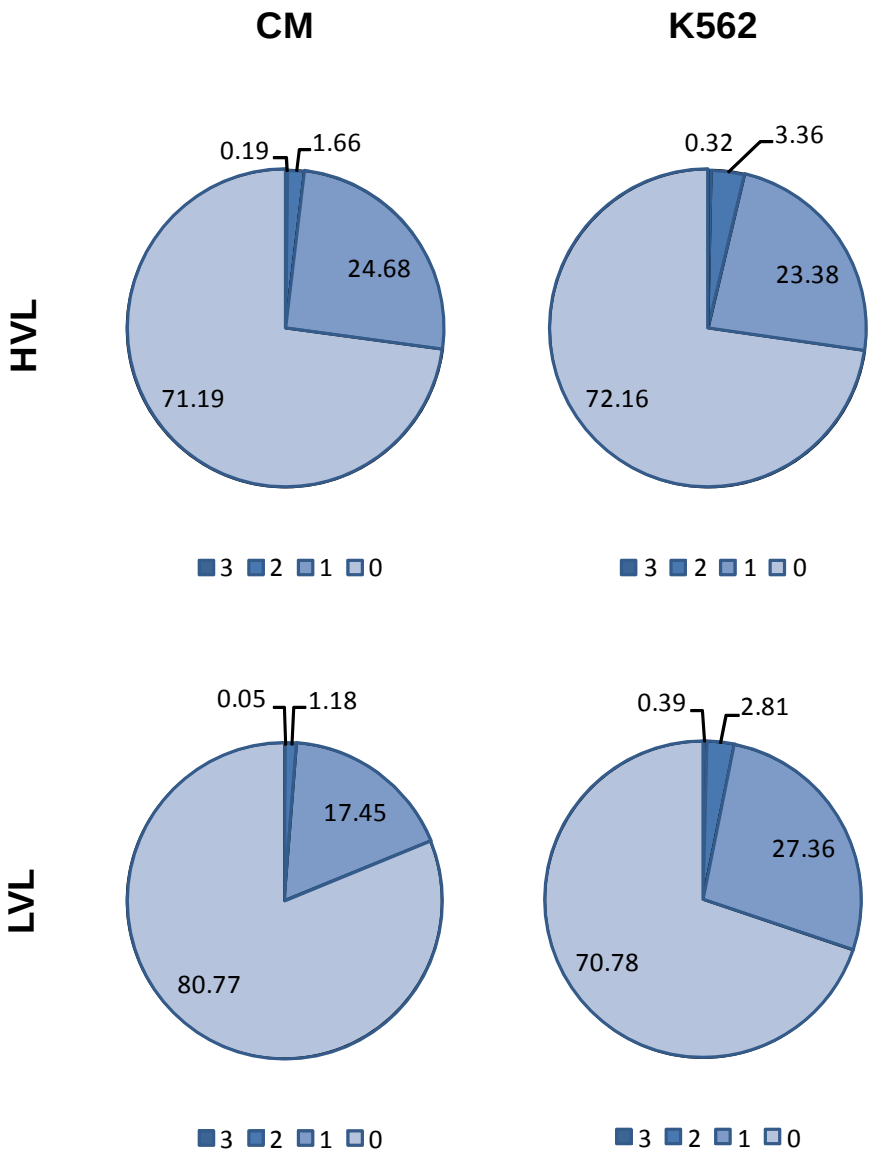

Figure 21: Number of NK cell functions are similar between high and low viral load patients. Frozen PBMC were thawed and incubated with culture medium for 6 hours to assess NK cell functions. NK cell functions examined include degranulation marker CD107a, NK cell-specific IFNγ and TNFα production. Pre-treatment spontaneous and K562-stimulated NK cell functions were evaluated in Caucasian and Aboriginal patients. The percentage of total NK cells with 0, 1, 2 or 3 functions are indicated as the median values for each cohort condition. Statistical significance of number of functions between cohorts was determined using the Mann-Whitney U test.

3.5 Liver damage

NK and NKT cells in the liver play a key role in innate defenses against viral infections, and thus are considered to protect again liver fibrosis in chronic HCV infections [137, 150]. However, NK cells also appear to be involved in liver damage during chronic HBV infection [120]. Thus we were interested in seeing the association between NK and NKT cell function and liver damage in this chronic HCV infection cohort. Liver fibrosis was assessed from biopsy samples according to the METAVIR scoring system. In addition, liver damage was measured by liver enzymes including ALT, AST, GGT and bilirubin.

3.5.1 NK cell cytotoxicity and fibrosis

To assess whether NK cell cytotoxicity correlated to fibrosis scores, cytotoxicity was measured using a standard four hour ^{51}Cr release assay. An E:T ratio of 12.5:1 was used for analysis as it was the intermediate value and was most likely to fall within the linear portion of the NK cell killing curve. We evaluated whether correlations existed between NK cell cytotoxicity and METAVIR fibrosis scores (Fig. 22). Spontaneous (n=20) NK cell cytotoxicity did not correlate with fibrosis score ($r=0.375$, $p=0.103$). Yet IFNα-stimulated (n=20) NK cell cytotoxicity was suggestive of a positive correlation with fibrosis, but this was not significant ($r=0.400$, $p=0.080$).

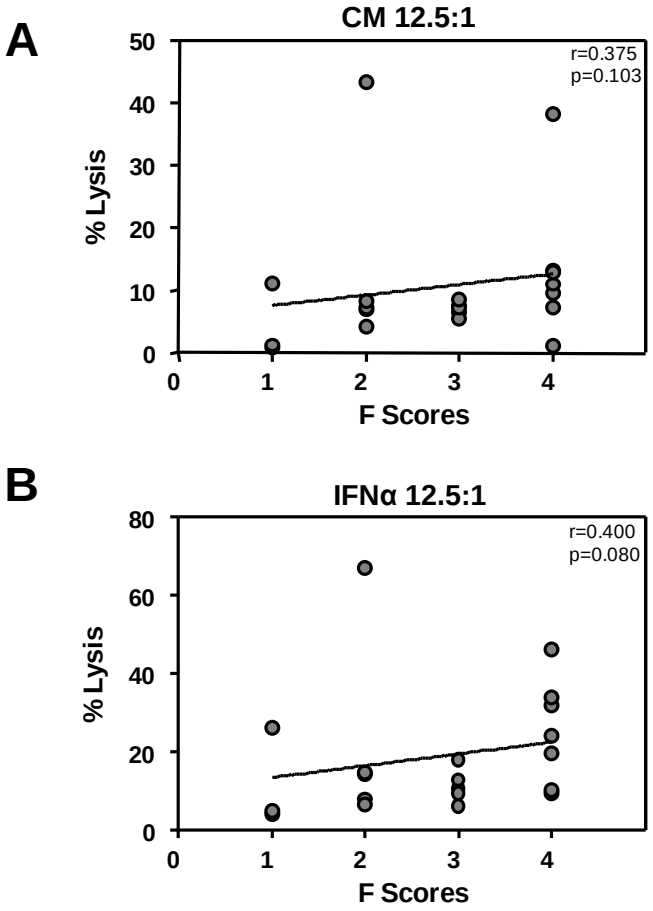

Figure 22: NK cell cytotoxicity was suggestive of a positive correlation with baseline fibrosis. PBMC were incubated overnight in culture medium (CM) and IFNα. NK cell cytotoxicity was measured by Cr-51 release assay. Fibrosis measured by METAVIR scoring system was plotted against NK cell cytotoxicity at E:T ratio of 12.5:1 with **A.** CM (n=20) or **B.** IFNα stimulation (n=20). Spearman rank correlation, r, is shown.

3.5.2 Fibrosis and other parameters

In addition to the ^{51}Cr release assay, PBMC were cultured with medium or with K562 targets for six hours. PBMC were stained for CD107a, TRAIL, PD-1 and intracellularly for TNFα and IFNγ via ICCS to evaluate these parameters against patient fibrosis scores. Analysis of these parameters against patient fibrosis yielded no significant correlations.

3.5.3 NK cell cytotoxiity and liver damage

We evaluated NK cell cytotoxicity to determine if cytotoxicity correlated with liver damage. NK cell cytotoxicity was assessed by ^{51}Cr release assay as previously described. Liver damage was measured by liver enzymes including ALT, AST, GGT and bilirubin. No significant correlations were observed between spontaneous and IFNα-stimulated NK cell cytotoxicity and liver enzymes (Table 4).

Table 4: NK cell cytotoxicity did not correlate with liver damage.

	CM 12.5E:1T		IFNα 12.5E:1T	
	r	p	r	p
ALT	0.113	0.554	0.072	0.707
AST	0.306	0.100	0.263	0.160
GTT	0.295	0.113	0.227	0.228
Bilirubin	0.278	0.136	0.287	0.124

3.5.4 Degranulation marker CD107a and serum AST

As an alternative method of assessing NK cell cytotoxicity, patient PBMC (n=21) were used in our ICCS protocol with culture medium or K562 target cells to indirectly measure cytotoxicity through the appearance of CD107a on the surface of NK cells. We examined whether any association existed between NK and NKT CD107a expression and serum AST. AST is an enzyme associated with liver parenchymal cells. Destruction of these cells leads to an increase in this enzymes concentration in the blood. AST is not specific to the liver, and is found in other cell types including red blood cells, and cardiac and skeletal muscle. Nevertheless, AST is a biomarker of liver injury [239].

Spontaneous CD107a expression within the total NK (Fig. 23a, r=0.372, p=0.097) and CD56dim (Fig. 23b, r=0.345, p=0.126) populations did not correlate with serum AST. In contrast, CD107a expression positively correlated with serum AST within the CD56bright population (Fig. 23c, r=0.535, p=0.013). There was a trend towards a positive correlation between CD107a expression within the NKT population and serum AST, but this was not significant (Fig. 23d, r=0.400, p=0.072).

Upon K562 stimulation, CD107a expression positively correlated with serum AST within the total NK (Fig. 23a, r=0.488, p=0.021) and CD56dim populations (Fig. 23b, r=0.435, p=0.043). On the other hand, the CD56bright population lost its significance (Fig. 23c, r=0.322, p=0.145). CD107a expression within the NKT population also positively correlated with serum AST (Fig. 23d, r=0.592, p=0.004)

HepG2 cells, a human liver carcinoma cell line, were also used as target cells in our ICCS protocol. Compared to K562 target cells, HepG2 cells more closely resemble the *in vivo*

interaction of immune cells with hepatocytes. HepG2 targets were used for 11 patient samples. Upon HepG2 stimulation, CD107a expression within the total NK (Table 5, $r=0.711$, $p=0.014$) and NKT populations ($r=0.691$, $p=0.019$) correlated with serum AST.

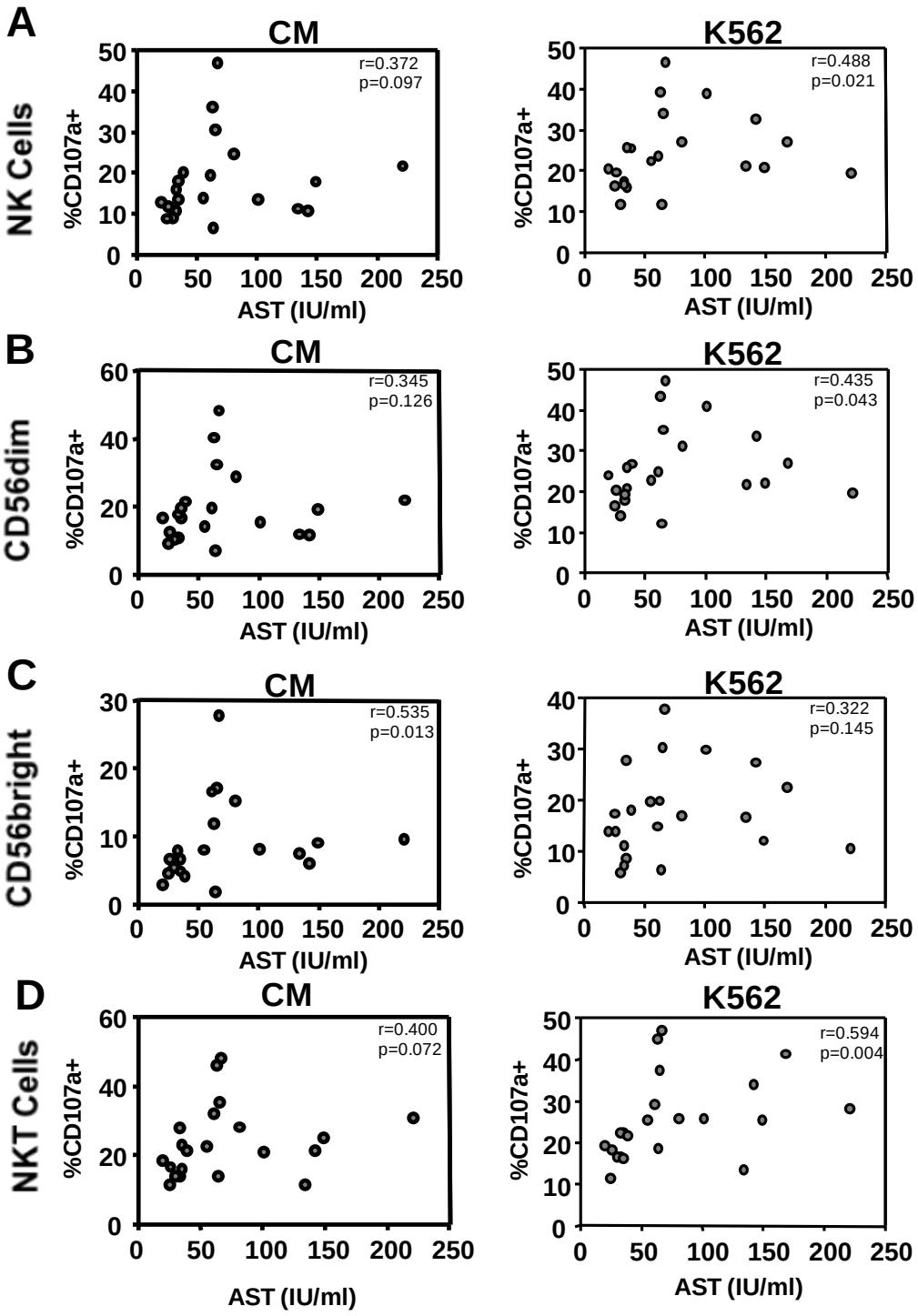

Figure 23: Increased expression of CD107a in NK, CD56dim and NKT cells upon K562 stimulation correlate with baseline serum AST. Frozen PBMC were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. Pre-treatment CD107a expression was assessed by flow cytometry and plotted against serum AST for **A.** all NK cells, **B.** CD56dim, **C.** CD56bright and **D.** NKT populations. Spearman rank correlation, r, is shown. $p<0.05$ is considered significant.

Table 5: CD107a expression correlates with liver damage upon HepG2 stimulation.

		CD107a	TRAIL	PD-1
ALT	NK	0.333	-0.045	0.138
	NKT	0.373	0.018	-0.269
AST	NK	0.711*	0.418	0.422
	NKT	0.691*	0.300	0.091
GTT	NK	0.632*	0.260	0.500
	NKT	0.629*	0.046	-0.128
Bilirubin	NK	0.612*	0.738†	0.795†
	NKT	0.396	0.273	0.569ϕ

* $p<0.05$
† $p<0.01$
Φ $p=0.068$

3.5.5 TRAIL expression and serum AST

Similar to evaluation of CD107a, TRAIL expression by ICCS was also assessed from patient PBMC cultured in medium or with K562 target cells to see any potential correlation with serum AST.

Spontaneous TRAIL expression within the total NK (Fig. 24a, r=0.433, p=0.05) and CD56bright (Fig. 24c, r=0.477, p=0.029) populations positively correlated with serum AST. In contrast, the CD56dim (Fig. 24b, r=0.313, p=0.167) and NKT populations (Fig. 24d, r=-0.057, p=0.806) did not. Upon K562 stimulation, TRAIL expression correlated with serum AST within the total NK (r=0.465, p=0.039) and CD56dim (r=0.454, p=0.034) populations. In contrast, TRAIL expression did not correlate with serum AST within the CD56bright (r=0.339, p=0.123) and NKT cell populations (r=0.164, p=0.477).

HepG2 targets cells were also used to evaluate any potential correlations between TRAIL expression and serum AST. Upon HepG2 stimulation, TRAIL expression did not correlate with serum AST within the total NK, CD56dim, CD56bright or NKT cell populations (Table 5).

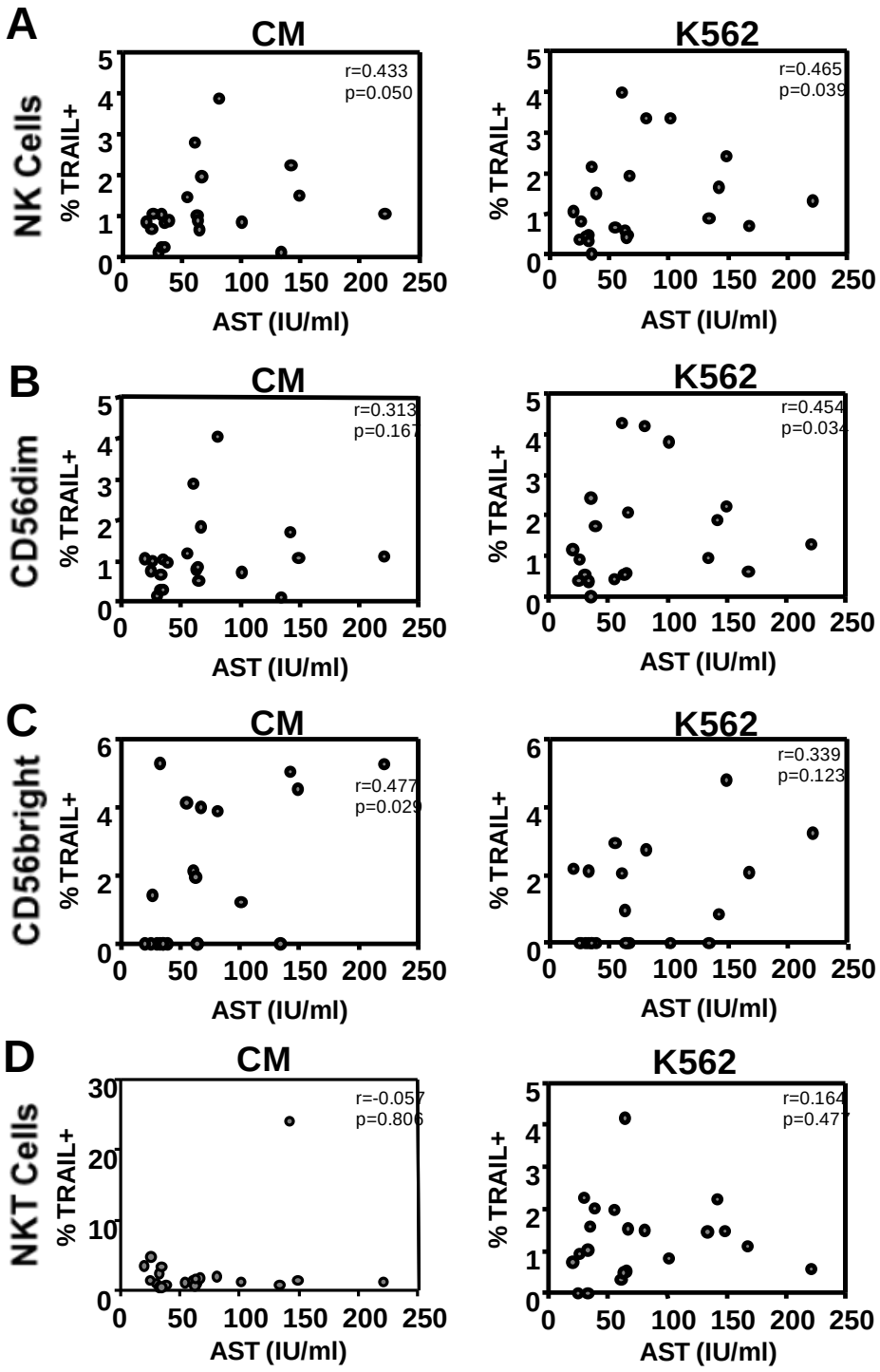

Figure 24: TRAIL expression within the total NK and CD56dim populations upon K562 stimulation correlate with baseline serum AST. Frozen PBMC were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. Pre-treatment TRAIL expression was assessed by flow cytometry and plotted against serum AST for **A.** all NK cells, **B.** CD56dim, **C.** CD56bright and **D.** NKT populations. Spearman rank correlation, r, is shown. $p<0.05$ is considered significant.

3.6 Treatment Outcomes

The exact mechanism by which current antiviral therapy eradicates chronic HCV infection has yet to be determined. The backbone of current antiviral therapy includes pegIFN. Since IFNα is a potent activator of NK cells, it is believed that NK cells may play a role in therapeutic clearance. Thus, we were interested to see if NK cell functions were beneficial during the course of antiviral therapy.

3.6.1 Study demographics between treatment outcome cohorts

Our patient cohort was separated based on their treatment outcomes. Patients who achieved at least a 2 log drop in HCV RNA at 3 months of treatment relative to baseline were defined as EVR+ while those who did not or had stopped treatment were defined as EVR-. Similarly, patients who remain HCV RNA free after six months of treatment were defined as SVR+ while patients who did not or who had stopped treatment for any reason were defined as SVR-. Treatment outcomes had limited influence on subject demographics and disease characteristics (Table 6). The SVR+ cohort had a slightly higher percentage of males, a higher percentage of Caucasians and a lower median age, but these were not significant. The distribution of genotype 1 infections was also comparable (SVR+ 75% versus SVR- 100%, p=0.096). Baseline viral loads, liver enzymes including ALT, AST, GGT and bilirubin, fibrosis scores and grades of inflammation also did not differ between treatment outcomes.

Table 6: Patient demographics of SVR+ and SVR- patients

Parameters	SVR+ (n=12)	SVR- (n=13)
Patient factors		
Male (%)	50	38
Age (median, range)	49 (23-59)	55 (40-65)
Caucasian (%)	75	62
Viral factors		
Genotype 1 (%)	75	100
Viral Load (log, IU/ml, median, range)	6.26 (1.74-7.10)	5.84 (3.66-7.33)
ALT (median, range)	79 (27-330)	55 (31-248)
AST (median, range)	62 (22-221)	81 (27-168)
GGT (median, range)	50.5 (10-172)	65 (26-361)
Bilirubin (median, range)	9.5 (4-20)	10 (4-25)
Fibrosis Scores (median, range)	2.5 (1-3)	.2.5 (1-4)
Grade of Inflammation (median, range)	2 (1-3)	2 (1-3)

3.6.2 NK cell cytotoxicity and EVR

Patients donated blood at baseline and at 3 months of treatment to assess NK cell cytotoxicity at these time points. Freshly isolated PBMC were incubated overnight in culture medium or culture medium supplemented with IFNα (1000 IU/ml). The following day, PBMC were used in a standard four hour ^{51}Cr release assay as previously described. Our patient cohort was divided into two groups based on their ability to achieve an EVR. We were interested in examining whether there were differences in NK cell cytotoxicity between EVR+ and EVR- patients.

Our analysis revealed that overnight incubation with IFNα increased NK cell killing in both the EVR+ (Fig. 25a, n=13, 1.42 fold, p=0.003) and EVR- (n=6, 1.58 fold, p=0.033) cohorts. In contrast, at 3 months of treatment, relative to CM alone, overnight incubation with IFNα did not increase NK cell killing in both EVR+ (n=15, 1.36 fold, p=0.648)) and EVR- (n=7, -1.14 fold, p=0.516) patients.

At baseline, limited differences between treatment cohorts were observed in spontaneous (p=0.251) and IFNα-induced (p=0.114) NK cell killing. Similarly, at 3 months of treatment our analysis revealed no differences between treatment cohorts in spontaneous (p=0.549) and IFNα-induced (p=0.438) NK cell killing.

A paired evaluation of baseline and 3 month samples from the same donor was performed. At baseline, a similar increase in NK cytotoxicity was observed upon overnight incubation of PBMC with IFNα relative to CM alone from both EVR+ (Fig. 25b, n=11, 1.42 fold, p=0.013) and EVR- (n=6, 1.58 fold, p=0.033) patients. In contrast, at 3 months, incubation of PBMC with IFNα relative to CM alone did not increase NK killing in either treatment cohort (EVR+: 1.33 fold, p=0.706; EVR-: -1.07 fold, p=0.591).

Further evaluation of paired samples at baseline revealed limited differences in spontaneous ($p=0.393$) and IFNα-induced ($p=0.209$) NK cell killing between the EVR+ and EVR- cohorts. Likewise, at 3 months of treatment, paired samples also had no differences in spontaneous ($p=0.763$) or IFNα-induced ($p=0.725$) NK cytotosicity between treatment cohort.

To further evaluate the impact of treatment on NK cell killing, the fold difference between cytotoxicity at 3 months relative to baseline was determined for paired samples. The fold change in spontaneous (Fig. 25c, $p=0.960$) and IFNα-induced ($p=0.451$) NK cell killing was similar between the EVR+ and EVR- cohorts.

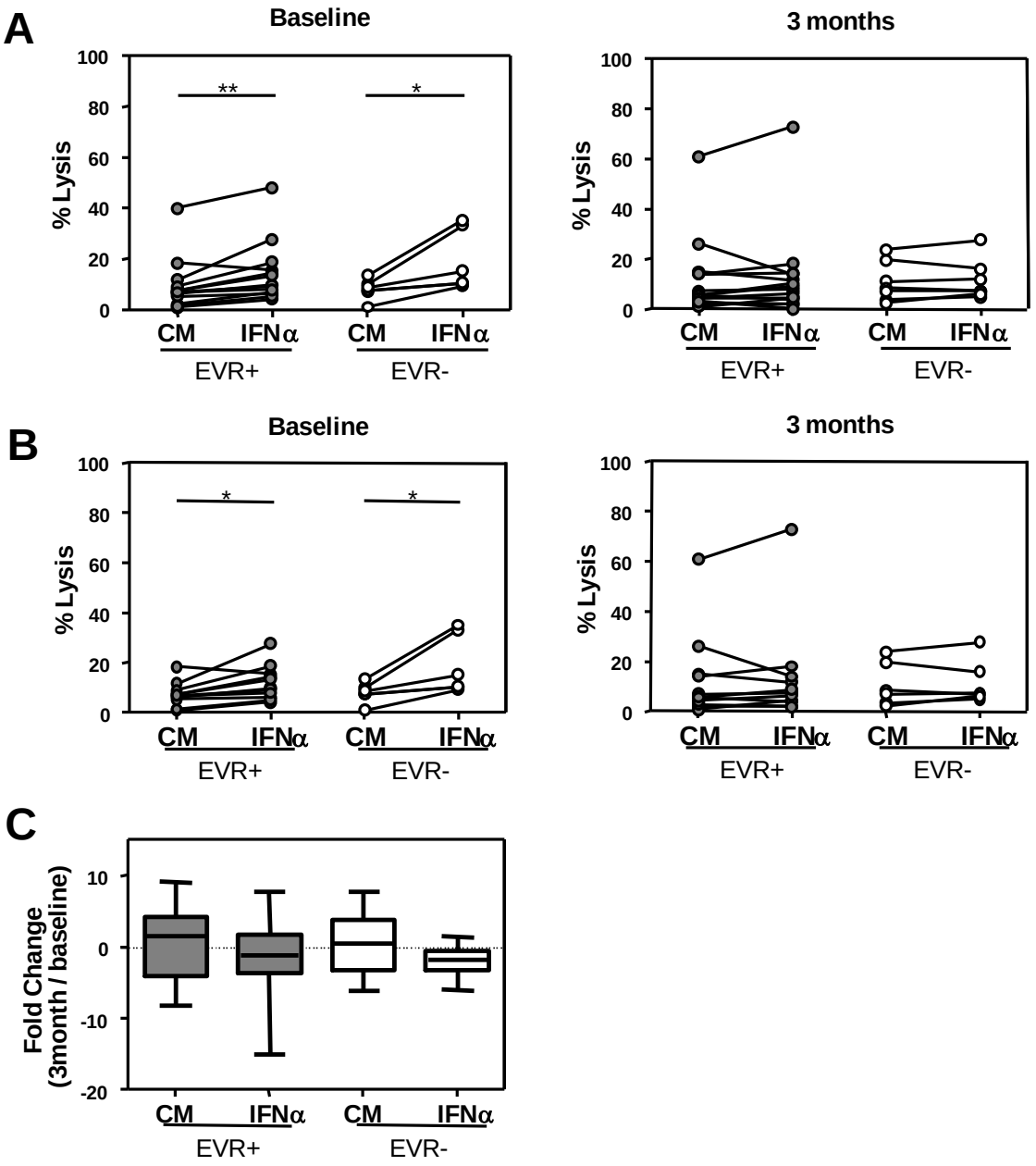

Figure 25: NK cell cytotoxicity does not differ between patients who achieve and do not achieve an EVR. PBMC were incubated overnight in culture medium (CM) and IFNα. NK cell cytotoxicity was measured by ^{51}Cr release assay. **A.** At E:T ratio of 12.5:1, similar NK cell cytotoxicity was observed between the EVR+ and EVR- cohorts at baseline and at 3 months of treatment. **B.** For patients with samples available at baseline and 3 months, a paired analysis was performed. **C.** Fold change in NK cell cytotoxicity from baseline to 3 months for each condition indicated. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and IFNα were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as *p<0.05, **p<0.01.

3.6.3 NK cell cytotoxicity and SVR

Different host and viral factors have been implicated with beneficial treatment outcomes. Please see Section 1.4.2 for these factors. We were interested to see if baseline NK cytotoxicity could predict treatment outcomes. Once again, PBMC were used in a standard four hour ^{51}Cr release assay as previously described after overnight incubation in culture medium or culture medium supplemented with IFNα (1000 IU/ml). Treatment cohorts were divided into SVR+ and SVR- for subsequent analysis.

This analysis revealed that overnight incubation with IFNα relative to CM alone increased cytotoxicity for both SVR+ (Fig. 26, n=10, 1.42 fold, p=0.013) and SVR- (n=7, 1.89 fold, p=0.018) cohorts. Between treatment cohorts, limited differences were seen in baseline spontaneous (p=0.536) and IFNα-induced (p=0.230) NK cell killing.

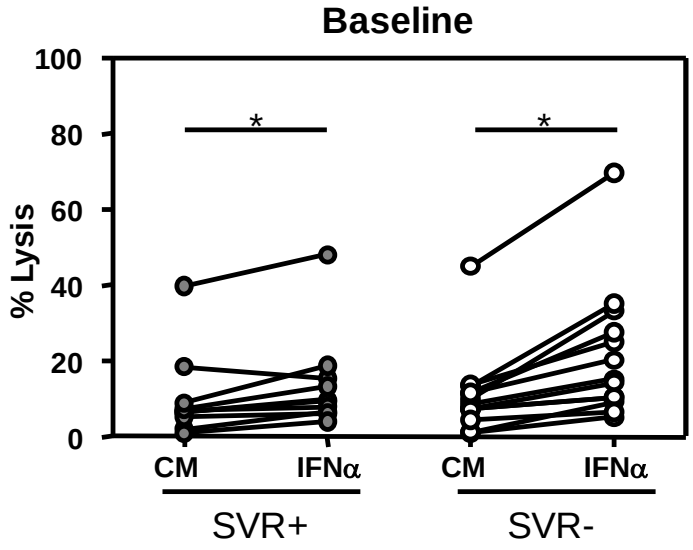

Figure 26: NK cell cytotoxicity does not differ between patients who achieve and don't achieve an SVR. PBMC were incubated overnight in culture medium (CM) and IFNα. NK cell cytotoxicity was measured by ^{51}Cr release assay. At 12.5E:1T ratios, similar NK cell cytotoxicity was observed between all SVR+ and SVR- patients at baseline. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and IFNα were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as *$p<0.05$.

3.6.4 CD107a, TRAIL and PD-1 expression and SVR

We were interested to observe if CD107a, TRAIL and PD-1 expression on the surface of NK cells could predict treatment outcomes. CD107a is a marker of degranulation that indirectly measures NK cell killing. The appearance of CD107a on the surface of NK cells correlated with the magnitude of HCV-specific T cells, which may be beneficial during treatment [238]. TRAIL upregulation following IFNα stimulation on NK cells associated with control of HCV infection [106]. Increased PD-1 expression on HCV-specific CTLs has previously been associated with failed SVR rates [235]. The appearance of CD107a, TRAIL and PD-1 was evaluated by our ICCS protocol as previously described.

Our analysis revealed that relative to CM alone, K562 stimulation did not increase CD107a expression from the SVR+ cohort within the total NK population (Fig. 27a, n=7, 1.43 fold, p=0.113). In contrast, K562 stimulation upregulated CD107a expression from the SVR- cohort (n=8, 1.39 fold, p=0.026). Subsequent evaluation between treatment outcomes showed limited differences in spontaneous (p=0.318) and K562-stimulated (p=0.336) CD107a expression within the total NK population.

Evaluation of TRAIL expression showed that K562 stimulation, relative to CM alone, could not upregulate TRAIL expression in either treatment cohort (Fig. 27b, SVR+: p=0.788; SVR-: p=0.502). When comparing TRAIL expression between the SVR+ and SVR- cohorts, no differences were observed in spontaneous (p=0.710) or K562-stimulated (p=0.613) TRAIL expression within the total NK population.

Similar to TRAIL expression, K562 stimulation could not upregulate PD-1 expression within either the SVR+ (Fig. 27c, p=0.479) or SVR- (p=0.535) cohorts relative to CM alone.

Spontaneous (p=0.805) and K562-stimulated (p=0.728) PD-1 expression within the total NK population was also equivalent between SVR+ and SVR- patients.

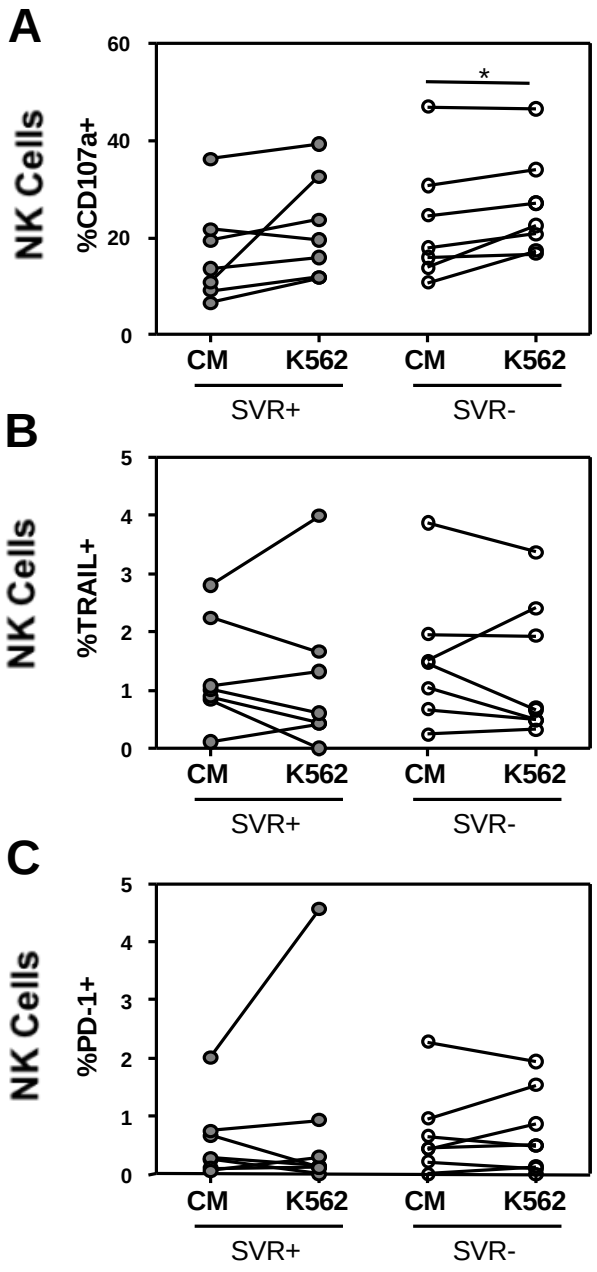

Figure 27: CD107a, TRAIL and PD-1 expression are not predictors of SVR. Frozen PBMC were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. **A.** CD107a expression, **B.** TRAIL expression and **C.** PD-1 expression within the total NK population was evaluated between the SVR+ and SVR- cohorts. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and K562 were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as *$p<0.05$.

3.6.5 NK cell cytokine production and SVR

NK cells can control viral replication indirectly through IFNγ production. Strong Th1 cytokine profiles have been associated with increased therapeutic clearance of chronic HCV infections [236, 237]. TNFα is such a cytokine produced by NK cells which contributes to the anti-viral state. We were interested to see if baseline NK cell-specific IFNγ and TNFα production could predict SVR. NK cell-specific IFNγ and TNFα production were determined by our ICCS protocol as previously described.

Our analysis revealed that relative to CM alone, stimulation of NK cells with K562 targets could not upregulate IFNγ production within the SVR+ (Fig. 28a, n=7, 3.88 fold, p=0.354) cohort. In contrast, it could within the SVR- (n=7, -1.04 fold, p=0.023) cohort. In between treatment cohorts, there were no differences in spontaneous (p=0.620) or K562-stimulated (p=0.867) NK cell-specific IFNγ production.

When evaluating NK cell-specific TNFα production, relative to CM, K562 stimulation upregulated TNFα production from the total NK population within the SVR+ cohort (Fig. 28b, 2.43 fold, p=0.046), but not within the SVR- (1.49 fold, p=0.140) cohort. Limited differences were observed in spontaneous (p=0.456) and K562- stimulated (p=0.779) NK cell-specific TNFα production between the SVR+ and SVR- cohorts.

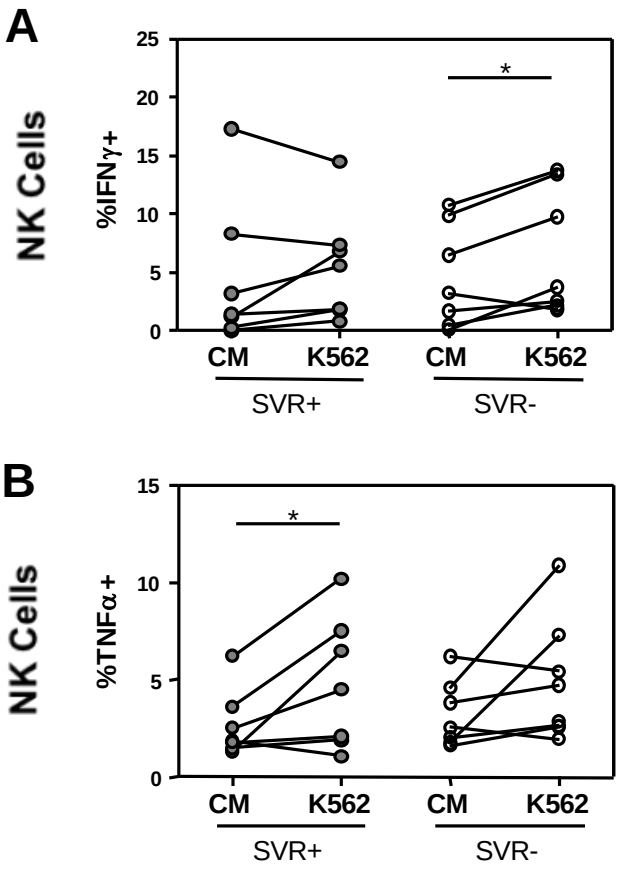

Figure 28: IFNγ and TNFα production from NK cells cannot predict SVR. Frozen PBMC were thawed and incubated with culture medium (CM) or K562 target cells for 6 hours. **A.** IFNγ production and **B.** TNFα production within the total NK population was evaluated between the SVR+ and SVR- cohorts. Lines indicate an individual patient's values for each condition. Within cohort significant differences between CM and K562 were determined by paired t test. Between cohort significant differences were determined by Mann-Whitney U test. Significant differences are indicated as *$p<0.05$.

3.6.6 NK cell functions and SVR

As another method to evaluate NK cell activity, the number of function displayed by NK cells within the SVR+ and SVR- cohorts were assessed. The NK cell functions of interest include the appearance of CD107a and NK cell-specific IFNγ and TNFα production. NK cells were divided according to the number of functions they displayed.

The number of NK cell functions were evaluated between the SVR+ and SVR- cohorts prior to the start of treatment (Fig. 29). Relative to CM, incubation with K562 target cells could not increase the number of NK cell functions within either treatment cohort. In addition, between the SVR+ and SVR- cohorts, no differences were observed in the spontaneous or K562-stimulatied number of NK cell functions.

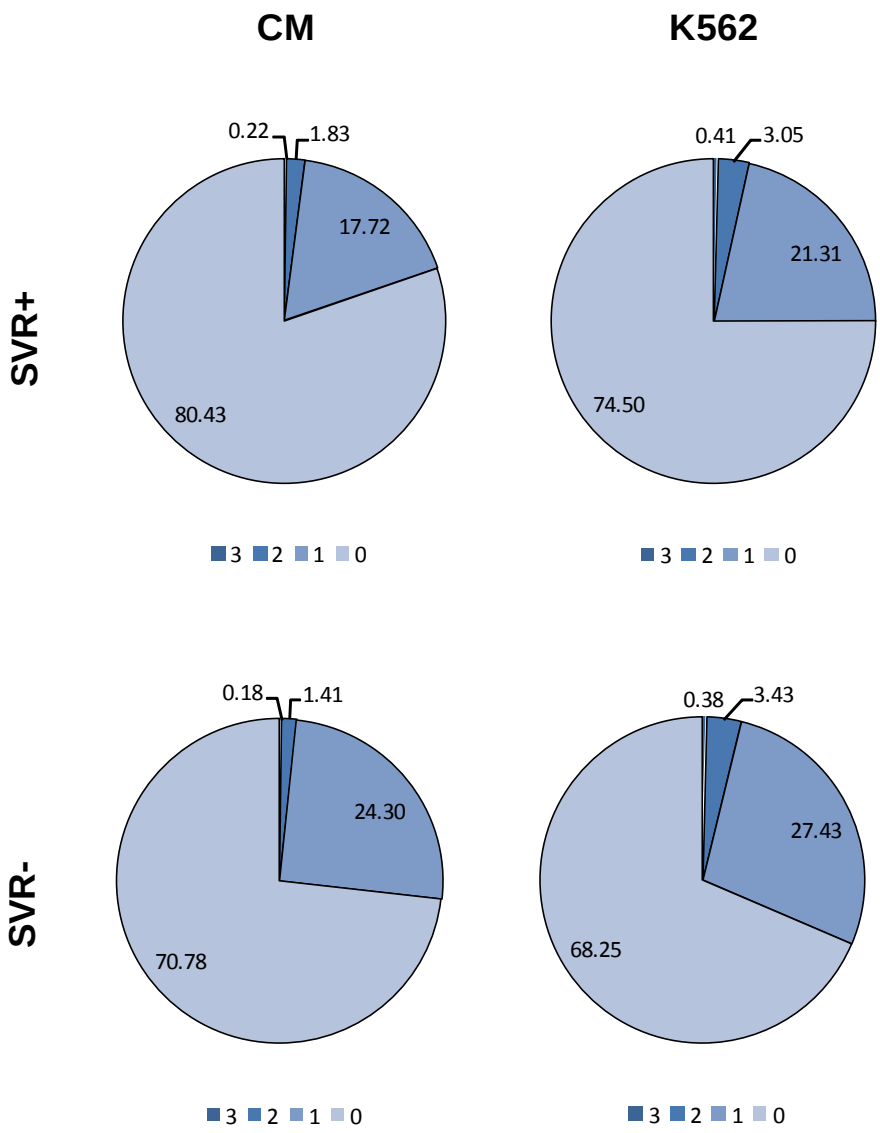

Figure 29: Number of NK cell functions are similar between SVR+ and SVR- patients. Frozen PBMC were thawed and incubated with culture medium for 6 hours to assess NK cell functions. NK cell functions examined include degranulation marker CD107a, NK cell-specific IFNγ and TNFα production. Pre-treatment spontaneous and K562-stimulated NK cell functions were evaluated between the SVR+ and SVR- cohorts. The percentage of total NK cells with 0, 1, 2 or 3 functions are indicated as the median values for each cohort condition. Statistical significance of number of functions between cohorts was determined using the Mann-Whitney U test.

4 Discussion

NK cell function was assessed between Caucasian and Aboriginal patients. Ethnicity had limited influence on baseline subject demographics and disease characteristics. When NK cell cytotoxicity was assessed two different ways between ethnic cohorts at baseline, no difference was observed. In contrast, at three months of treatment, Caucasian patients had increased spontaneous and IFNα-induced NK cell cytotoxicity compared to Aboriginal patients. Baseline PD-1 expression also did not differ between Caucasian and Aboriginal patients. Caucasian NK, NKT and T cells could produce more IFNγ spontaneously and upon K562 stimulation when compared to their counterparts within Aboriginal patients. Only NK cells from Aboriginal patients could upregulate IFNγ production upon K562 stimulation. NK cell-specific TNFα production did not differ between ethnicities. However, in contrast to IFNγ production, Caucasian NK cells could upregulate TNFα production upon K562 stimulation. Lastly, the number of NK cell functions were similar between Caucasian and Aboriginal patients at baseline.

NK cell function was also assessed against baseline viral loads. NK cell cytotoxicity negatively correlated with baseline viral load whereas CD107a and NK cell-specific IFNγ production positively correlated with viral loads. Interestingly, CD107a and NK cell-specific IFNγ production correlated with each other within the CD56bright NK cell population. PD-1 expression correlated with baseline viral loads upon K562 stimulation. Lastly, 1 spontaneous NK cell function positively correlated and 0 spontaneous functions negatively correlated with baseline viral loads whereas all K562-stimulated functions had no relationship with baseline viral loads.

We evaluated NK cell function too see if it reflected liver damage measured by fibrosis scores and serum liver enzymes levels. NK cell cytotoxicity measured by the ^{51}Cr release assay positively correlated with fibrosis, but not with any liver enzymes. In addition, spontaneous CD107a and TRAIL expression within the CD56bright population correlated with serum AST whereas K562-stimulated CD107a and TRAIL expression within the total NK and CD56dim populations correlated with serum AST. Spontaneous CD107a expression within the NKT population also correlated with serum AST. Lastly, HepG2 stimulation strengthened the relationships between NK cell function and liver damage. CD107a expression within the total NK cell population correlated with serum AST, GTT and bilirubin. TRAIL expression within the total NK cell population also correlated with serum bilirubin. In addition, CD107a expression within the NKT population correlated with serum AST and GTT.

Last of all, we examined if NK cell functions at baseline could predict EVR or SVR within our patient population. Limited differences in subject demographics and disease characteristics were apparent between treatment cohorts at baseline. In addition, NK cell cytotoxicity and cytokine production at baseline could not predict either EVR or SVR. Interestingly, SVR- patients could upregulate NK cell-specific IFNγ production at baseline whereas SVR+ patients could upregulate NK cell-specific TNFα production at baseline. The number of NK cell functions were also similar between SVR+ and SVR- patients at baseline.

4.1 NK cell function in Caucasian and Aboriginal patients with chronic HCV infections

4.1.1 Differences in NK cell function between ethnic cohorts

Spontaneous clearance of the acute HCV infections has been shown to vary across different ethnicities [10-12, 122, 123]. Specifically, Aboriginal ethnicity is associated with enhanced spontaneous clearance relative to Caucasian and African-American populations [10-12,

122]. The Rempel lab has been investigating different factors which may account for the enhanced spontaneous clearance within the Aboriginal population. These include genetic and functional studies that show that Aboriginals have an enhanced pro-inflammatory microenvironment because of the role of specific KIR genes in promoting HCV clearance [134, 240, 241]. Dr. Rempel's lab also investigated the KIR genotypes in the Aboriginal and Caucasian populations. They demonstrated that Aboriginal KIR clusters displayed a greater immune activating phenotype due to a greater presence of genes from the B haplotype situated within the telomeric region of the KIR gene cluster relative to Caucasian counterparts. This was accompanied by a decrease in the presence of genes of the B haplotype located within the centromeric region [127]. This was the similar KIR profile observed for HCV clearance versus chronicity. KIR genes participate in regulating NK cells along with certain T cell subsets. Taken altogether, these suggest that Aboriginals have a 'natural" advantage' in NK cell function. The role of NK cells in chronic HCV infections has previously been discussed (see Section 1.3.8). Our first objective in this study was to evaluate whether NK cell functions differ between Caucasian and Aboriginal patients with chronic HCV infections.

In chronic HCV infected patients, subject demographics and disease characteristics did not differ between ethnic cohorts (Table 1). The Caucasian cohort had a slightly lower percentage of males and genotype 1 infections when compared to the Aboriginal cohort. This apparent 'treatment advantage' in Caucasians may contribute to slightly higher (but statistically similar) EVR and SVR compared to Aboriginals. When evaluating genotype 1 infections alone, SVR was achieved in 47% of Caucasians and 44% of Aboriginals ($p>0.05$). This observation agrees with previous published results that found comparable SVR rates between non-Aboriginal and Aboriginal people infected with genotype 1 virus [199]. The marginally higher rate of treatment

discontinuation within the Aboriginal cohort is likely an artifact since in a previous study in Dr. Rempel's lab found that while 6 Caucasian men discontinued treatment, no Aboriginals did (personal communication, J. Rempel)

To determine whether NK cell function differed between Caucasian and Aboriginal HCV infected individuals, NK cell activity was examined. We did not find a difference in NK cell activity between ethnic cohorts based on similar NK cell cytotoxicity. NK cell cytotoxicity was assessed two ways; through a standard four hour ^{51}Cr release assay and through CD107a and TRAIL expression from our ICCS protocol, both of which have been previously described. No differences were observed in spontaneous or IFNα-induced cytotoxicity in the ^{51}Cr release assay (Fig. 2). Likewise, no differences were seen in spontaneous or K562-stimulated CD107a or TRAIL expression from the total NK, CD56dim (associated with cytotoxicity) and CD56bright (associated with cytokine production) populations (Fig. 5). Unfortunately, due to widespread lymphopenia within our study cohort at 3 months of treatment, there were not enough PBMC to use within our ICCS assay at this time point. It appears that the main mechanism of killing within our ^{51}Cr release assay is through the perforin/granzyme B pathway. This is indicated by the modest expression of CD107a and little expression of TRAIL. Taken altogether, our data suggests that the natural genetic advantage (based on the KIR data) Aboriginals have in spontaneously clearing HCV infection is not apparent in chronic HCV infection. This may result from interaction of the HCV virus with NK cells to inhibit the natural advantage in Aboriginals or from a selection bias in our Aboriginal cohort where only individuals who do not possess the advantageous KIR genes develop a chronic HCV infection.

Following three months of treatment, Caucasian patients have increased NK cell cytotoxicity compared to Aboriginal patients (Fig. 3). This increase does not appear to be beneficial during

treatment for chronic HCV as both ethnic cohorts have similar abilities to achieve EVR (Table 2). Subsequent analysis using paired patients, where cytotoxicity results were available at baseline and at 3 months of treatment, showed similar trends are observed in NK cytotoxicity although some significance is lost due to a decrease in sample size (Fig. 3).

The other NK cell activity we evaluated was cytokine production including the percent of cells producing IFNγ and TNFα. IFNγ and TNFα gene polymorphisms have been associated with differential expression of each respective cytokine. For IFNγ, one such allelic polymorphism is at the +874 loci [242, 243]. More polymorphisms exist within the TNFα promoter region, with one notable polymorphism at the -308 loci [244, 245]. One study reports the frequency of these allelic polymorphisms between Canadian Aboriginals and Caucasians [246]. They found that when compared to Caucasians, individuals of Dené, but not Cree ancestry, had a higher frequency of the A allele which is associated with low IFNγ production. Also, when compared to Caucasians, individuals of Dené and Cree ancestry had a significantly higher frequency of the G allele at the -308 location of the human TNFα promoter which is associated with low TNFα production [246]. In addition, it has been reported that when compared to Caucasians, Canadian Aboriginal individuals have a higher frequency of cytokine single-nucleotide polymorphisms favouring a low production of TNFα, IFNγ and IL-10 and high production of IL-6 as compared to a Caucasian population [241]. Taken altogether, this suggests that Caucasians and Aboriginals have unique cytokine genotype profiles. Supporting this observation, our Caucasian cohort produced significantly more IFNγ spontaneous and upon K562 stimulation compared to our Aboriginal cohort (Fig. 8). This difference in IFNγ production was not restricted to the NK cell populations as it was also observed within the NKT and T cell populations. Also supporting the above observation but in contrast to IFNγ production, TNFα production was similar between

ethnicities (Fig. 9). The difference in cytokine production between our ethnic cohorts may reflect the genetic bias of Aboriginal patients that develop chronic HCV infections.

To determine whether NK cell exhaustion played a role in our Caucasian and Aboriginal cohorts, PD-1 expression was evaluated on the total NK, CD56dim and CD56bright populations (Fig. 7). Exhaustion marker PD-1 has been shown to inhibit T cell functions and has been implicated in several viral infections [247-249]. Accordingly, chronic HCV infections are characterized by upregulation of PD-1 on HCV-specific CTLs in both the peripheral and intrahepatic compartments when compared to individuals who spontaneously recovery from acute HCV infections [250-252]. Additionally, PD-1 expression on NK cells from individuals with chronic HCV infections was found to be significantly higher compared to healthy individuals [235]. We did not observe a difference in PD-1 expression from any subset of NK cells between our Aboriginal and Caucasian cohorts. This observation agrees with a previous report that found no difference in PD-1 expression between ethnicities (Caucasian and African-American) [235]. Thus, it appears that NK cell exhaustion through PD-1 expression does not play a role within our study cohort.

The number of NK cell functions was also evaluated between the Caucasian and Aboriginal cohorts (Fig. 10). The number of NK cell functions have previously been shown to play a role in spontaneously resolving acute HCV infection [238]. We had hypothesized that Aboriginal patients would have NK cells with more functions compared to Caucasians due to unique KIR clusters that display a greater immune activating phenotype [127]. Our data does not support this hypothesis. NK cells from both ethnicities displayed similar percentages of functions. We are unsure as to whether this is a consequence of HCV infection within the Aboriginal patients or the selection bias towards Aboriginals that develop a chronic HCV infection.

4.1.2 Stimulation of NK cells between ethnic cohorts

An important factor we examined in this study was how a HCV infected patient's NK cells responded to stimulation. Within our ^{51}Cr release assay, PBMC were incubated overnight in culture medium or culture medium supplemented with IFNα whereas our ICCS protocol incubated PBMC for 4 hours prior to staining in culture medium or K562 targets. Within the ^{51}Cr release assay, NK cells from both patient cohorts are equally responsive to overnight IFNα stimulation as indicated by the increase in killing the following day (Fig. 3). This suggests that NK cells in chronic HCV infection are not fully stimulated as they can react to exogenous IFNα, a potent activator of NK cells. Caucasians have an increase in the fold change in IFNα-induced killing compared to Aboriginals suggesting that Aboriginal patients appear to be more refractory to in vitro IFNα stimulation when compared to Caucasian patients. Also following three months of treatment, relative to spontaneous killing, NK cells from both patient cohorts fail to increase IFNα-induced killing at 3 months of treatment (Fig. 3). This finding agrees with previously published results where NK cells become refractory to further in vitro IFNα stimulation after 1 week of pegIFN therapy and remained low for a further 11 weeks [253].

Within the ICCS protocol, not only did the total NK and CD56dim populations increase CD107a expression upon K562 stimulation from both ethnicities, but the CD56bright population did as well (Fig. 5). The observation that CD56bright NK cells can have markers of degranulation has previously been reported [254, 255]. In contrast, TRAIL expression could not be upregulated upon K562 stimulation relative to culture medium alone within all the examined NK populations for either ethnic cohort (Fig. 6). Another major stimulator of NK cells, IFNα, has been shown to upregulate TRAIL expression on both CD56dim and CD56bright NK cells [106]. Taken altogether, our results suggest other mediators, not cellular contact between NK cells and the

MHC-devoid K562 target cells, are required to upregulate TRAIL expression on NK cells. Similar to TRAIL expression, Caucasians and Aboriginals could not upregulate PD-1 expression upon K562 stimulation relative to culture medium alone suggesting that further stimulation of NK cells in chronic HCV infection does not cause NK cell exhaustion (Fig. 7). NK cell-specific IFNγ production increased upon K562 stimulation within the total NK and CD56dim populations only within the Aboriginal cohort (Fig. 8) whereas NK cell-specific TNFα production increased upon K562 stimulation predominantly within the Caucasian cohort (Fig. 9). These different cytokine responses from NK cells may be a factor contributing to disease susceptibility and resistance between these two populations.

4.1.3 Summary of NK cell function in Caucasian and Aboriginal patients with chronic HCV infections

Taken altogether, our data suggests that the natural genetic advantage (based on the KIR data) Aboriginals have in spontaneously clearing HCV infection is not apparent in chronic HCV infection. This may result from interaction of the HCV virus with NK cells to inhibit the natural advantage in Aboriginals or from a selection bias in our Aboriginal cohort where only individuals who do not possess the advantageous KIR genes develop a chronic HCV infection. The Rempel lab is currently investigating these two hypotheses by conducting genetic studies examining KIR and HLA genes within Caucasians and Aboriginals with chronic HCV infections.

4.2 Viral loads and NK cell function

4.2.1 NK cell functions between high and low viral

Controversy surrounds whether or not HCV can inhibit NK cell function. Numerous ex vivo studies have tried to evaluate NK cell function using PBMC from patients with chronic HCV infections only to yield conflicting results [109, 139, 142, 146]. In order to address these conflicting results, several studies tried to identify potential interaction mechanisms between NK cells and the hepatitis C virion. Initial mechanistic studies using immobilized recombinant HCV envelope proteins to investigate the effect of HCV envelop proteins on NK cell function showed that the envelop protein E2 inhibits NK cells in vitro by binding to CD81 on the surface of NK cells [93, 94]. However, this recombinant E2 protein does not represent naturally occurring E2 proteins which are found in a lipid bilayer enveloping the HCV virion. This problem was solved with the development of cell culture systems that can generate infectious HCV virions [20, 256]. These cultures systems provide a tool to study HCV replication and pathogenesis. Using these infectious cell culture systems, others have shown that naturally expressed E2 on infectious HCV particles do not inhibit NK cell functions unless the virions themselves are immobilized and concentrated on the surface of a plate [257, 258]. When this occurs, there is inhibited IFNγ production by IL-12 activated NK which was mediated through engagement of cellular CD81 by HCV virion displayed E2 [257]. Furthermore, since the discovery of immunomodulatory effects of HCV proteins on innate immune responses of infected hepatocytes, it has been suggested that HCV may control NK cell functions through regulatory pathways of infected hepatocytes [259]. This was successfully shown in a study that investigated whether HCV can modulate NK cell functions through direct interactions with HCV-infected hepatocytes [260]. They found that direct cell-to-cell contact between infected hepatocytes and NK cells not only inhibited NK cell

cytotoxicity, but IFNγ secretion as well. Also, the reduction in cytotoxicity correlated with reduced expression of NK cell activation receptors including NKG2D and NKp30 on the surface of NK cells. Thus although there is no direct evidence that NK cells are inhibited in the HCV infected liver, there is evidence suggesting that the functional capacity of liver-infiltrating NK cells that interact with HCV infected hepatocytes may be reduced. It is possible that these reduced functional capacity, liver-infiltrating NK cells may migrate back into the peripheral blood.

We were interested to investigate any potential relationships between NK cell function and viral load within our HCV infected individuals. Using the ^{51}Cr release assay, we found a significant negative correlation between spontaneous cytotoxicity and baseline viral loads (Fig. 11). In addition, we found that IFNα-stimulated cytotoxicity was suggestive of a negative correlation with baseline viral loads ($p=0.081$). In contrast, we found that spontaneous CD107a expression, evaluated by our ICCS protocol, positively correlated with baseline viral loads (Fig. 12). This correlation was lost upon K562 stimulation. The discrepancy between our cytotoxicity results from the chromium release assay and CD107a assessment disagreed with another study that examined this relationship where the appearance of CD107a correlates with NK cell cytotoxicity in healthy individuals [261]. These contradicting results, however, can be explained. To our knowledge, no one has examined the relationship between cytotoxicity as measured by a ^{51}Cr release assay and by CD107a expression in HCV infected individuals. CD107a expression may not correlate with target cell lysis in individuals with chronic HCV infections. Another possible explanation involves our experimental design. The ^{51}Cr release assay using fresh PBMC measured NK cell killing through direct target cell lysis. The ICCS protocol using previously frozen and thawed PBMC measured NK cell killing indirectly through the appearance of

CD107a on the NK cell surface. The freezing process of PBMC has been shown to reduce NK cell cytotoxicity measured through a ^{51}Cr release assay [262]. Furthermore, the same group also demonstrated that incubation of thawed cells with culture medium increased the cytotoxic potential unevenly. Thus we believe that the CD107a expression in our ICCS assay is measuring cytokine secretion and not NK cell killing. Others have shown that CD107a not only correlated with target cell lysis, but also with cytokine secretion [233]. This observation is further strengthened as CD107a expression was suggestive of a positive correlation with NK cell specific IFNγ production, especially within the CD56bright subset (Fig. 18). Taken altogether, our data suggests that viral loads negatively correlate with NK cytotoxicity and positively correlate with IFNγ production.

The correlations seen between NK cell function and viral loads may aid in the pathogenesis of HCV infection. These observations come from recent studies in the lymphocytic choriomeningitis virus (LCMV) model that suggest NK cell cytotoxocity plays a regulatory role in controlling the inflammatory cascade virus-specific CD4 and CD8 T cell responses [263, 264]. LCMV infections in murine hosts are often used to study the immune responses generated during the acute and chronic phases of infection. One study examined the pathogenesis of different doses of LCMV infection in the presence or absence of NK cells [263]. NK cell cytotoxicity was shown to be beneficial to the host after high-dose LCMV infection as they killed activated $CD4^+$ T cells which help to eliminate the $CD8^+$ T cell response. This resulted in CD8 T cell exhaustion, allowing LCMV to persist and the host to survive with minimal immunopathology. With medium-dose LCMV infection where the viral dose does not cause CD8 T cell exhaustion when NK cells eliminate CD4 T cells resulting in chronic infection and widespread immunopathology, NK cell killing is not beneficial. At low-dose LMCV infection, NK cells do not affect viral

persistence of immunopathology as the virus is cleared in the presence of absence of NK cells. Thus, it has been suggested that NK cells function as 'rheostats' for virus-specific T cell responses [263]. However, if NK cell cytotoxicity is inhibited by the HCV virus, then increased viral loads may cause NK cells to not sufficiently kill CD4 T cells. This might result in greater virus-specific CD8 T cell frequencies and function along with greater immunopathology.

It was interesting to see the association between viral load with NK cell killing and IFNγ production (Fig. 16). At low viral loads, NK cells have high killing potential and low cytokine production. In contrast, at high viral loads, NK cells switch to low killing potential and high cytokine production. This "switch" between the two main functions of NK cells does make sense. At low viral loads, NK cells can focus on killing individual infected cells to control the infection. At high viral loads, NK cells would be better off producing IFNγ which has broader anti-viral effects. Although we are unsure as to the cause of this "switch" between the two main functions of NK cells, it is possible that this is due to the balance between activation and inhibitory receptors (covered in Section 1.3.4).

The role of PD-1 expression on NK cells from HCV infected individuals has recently been identified [235]. Although there was no significant correlation between spontaneous PD-1 expression on NK cells with viral loads, further stimulation of NK cells with K562 targets had PD-1 expression that correlated with viral loads (Fig. 14). It may be that further stimulation is required to see the association between PD-1 expression and HCV viral load. Furthermore, higher PD-1 expression on NK cells was observed in patients with high viral loads compared to low viral loads. Others have observed similar trends in PD-1 expression on NK cells within Epstein-Barr virus infections [265]. It is possible that PD-1 on NK cells is a potential regulatory mechanism responsible for NK cell functional abnormalities seen during chronic viral infections.

Mechanistic studies examining the role of PD-1 expression on NK cell function within HCV infected patients have yet to be done. However, a group examining this in patients with post-transplantation lymphoproliferative disorders caused by Epstein-Barr virus have found that in their *in vitro* model, blocking PD-1 with an antibody only partially restored IFNγ production but not CD107a expression [265]. They suggest that cytotoxicity and IFNγ may be differently regulated in their setting. This observation supports our own conclusion that others factors such as the cytokine milieu and engagement of receptors with their corresponding ligands may control the two main functions of NK cells in chronic HCV infections.

4.2.2 NK cell function between high and low viral loads

High baseline viral loads have previously been associated with worse therapeutic outcomes in particular for genotype 1 infections [173-175]. These studies define high viral loads as >800,000 IU/ml and low viral loads as ≤800,000 IU/ml. Our study cohort was divided into two groups to assess NK cell function between high and low viral loads. In chronically HCV infected patients, viral loads had limited effect on subject demographics and disease characteristics (Table 3). The HVL cohort had a slightly higher percentage of males and more genotype 1 infections when compared to the LVL cohort. Despite these factors that according to the literature should reduce EVR and SVR rates [173-175], the HVL cohort have higher (but statistically similar) EVR and SVR rates compared to the LVL cohort.

Since NK cell killing negatively associated with viral loads, it is expected that the HVL cohort would have lower NK cytotoxicity compared to the LVL cohort (Fig. 11c). However, it is interesting to note that NK cell from both the high and low viral load cohorts could increase their cytotoxicity upon overnight stimulation with IFNα. This suggests that viral load does not

interfere with the ability of NK cells to respond to stimulation and may help to explain why a form of IFNα remains the backbone of antiviral therapy.

NK cells in patients with persistent HCV infections had higher PD-1 expression when compared to healthy controls suggesting that PD-1 may play a role in suppressing NK function during chronic HCV infection [235]. We observed that CD56dim NK cells had higher spontaneous and K52-stimulated PD-1 expression in HVL patients when compared to LVL patients (Fig. 15). This suggests that NK cells from HVL patients experience more exhaustion compared to LVL patients. This may play a role in the decreased cytotoxicity observed within the HVL cohort (Fig. 11c). The CD56bright NK also had increased PD-1 expression upon K562 stimulation in HVL patients compared to LVL patients. This did not result in a corresponding decrease in IFNγ production (Fig. 17b). Also, spontaneous PD-1 expression within the CD56bright population did not differ between viral load cohorts whereas spontaneous IFNγ production from CD56bright cells was higher from HVL patients compared to LVL patients. These inconsistencies between PD-1 expression and NK cell functions suggest that PD-1 does not affect NK cell function.

4.2.3 Viral load and NK cell function summary

NK cell cytotoxicity negatively correlated with baseline viral loads (Fig. 11). This may reflect the ability of HCV-infected hepatocytes to inhibit NK cell function. This inhibition in NK cell function may also play a role in HCV pathogenesis. In contrast to NK cell cytotoxicity, NK cell-specific IFNγ production positively correlated with baseline viral loads (Fig. 16). This suggests some plasticity within the NK cell response which is dependent upon viral load in an attempt to better control viral replication. Lastly, this difference in response at varying viral loads may be dependent upon receptors founds on the surface of NK cells including the expression of exhaustion marker PD-1.

4.3 NK cell function and liver damage

4.3.1 NK cell function and fibrosis

Current evidence suggests that hepatic fibrosis is driven primarily by hepatic inflammation in response to liver injury. The complex mechanisms that drive chronic liver injury to fibrosis are not fully understood. However, mounting evidence suggests that hepatic stellate cell (HSC) activation during liver injury is vital in the development of liver fibrosis [266-268]. HSC activation is controlled by many different factors including different cytokines, growth factors and immune cells [266, 268, 269]. NK cells can kill activated HSC directly via cytotoxicity or indirectly via IFNγ production [270-272]. Furthermore, three studies from one group suggest that activated HSCs can stimulate NK cell activation which in turn will kill activated HSC to inhibit liver fibrosis [270, 273, 274]. Thus NK cells from individuals with high fibrosis scores may demonstrate greater cytotoxic potential. Our results agree with this hypothesis as NK cytotoxicity measured from our ^{51}Cr release assay was suggestive of a positive correlation with fibrosis (Fig. 22). Other markers of NK killing including CD107a and TRAIL expression did not correlate to fibrosis scores within our study cohort.

In contrast to NK cells, NKT cells can be pro-fibrotic [275, 276] or anti-fibrotic [277, 278]. No relationship was observed between NKT markers of killing and fibrosis scores suggesting that within our cohort, only NK cells, not NKT cells, reflect fibrosis in HCV-infected individuals.

4.3.2 NK cell function and liver enzymes levels

Since HCV has been shown to replicate non-cytopathically [279], it has been suggested that immune responses play an integral role in HCV-related liver damage through destruction of hepatocytes. This was observed in a longitudinal study of chronic HBV infection where

correlations were observed between flares of liver inflammation and fluctuations in IL8, IFNα, and NK cell expression of TRAIL directly *ex vivo* [120]. This same study also shows that NK cells can lyse human hepatocytes which contribute to HBV-related liver damage. In the context of HCV, some studies have reported a relationship between elevated ALT levels and the presence of intrahepatic CD8+ T cells [280, 281], but these associations have not been a consistent finding. We found no correlation between the portions of NK cells with any of the four measured liver enzymes (data not shown). This result agrees with another study that found no significant correlations between the proportion of NK cells and ALT levels [282]. Thus the number of NK cells does not reflect liver damage measured by liver enzymes.

NK cells have been shown to eliminate HCV-infected hepatocytes via perforin/granzyme and TRAIL mediated mechanisms [106, 283]. Bystander killing via perforin/granzyme B and other killing mechanisms may play a role in HCV-mediated liver damage. This observation is supported as within hours after starting therapy for chronic HCV infections when NK cell degranulation and TRAIL production peak, there is a corresponding slight rise in serum ALT levels [253, 284]. When evaluating NK cell functions, no correlations were present between NK cell cytotoxicity and liver enzymes (Table 4). In contrast, TRAIL expression within the total NK and CD56bright populations positively correlated with AST (Fig. 24). The TRAIL pathway of killing is not the predominate mechanism of killing within the chromium release assay thus this difference may not be seen within the assay, or may not play a major role compared to other killing mechanisms. Taken altogether, our data suggests that NK killing potential does not affect liver enzyme levels in the blood. On the other hand, CD107a expression within the CD56bright population correlated with AST (Fig. 23). Similar observations were seen within the total NK, CD56dim and NKT populations but these were not significant. Stimulation with K562 targets

strengthened these observations thus CD107a is suggestive of a positive correlation with serum AST. However, no correlations were present between NK cell specific TNFα and IFNγ production and serum AST. Since CD107a not only correlates with target cell lysis, but also with cytokine secretion [233], it appears that NK cell activity through secretion of mediators other than TNFα and IFNγ reflect the degree of liver damage measured by serum AST within our study cohort. NK cell activity did not correlate with any other measured liver enzyme suggesting that NK cells do not reflect liver damage through measurement of ALT, GGT or bilirubin.

4.3.3 NK cell function and liver damage summary

These results indicate that NK cell killing reflects the degree of fibrosis within our study cohort. In addition, the data suggests that NK cells may reflect some degree of liver damage indicated by elevated serum AST. Both unstimulated CD56dim NK cell and stimulated CD56bright NK cell CD107a and TRAIL expression correlated with serum AST. Thus NK cell functional tests may be used to assess the degree of liver damage in chronically HCV-infected individuals.

4.4 The role of NK cells in treatment outcomes

4.4.1 Baseline NK cell functions cannot predict EVR

Pegylated IFN and ribavirin have been standard of care anti-viral therapy for chronic HCV infection for over a decade. At best, it is effective in 50% of patients infected with genotype 1 infections. Pegylated IFN and ribavirin anti-viral therapy is associated with toxicity, including neuropsychiatric morbidity, cytopenias, rashes and influenza-like symptoms [285-288]. Even with the addition of direct acting antivirals, tolerability remains an issue due to adverse clinical

events. Thus there is much interest in identifying viral and host factors that could predict HCV treatment outcomes.

NK cell cytotoxicity at baseline did not differ between patients who subsequently developed an EVR and those who did not (Fig. 25). In addition, at 3 months of treatment when EVRs are assessed, there were no differences in NK killing between treatment cohorts. As was noted within the previous Caucasian and Aboriginal chapter of the discussion, this finding agrees with previously published results where NK cells become refractory to further in vitro IFNα stimulation after 1 week of pegIFN therapy [253]. Taken altogether, this suggests that NK cytotoxicity does not directly play a role in the development of an EVR.

4.4.2 Baseline NK cell functions cannot SVR

Within our treatment cohort, there were limited differences between subject demographics and disease characteristics (Table 6). Individuals who achieved a SVR had a slightly lower (but not significant) median age compared to those who didn't achieve a SVR. This observation agrees with previously published results that found that younger individuals respond better to current antiviral therapy [152, 153, 172]. The lower prevalence of genotype 1 infections within the SVR+ compared to the SVR- cohort reflects the difficulty in achieving successful outcomes with this viral genotype. All patients who were genotypes 2/3 achieved SVR.

During HCV infection, chronic exposure of NK cells to endogenous IFNα results in increased STAT1 expression and preferential STAT1 phosphorylation over STAT4 phosphorylation [253, 289] resulting in increased cytotoxicity rather than IFNγ production [107, 109]. IFNα can be produced by pDC and Kupffer cells that sense HCV RNA [290, 291]. This connected increase in pSTAT1-dependent cytotoxicity and decrease in pSTAT4-dependent IFNγ production can be seen *in vitro* when NK cells from healthy donors are stimulated with IFNα [109] and is further

enhanced when chronic HCV infected individuals undergo IFNα-based therapies [253, 284]. These events correlate with STAT1 phosphorylation and reduction in HCV titer during the first 48 hours of therapy which mark the first phase of the virological response [253]. In contrast, NK cells from individuals with slow first-phase HCV RNA decline have lower pSTAT1 levels [253]. Treatment responders show greater levels of NK cell degranulation for at least the first 12 weeks of treatment compared to non-responders [253]. Patients who develop a SVR also show enhanced NK cytotoxicity [292].

We did not find any differences between the frequencies of total NK, CD56dim or CD56bright cells between SVR+ and SVR- patients. In contrast, others have found differences prior to the commencement of treatment. Patients who subsequently developed a SVR had increased frequencies of CD56dim NK cells while those who did not had increased frequencies of CD56bright NK cells [292, 293]. However, neither of these groups looked directly at NK cell cytotoxicity. One of these groups observed that NK cells from SVR+ patients displayed a greater perforin content than SVR- patients suggesting that cytotoxicity may be increased within the SVR+ patients [292]. But increased perforin content does not necessarily infer increased cytotoxix potential. Our results that measured cytotoxicity directly through a standard four hour ^{51}Cr release assay, found no differences in NK killing potential between treatment cohorts prior to starting treatment (Fig. 26). CD107a and TRAIL expression supported the above conclusion as they were also similar between treatment outcomes prior to starting treatment (Fig. 27).

TNFα and IFNγ NK cell-specific cytokine production could not predict treatment outcomes within our treatment cohort (Fig. 28). Interestingly, NK cells from SVR+ patients responded to further stimulation to upregulate TNFα production. In contrast, NK cells from SVR- patients

upreguated IFNγ production upon further simulation. This suggests that TNFα, and not IFNγ, is important to develop a SVR on treatment.

4.4.3 The role on NK cells in treatment outcomes summary

Our results suggest that NK cell function does not directly play a role in therapeutic outcomes of chronic HCV infection. However, it is possible that they indirectly participate during antiviral therapy. NK cell killing is refractory to further IFNα stimulation after 3 months of pegylated IFN and ribavirin therapy suggesting that the exogenously administered IFNα does exert a role on NK cells. Strong, multi-specific T cell responses have been shown to clear the virus [56-58], thus it is possible that NK cells aid in the development of this adaptive immune responses. Furthermore, NK cells have been shown to eliminate HCV-infected hepatocytes via perforin/granzyme and TRAIL mediated mechanisms [106, 283]. Thus NK cells may contribute to clearing infected hepatocytes to eliminate viral reservoirs during treatment.

4.5 Conclusion

In conclusion, these findings suggest limited differences in NK cell functions between Caucasian and Aboriginals with chronic HCV infections. Although higher NK cell cytotoxicity was observed in Caucasian patients at 3 months of treatment relative to their Aboriginal counterparts, it does not appear to affect the ability of each ethnic cohort to achieve EVR. Whether these results are due to the interaction of the HCV virus with NK cells to inhibit an apparent natural advantage in Aboriginals, or from a selection bias in our Aboriginal cohort where only individuals who do not possess the advantageous KIR genes develop a chronic HCV infection is currently under investigation within the Rempel lab. These findings also suggest that viral loads affect NK cell function. NK cell cytotoxicity associated with low viral loads while IFNγ production associated with high viral loads. This may play a role in further viral control

along and may help to explain the pathogenesis of chronic HCV infection. In addition, certain NK cell functions may reflect liver damage measured by fibrosis and liver enzymes. Further work is required to evaluated whether or not NK functional assays may be used as biomarkers for liver damage. Lastly, NK cell functions prior to starting treatment for chronic HCV infection could not predict treatment outcomes suggesting that they do not directly play a role in therapeutic outcomes. Further research is necessary to identify specific NK cell phenotypic and functional properties that may play a role in treatment outcomes.

4.6 Considerations and limitations

The biggest limiting factor in this study was the small samples size. It is difficult to interpret data with a limited sample size. In addition, this gives us less power in our statistical analyses and therefore some significance in our results may be missed.

With the development of direct-acting antivirals to better treat genotype 1 infections in combination with pegIFN and ribavirin, it is difficult to interpret these results in the context of evolving treatment options. With these new antivirals, SVR rates are continuing to climb not only in treatment-naïve individuals, but also in treatment-experienced individuals. Further research is required to assess the role of NK cells with this new treatment regime.

Lastly, the NK cells we examined in this study came from peripheral blood. These may not be the most important NK cell in the development of a chronic HCV infection. Several studies have already emphasized differences between intrahepatic and peripheral blood NK cells in the context of HBV and HCV. Intrahepatic NK cells may behave differently compared to peripheral NK cells due to the tolerogenic environment of the liver. However, due to difficulties associated with organ immunology such as limited cell numbers and lack of healthy donors/controls, other alternative solutions to study the role of NK cells in chronic HCV must be sought.

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