ANTAGONIZING A KILLER: HEPATITIS C VIRUS-INFECTED CELLS INHIBIT NATURAL KILLER CELLS

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Antagonizing a Killer: Hepatitis C Virus-Infected Cells Inhibit Natural Killer Cells

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Abstract

Hepatitis C virus (HCV) infects at least 3% of the global population, 80% of those will develop chronic HCV infection, which can lead to liver scarring and hepatocellular carcinoma. Natural killer (NK) cells provide a rapid defense against viral infections, thus, evasion of NK cell functions may be key for the establishment of viral persistence and chronic infection. Here, we show that HCV-infected cells directly interact with, and dampen NK cell cytokine and cytotoxic functions, independent of cytopathic effects exerted by HCV infection of the human hepatoma cells. We observed no change in expression levels of NK cell activating receptors, NKG2D, NKp46 or CD16 on NK cells exposed to HCV-infected cells, nor of human histocompatibility-linked leukocyte antigen (HLA)-E on HCV-infected compared to uninfected cells. However, surface expression of the natural cytotoxicity receptor NKp30 was reduced and infection of Huh-7.5 cells with HCV increased surface binding of an NKp30-IgG_1 Fc_γ fusion protein, suggesting up-regulation of an antagonistic NKp30 ligand on HCV-infected cells.

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Attribution

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Abbreviations

°C	degrees Celsius
%	percentage
~	approximately
‘	prime
α	alpha
aa	amino acid
ADCC	antibody-dependent cell-mediated cytotoxicity
AL-uPA	albumin-urokinase type plasminogen activator transgene
amp	ampicillin
AP	alkaline phosphatase
APC	allophycocyanin
ATCC	American Type Culture Collection
β	beta
BAT3	HLA-B-associated transcript 3
BLCL	B lymphoblastoid cell line
BSA	bovine serum albumin
BTLA	B and T lymphocyte attenuator
BVDV	bovine viral diarrhea virus
C	carboxy
CD	cluster of differentiation
cDNA	complementary DNA
CELISA	cell-based enzyme-linked immunosorbent assay
CLDN1	claudin-1
CPE	cytopathic effect
Cr	Chromium
CTLA-4	cytotoxic T lymphocyte-associated protein 4
Da	Dalton
DAP10	DNAX-activating protein of 10kDa
DAP12	DNAX-activating protein of 12kDa
DAPI	4’,6-diamidino-2-phenylindole
DC	dendritic cell
DISC	death-inducing signaling complex
DENV	dengue virus
dH$_2$O	deionised molecular biology grade water
DMEM	Dulbecco’s modified Eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ds	double-stranded
E	envelope
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EMCV	encephalomyocarditis virus

EGFR	epidermal growth factor receptor
EphA2	ephrin receptor A2
ER	endoplasmic reticulum
ERK	extracellular signal-related kinases
ESCRT	endosomal sorting complex required for transport
E:T	effector to target ratio
EtBr	ethidium bromide
FA	formaldehyde
FasL	Fas ligand
FasR	Fas receptor
FADD	Fas-associated death domain
FCS	fetal calf serum
FDA	Food and Drug Administration
ffu	focus-forming unit(s)
FITC	fluorescein isothiocyanate
γ	gamma
g	gram(s)
g	gravitational force
Gal-9	galectin-9
GTP	guanosine-5'-triphosphate
HA	hemagglutinin
HAV	hepatitis A virus
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCl	hydrochloric acid
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HCVcc (virus)	HCV cell culture-derived virus
HCVpp	HCV pseudoparticle
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHLA2	human endogenous retrovirus long terminal repeat-associating protein 2
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HLA-C1	human leukocyte antigen C group 1
HVR	hypervariable region
IC	isotype control
IF	immunofluorescence
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRES	internal ribosome entry site
ITAM	immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibitory motif

IU	international units
JFH	Japanese fulminant hepatitis
k	kilo
kb	kilobase
KIR	killer immunoglobulin-like receptor
l	litre(s)
LB	lysogeny broth
LBD	ligand binding domain
Lck	lymphocyte-specific protein tyrosine kinase
LD	lipid droplet
LDL	low-density lipoprotein
LEL	large extracellular loop
LVP	lipoviroparticle
M	molar
MCMV	murine cytomegalovirus
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MOI	multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide
N	amino
n	nano
NaCl	sodium chloride
$Na_2{}^{51}CrO_4$	sodium chromate
NCR	natual cytotoxicity receptor
NK	natural killer
NKG	natural killer group
NPC1L1	Niemann-Pick C1-like 1
NS	non-structural
NTPase	RNA-stimulated nucleoside triphosphatase
OCLN	occludin
P	phosphorylation
PBMC(s)	peripheral blood mononuclear cell(s)
PBS	phosphate-buffered saline
PD-1	programmed cell death protein 1
PD-L1	programmed cell death ligand 1
PE	phycoerythrin
peg	pegylated
PerCP	peridinyll-chlorophyll protein
pH	potential of hydrogen
PI	propidium iodide
P/S	penicillin/streptomycin
PtdSer	phosphatidylserine
Rag	recombination-activating gene
RC	replication complex

RIG-I	retinoic-acid-inducible gene I
rIL	recombinant interleukin
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI 1640	Roswell Park Memorial Institute medium 1640
SCID	severe combined immunodeficiency
SEM	standard error of the mean
S.O.C.	super optimal broth with catabolite repression
SRBI	scavenger receptor class B type I
SH2	Src homology 2
ss	single-stranded
SVR	sustained virological response
Syk	spleen tyrosine kinase
TAPA1	target of antiproliferative antibody 1
TCR	T cell receptor
T_h1	T helper 1
TIM-3	T cell immunoglobulin mucin-3
TLR	toll-like receptor
TMD	transmembrane domain
TNF	tumour necrosis factor
TGF-β	transforming growth factor-β
Tris	tris(hydroxymethyl)aminomethane
μ	micro (10^{-6})
UTR	untranslated region
UV	ultraviolet
VLDL	very low density lipoprotein
WNV	West Nile virus
wt	wild-type
ζ	zeta
ZAP70	zeta-chain associated protein kinase of 70 kDa
Z-VAD-FMK	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

Commonly Used Icons

- Activating ligand
- Antibody
- Antigen
- Apoptotic cell
- CD16 receptor
- CD56 receptor
- CD81 receptor
- CD160 receptor
- C-lectin domain
- DAP-10 homodimer
- DAP-12 homodimer
- Endoplasmic reticulum
- Envelope
- FcεRIγ homodimer
- FcεRIγ/ζ chain heterodimer
- Glycosylphosphatidylinositol anchor
- HCV core protein
- HCV envelope 1 protein
- HCV envelope 2 protein
- HCV particle
- Immunoglobulin domain
- Immunoreceptor tyrosine-based activating motif
- Immunoreceptor tyrosine-based inhibitory motif
- KIR3DS1 receptor
- Lipid droplet
- Major histocompatibility complex class I
- Natural killer cell
- NKG2A/CD94 receptor

Major histocompatibility complex class I

Natural killer cell

NKG2A/CD94 receptor

NKG2D receptor

NKp30 receptor

NKp44 receptor

NKp46 receptor

Peptide

Ribosome

Scavenger receptor class B1

Single-stranded RNA

Stressed target cell

Target cell

Tim-3 receptor

Very low density lipoprotein

ζ chain homodimer

One Letter Amino Acid Abbreviations

Amino acid	One letter code
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

1 Introduction

1.1 Host Invasion, Immune Evasion

Human immunity has been classically divided into innate and adaptive responses. The adaptive immune response has been characterized as being solely mediated by B and T cells, as progenitors of these lymphoid cells undergo recombination-activating gene (Rag)-dependent antigen-specific gene rearrangement to produce a diverse repertoire of antigen-specific receptors (1). Innate immunity is comprised of cells and mechanisms that recognize and respond to pathogens in a generic, non-specific manner, providing the first line of defense (2). Viral persistence is dependent upon successful evasion of host immune defenses. This coexistence of viruses and hosts induces evolutionary pressure on the host immune system as well as on viral genes, forcing viruses to develop ways to evade host recognition.

Viral evasion strategies can include the synthesis of decoy proteins that inactivate immune responses, such as innate toll-like receptor (TLR) sensing (2). For example, hepatitis C virus (HCV) proteins, such as the non-structural protein 3 (NS3) can facilitate evasion of the innate immune response through disruption of TLR adaptor molecules (3). Viruses can also interact with and disrupt host humoral immune responses, via antigenic variation, and cellular immune responses and/or effector functions (2). Documented HCV evasion strategies involve diminished type I interferon (IFN) responses (4-9), impaired cytotoxic CD8$^+$ T cell activity and suppression of CD4$^+$ T helper (T_h)1 responses (10).

To escape the adaptive T cell response, viruses may also encode genes to produce proteins, termed immunoevasins, which ultimately prevent the cell surface display of viral peptides in complex with major histocompatibility complex class I (MHC-I) molecules for T cell recognition (2). Although down-regulation of cell surface MHC-I expression upon viral infection will impair T cell recognition, these cells would subsequently become sensitive to natural killer (NK) cell-mediated lysis. To usurp NK cell recognition and function, viruses, such as human cytomegalovirus (HCMV), will encode proteins (i.e. UL14 and UL141) that retain ligands for NK activating receptors inside infected cells (11, 12).

As with HCMV, human immunodeficiency virus (HIV) also down-regulates MHC-I surface expression upon infection, allowing HIV-infected cells to escape T cell recognition yet remain resistant to NK cell cytolysis (13). HIV is thought to accomplish this through selective down-regulation of certain MHC-I complexes while preserving those interacting with inhibitory NK cell receptors (14, 15). The emergence of protective NK cell killer immunoglobulin receptors (KIR):MHC-1 allelic combinations in the context of HIV infection also provides evidence that NK cells play a role in the control of early infection (16, 17). Recently, association studies have revealed HCV-infected individuals have a higher probability of spontaneous clearance with a certain combination of NK cell KIR and human leukocyte antigen (HLA) alleles (discussed in Section 1.4) (18-22). Thus, observations from multiple studies of NK cells in the context of viral infections indicate that viruses can evade immune pressure by generating variants that modulate recognition of infected cells by NK cells. Here, we specifically consider the effects HCV-infected cells have upon NK cells.

1.2 Hepatitis C Virus

1.2.1 Hepatitis C Virus Infection

HCV has been estimated to infect 3% of the world's population (23). In the absence of effective treatment, approximately 80% of individuals exposed to and infected with HCV fail to mount an immune response adequate for viral clearance, develop chronic infection, and suffer an increased risk of liver fibrosis and hepatocellular carcinoma (24-26). Only 20-30% of individuals infected with HCV will spontaneously clear the virus. However, emerging evidence suggests that HCV ribonucleic acid (RNA) persists in serum at levels undetectable by currently used clinical assays (27). The mechanism by which HCV infection is spontaneously cleared is unknown, and a better understanding of both the clearance process and of viral strategies underlying immune escape is necessary for future vaccine development and management of infection.

Currently, there are seven major genotypes of HCV that differ in their nucleotide sequence by 30-35% (28). Treatment efficacy varies with genotype,

genotype 1 being the least responsive to treatment. Prior to 2011, combination treatment for HCV with pegylated interferon-alpha (peg-IFN-α) and ribavirin produced a sustained virological response (SVR) in 40-60% of genotype 1 patients (reviewed in [29]). SVR can be improved to 60-80% for individuals infected with genotype 1 HCV with introduction of one of the recently Food and Drug Administration (FDA)-approved HCV NS3/4A protease inhibitors, telaprevir or boceprevir (30). Although these new treatments are promising, methods to limit the emergence of drug-resistant quasispecies and/or a prophylactic vaccine to prevent HCV infection are needed.

1.2.2 Hepatitis C Virus Experimental Systems

A non-A, non-B hepatitis virus was first identified and its cDNA cloned in 1989 (31), however, efficient culture of HCV *in vitro* remained elusive. In 1997, Rice's lab discovered that the original HCV clone lacked the 3'-untranslated region (UTR) necessary for replication in cell culture, and subsequently produced a full-length functional cDNA clone (32, 33). The study of HCV replication in cell culture was made possible by Bartenschlager's group in 1999 following their development of the bicistronic HCV replicon system containing a neomycin resistance gene. Neomycin is expressed under the control of the HCV internal ribosome entry site (IRES), whereas the non-structural NS3-NS5B proteins are under the control of the encephalomyocarditis virus (EMCV) promoter, thereby allowing maintenance of HCV RNA following electroporation of the replicon into the human hepatoma, Huh-7, cell line (34). The Huh-7 cell line was originally established from hepatoma tissue removed from a 57-year old Japanese male with hepatocellular carcinoma (35).

Progress in understanding the HCV life cycle and developing therapeutic interventions against this pandemic were also hampered by the lack of a small animal model. In 2001, Mercer *et al.* succeeded in developing chimeric human/murine livers by transplanting human hepatocytes into severe combined immunodeficient (SCID) mice harbouring an albumin-urokinase type plasminogen activator transgene (AL-uPA) (36), a gene that encodes a serine protease involved in cell migration and tissue remodeling (37, 38). Targeting the liver with the AL-uPA transgene causes cytopathic effects in hepatocytes expressing AL-uPA thereby allowing hepatocytes that do not express the transgene to repopulate the liver (39, 40). These transgenic mice develop HCV infection with high viral loads when inoculated with sera from HCV-infected individuals.

Although this was the first small animal model for the *in vivo* study of HCV, it was not adequate for the study of innate or adaptive immune responses or pathogenesis. Prior to the development of the SCID/uPA murine model, chimpanzees were, and continue to be used for the *in vivo* study of HCV vaccine development and some aspects of innate and adaptive immune responses to HCV infection. Although they reproduce many characteristics of HCV infection in humans, such as protective $CD4^+$ and $CD8^+$ T cell responses to HCV infection

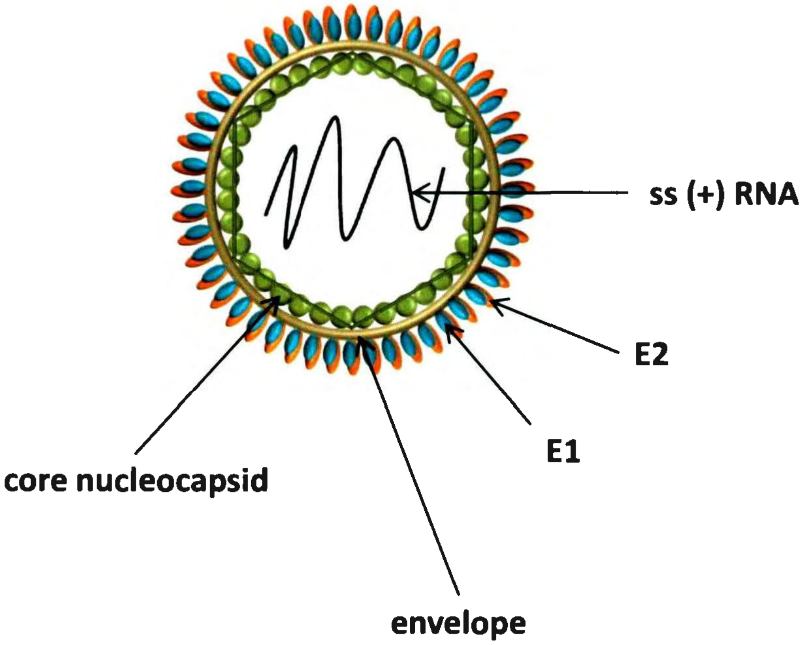
(41, 42), they are an endangered species and their use in medical research is strictly limited.

The lack of both a small animal model to study *in vivo* HCV infection and the inability to reproduce the complete HCV life cycle *in vitro* hampered the examination of viral entry, assembly and general virus-cell interactions. The HCV pseudoparticle (HCVpp) system developed in 2003 by Bartosch and Cosset allowed the study of viral glycoprotein functions during entry (43). In the HCVpp system, recombinant retroviruses containing unmodified HCV glycoproteins are produced following co-transfection of plasmids encoding HCV E1/E2 proteins, retroviral Gag-Pol proteins, a packaging component and a retrovirus-derived reporter gene to detect infection into human 293T cells (43). It was not until 2001, 12 years after the discovery of HCV, that Kato and Wakita isolated an HCV genotype 2a clone from a Japanese patient with fulminant hepatitis (Japanese fulminant hepatitis 1, JFH-1) (44). This clone was capable of *in vitro* replication (45) in the permissive human hepatoma cell line, Huh-7.5, a subclone of Huh-7 cells (46-48). More importantly, JFH-1 permitted the study of the complete HCV life cycle in cell culture for the first time, since genomes transfected into Huh-7.5 cells transcribed and replicated the viral genome, assembled virions and released infectious HCV particles, termed HCVcc (cell culture-derived HCV) (46-49). Furthermore, HCVcc particles are capable of infecting HCV-susceptible animal models (47, 49).

1.2.3 Hepatitis C Virus Structure and Protein Functions

HCV is an enveloped, positive-sense RNA virus of the *Hepacivirus* genus within the *Flaviviridae* family (3). The uncapped 9.6 kb RNA genome includes 5'- and 3'-UTRs with the 5'-UTR being necessary for effective RNA replication and containing an IRES to mediate cap-independent translation (50, 51). The 5'-UTR is conserved among genotypes and is believed to be integral to the replication, stability and packaging of HCV RNA into intact particles (51).

The HCV genomic RNA is translated into a polyprotein by the host ribosomal machinery and cleaved co- or post-translationally by host and viral proteases to form structural proteins, which comprise the virus particle (core, envelope glycoproteins E1 and E2), p7, and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) which aid in either virion assembly and/or viral replication (3, 46). Viral replication occurs within replication complexes (RCs), which are formed from altered endoplasmic reticulum (ER) membranes and contain viral and cellular proteins essential for viral replication (52). The HCV core multimers form the nucleocapsid in which the HCV RNA genome is encased. The nucleocapsid is surrounded by an envelope derived from ER membrane in which E1 and E2 are embedded (53) (Figure 1.1).


ss (+) RNA
E2
E1
core nucleocapsid
envelope

1.2.3.1 HCV Structural Proteins

The structural proteins comprise the physical structure of the virus particle. This section will briefly discuss the known functions of each protein.

1.2.3.1.1 HCV Core Protein

Immature core protein stems from the N-terminus of the HCV genome and is cleaved by signal peptidase, and then further cleaved by signal peptide peptidase to yield a mature core protein of 21 kilodalton (kDa) (54). Mature core protein consists of two domains: a highly basic N-terminal domain (D1) that associates with HCV RNA, and a hydrophobic C-terminal domain (D2), which mediates an association between core and lipid droplets (LDs) (55). This core-LD association is necessary for infectious HCV production, since abrogation of this interaction inhibits virus production (56, 57). However, it is currently unclear whether capsid oligomerization and assembly occur on ER membranes or the LD surface (55, 57, 58).

1.2.3.1.2 HCV E1 and E2 Glycoproteins

Infectious HCV particles possess E1 (~35 kDa) and E2 (~70 kDa) glycoproteins embedded in a lipid bilayer surrounding the core nucleocapsid (59). HCV envelope proteins are required for viral entry into host cells via interaction between E1/E2 and several host cell receptors (43). The N-linked glycosylated envelope glycoproteins are type I transmembrane proteins that form non-covalent heterodimers on the virus particle (60). The N-terminus consists of the entry determinants for viral infection of host cells whereas the C-terminus is responsible for the heterodimerization of the glycoproteins and contains ER-retention signals that may anchor the glycoproteins in the envelope (61). A major neutralizing epitope has been identified as the hypervariable region (HVR) 1 located at the N-terminus of E2 (62, 63). HCV entry is mediated through interaction with host scavenger receptor class B type I (SRBI) and domains I and III of CD81 (also termed target of antiproliferative antibody 1 [TAPA1]) (64, 65). Although it is thought that HCV E1 and E2 form heterodimers on the virus surface, the glycoproteins could undergo a pH-dependent conformational rearrangement to facilitate a fusogenic trimeric form as occurs with other *Flaviviridae* (i.e. dengue virus [DENV] or West Nile virus [WNV]) (61, 66, 67). This trimeric conformation would expose the fusion tip, of which E1 is thought to be involved, enabling delivery of the HCV RNA genome into the cytoplasm of the newly-infected cell (3, 68, 69).

1.2.3.1.3 HCV p7 Polypeptide

The ~7 kDa p7 polypeptide is found at the junction between the structural and non-structural proteins and is cleaved by host signal peptidase (3). Although the precise structure and function of p7 have yet to be unequivocally determined, it is essential for HCV infectivity and may form homopolymeric complexes with ion channel capabilities (70), similar to influenza virus M2 protein (71). In addition, p7 may also contribute to viral assembly (72, 73) through interaction with other HCV proteins such as E1 and NS2.

1.2.3.2 HCV Non-Structural (NS) Proteins

The non-structural proteins are critical for viral replication and assembly but are not physical components of the virus particle. This section will briefly discuss the known functions of each protein.

1.2.3.2.1 NS2

Dimeric NS2 (~23 kDa monomer) is a transmembrane protein with C-terminal cysteine protease activity responsible for self-cleavage of the protein from the adjacent NS3 (74, 75). NS2 is essential for virus assembly, though the mechanism by which NS2 acts during this process remains to be determined (76-78).

1.2.3.2.2 NS3 and NS4A

Like NS2, the ~70 kDa protein NS3 also catalyzes polyprotein processing. The N-terminal serine protease domain heterodimerizes with NS4A (16 kDa) to direct NS3 to the ER where, via serine protease activity, it cleaves the polyprotein boundaries between NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B (3). The C-terminal domain has RNA helicase and RNA-stimulated nucleoside triphosphatase (NTPase) activity (79), which contributes to RNA unwinding and also viral replication and assembly (80). NS4A co-localizes with RCs, supporting its professed role in viral replication (81). Jones *et al.* demonstrated an interaction between core and NS3 proteins that is essential for virus assembly (82). NS3 has also been shown to usurp host innate immune responses by cleaving important adaptor molecules (Section 1.1).

1.2.3.2.3 NS4B

One of the lesser characterized HCV proteins is the highly hydrophobic NS4B (27 kDa), but it is believed to be an integral oligomeric membrane protein (83). Evidence suggests that NS4B induces ER membrane convolutions, commonly referred to as the 'membranous web', forming RCs in concert with other viral and host proteins in order to support HCV RNA replication (52, 84). NS4B may contribute to *Flaviviridae* cytopathicity, however, the studies suggesting this were performed in the context of bovine viral diarrhea virus (BVDV) and remain to be confirmed in the context of HCV infection (85).

1.2.3.2.4 NS5A

The NS5A protein has varied interactions with other viral and cellular proteins involved in viral replication and assembly, has no known enzymatic activity, and is found at 56 kDa when hypophosphorylated or 58 kDa when hyperphosphorylated (3). The NS5A protein is a dimeric zinc-binding metalloprotein comprised of three domains (D) (86, 87), with DI being important for cellular membrane attachment and containing the helix that interacts to and binds core at LD surfaces (88, 89). It is proposed that DI and DII contain RNA-binding residues that aid both RNA replication (90-93) and the switch between viral translation and replication (94). Most recently, DIII has also been implicated as having low *in vitro* RNA binding capacity (95). The interaction between NS5A DIII and core protein has also been shown to be essential for viral assembly (96). In addition, the degree of NS5A phosphorylation is thought to balance viral RNA replication, as decreasing the hyperphosphorylation of NS5A tends to increase RNA replication (90, 92).

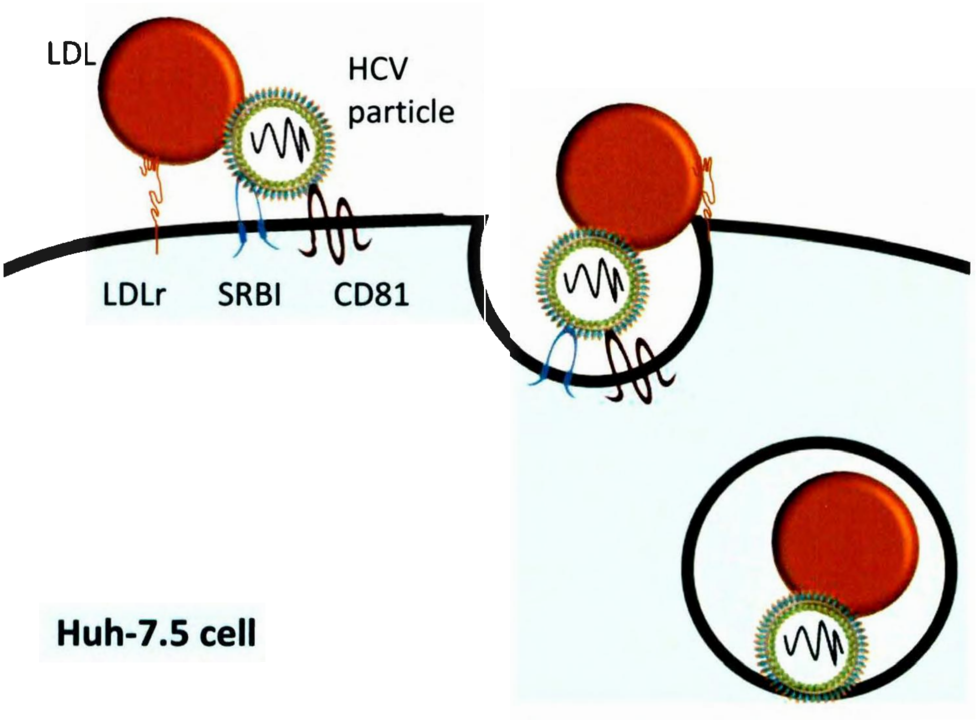
1.2.3.2.5 NS5B

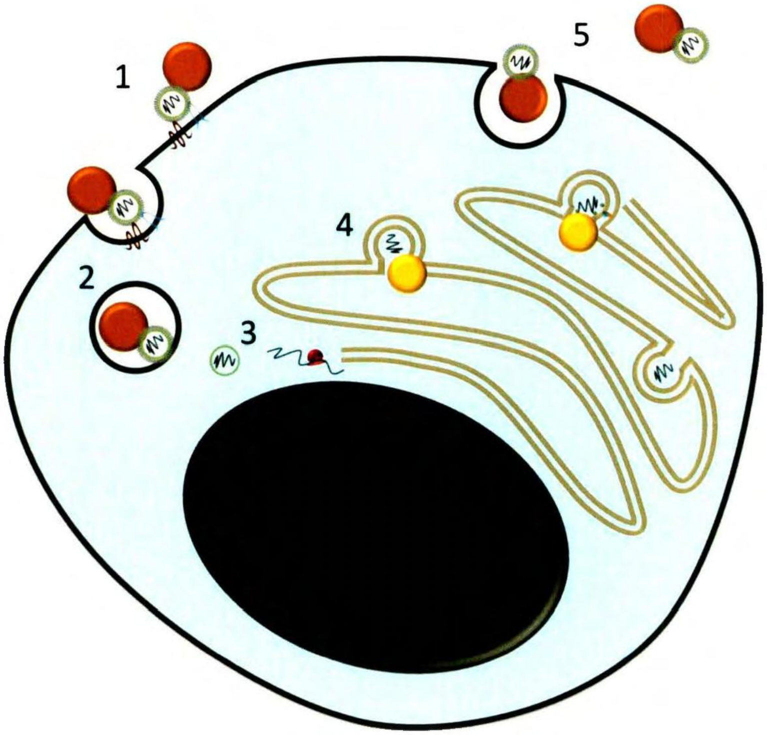
Viral RNA replication is mediated through the catalytic subunit of the 68 kDa NS5B protein, the RNA-dependent RNA polymerase (3). In comparison with other polymerases, HCV NS5B has the classical right hand structure complete with finger, palm and thumb domains with the active site between the finger and thumb domains (97-99). Palm domain catalytic residues can align the RNA template strand and nucleotide triphosphates for transcription (100).

1.2.4 Hepatitis C Virus Life Cycle

Entry of HCV into permissive hepatocytes requires multiple host co-receptors including CD81 (43, 101-104), SRBI (105-107), occludin (OCLN) (108-110) and claudin-1 (CLDN1) (111, 112). The entry process, including cell binding, clathrin-mediated endocytosis and low pH endosomal fusion, is mediated primarily by HCV E1 and E2, which interact with conserved residues within both host SRBI (107) and the large extracellular loop (LEL) of tetraspanin CD81 (113-116) (Figure 1.2). Viral association with high density lipoproteins will enhance viral entry, providing there is adequate SRBI expression on hepatocytes (43, 117, 118). High levels of SRBI receptors occur on hepatocytes and are important for the uptake of cholesterol from lipoproteins. The low density lipoprotein receptor (LDLr) also may be involved in viral entry through interaction with lipoprotein particles (3). Most recently, epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2) and the Niemann-Pick C1-like 1 (NPC1L1) cholesterol uptake receptor have been identified as host cofactors for HCV entry (119, 120). In addition to CD81 and SRBI, CLDN1 expression and binding to HCV E2 is essential for viral entry into permissive hepatocytes (112). Moreover, Harris *et al.* have demonstrated lateral CD81:CLDN1 complexes are important for viral entry (121, 122).

Post-attachment, it is believed that HCV endocytosis occurs in a clathrin-dependent process with successful entry and subsequent fusion dependent on adequate acidification of the endosome to trigger pH-dependent fusion (123) (Figure 1.3). The open reading frame of the positive sense, uncapped HCV RNA genome is translated via the 5'-IRES by host machinery, and co- and post-translationally modified by viral and host proteases to yield the 10 mature viral


LDL
HCV particle
LDLr
SRBI
CD81
Huh-7.5 cell



proteins to enable negative RNA strand synthesis (3). Both host and mature HCV proteins localize to ER membranes to comprise RCs that replicate positive sense genomic HCV RNA into the negative strand template. Subsequently, positive sense HCV genomes are produced, translated into viral proteins and assembled into mature infectious virus particles (3).

Although the precise mechanism by which HCV assembly occurs is unknown, it is thought that viral RNA and core protein accumulate on host LDs, possibly via a core:NS5A interaction (124). HCV E1 and E2 heterodimerize and migrate, along with p7, to the assembly site, possibly through NS2 interactions (125, 126). Viral capsids form and bud into the ER lumen in a manner that incorporates apolipoprotein E (55, 127) then mature into infectious particles.

The very low density lipoprotein (VLDL) assembly pathway is thought to provide a means for viral maturation and egress, however, as with virus assembly, viral egress is also enigmatic. Data suggest that the release of infectious HCV particles relies on components of the endosomal sorting complex required for transport (ESCRT) pathway (128). Circulating mature virus particles released into the extracellular environment in HCV-infected individuals are complexed with VLDL (129, 130). This process and the ensuing host immune response are characteristic of liver disease.

The progression of liver disease in those chronically infected with HCV is usually a process involving inflammation and deposition of collagen, which leads to hepatocellular fibrosis and, in some instances, cirrhosis. Although HCV is not directly cytopathic, it can trigger host immune responses, such as cytokine production and hepatocyte apoptosis (reviewed in [131, 132]).

1.2.5 Hepatitis C Virus-Associated Cytopathic Effects

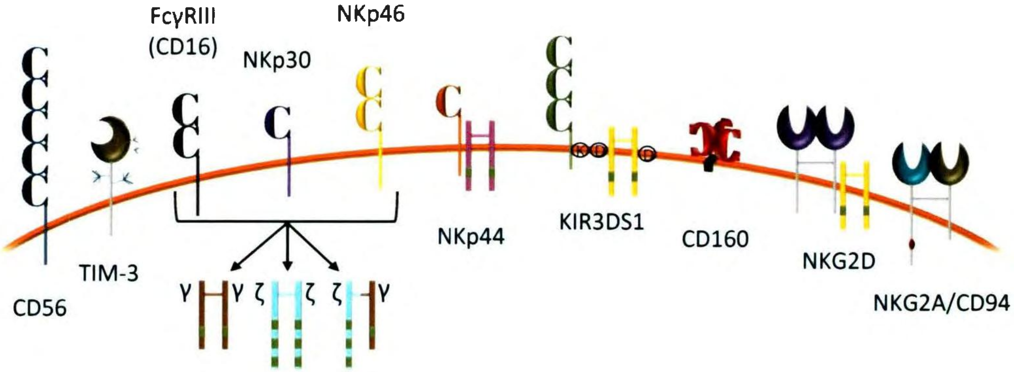
In mammalian cells, flaviviruses cause cytopathic effects (CPEs) via apoptotic mechanisms. For example, DENV serotype 2 (DENV-2) and WNV NS3 proteins (133-135), as well as overexpression of HCV NS4A and/or NS4B have been implicated in causing CPEs, the latter likely from ER stress due to the membranous web and RC formation (85, 136, 137). Using a novel plaque assay, Sekine-Osajima *et al.* demonstrated that JFH-1 replication results in large-scale cell death (138). Subsequent mutagenesis studies by Mishima *et al.* on HCV NS5A and NS5B showed that enhanced viral replication positively correlated with increased CPE (138, 139). Although preliminary, these findings may have implications for the development of liver steatosis, cirrhosis or hepatocellular carcinoma.

1.3 Natural Killer Cells

1.3.1 Natural Killer Cell Innate and Adaptive Immune Responses

Comprising 5-20% of peripheral blood lymphocytes, NK cells can be found enriched in liver and uterine tissues (140), and provide inherent, tissue-resident defense against transformed cells and many pathogens, including viruses. Classically defined as cytolytic lymphocytes after their discovery in the 1970's (141, 142), NK cells are categorized as innate immune effector cells as they do not undergo gene rearrangement, thus, recognize and target infected or transformed cells through a diverse assortment of germ-line encoded receptors (143) (Figure 1.4). NK cells have the innate capacity to recognize and kill target cells with down-regulated surface MHC-I molecules, a process that occurs during many viral infections (143). Although originally categorized as innate immune cells, it was recently suggested that NK cells demonstrate immunological memory, a feature predominately associated with adaptive immunity (144).

There are four phases ascribed to cells of the adaptive immune system. In response to an encounter of a naïve T cell with its cognate ligand, the pathogen-specific T cell population proliferates and expands, resulting in effector cells (145, 146). Following a period of effector activity, these activated T cells will undergo apoptosis, a phase termed 'contraction' (147, 148). Stable, long-lived memory T cell populations (145, 146, 149) will remain in both lymphoid and non-lymphoid


FcγRIII
(CD16)
NKp30
NKp46
CD56
TIM-3
γ
γ
ζ
ζ
ζ
γ
NKp44
KIR3DS1
CD160
NKG2D
NKG2A/CD94

tissues (150, 151) to provide defense against previously encountered pathogens. In response to an encounter with their cognate antigen, memory T cells enter the final phase and undergo a robust expansion against antigenic challenge (152).

Although not extensively documented, recent evidence suggests that, like adaptive immune cells, some NK cells can generate memory cells against previously-encountered haptens or pathogens after initial exposure (144). Using the murine cytomegalovirus (MCMV) infection model, Sun *et al.* documented NK cells undergoing clonal-like expansion, persisting in lymphoid and non-lymphoid tissues, and then mediating a more efficacious effector function response upon re-encounter with the same virus, all traits of the adaptive immune response (153, 154). A similar response has been noted in human studies revealing that Hantavirus, HCMV and HIV infections result in increased circulating NKG2C$^+$ NK cell subsets (155-157). The precise mechanisms by which NK cells develop and maintain immunological memory remain to be determined, but it appears to involve selective expansion of NK cells with a particular receptor repertoire in response to antigenic challenge.

1.3.2 Natural Killer Cell Functions

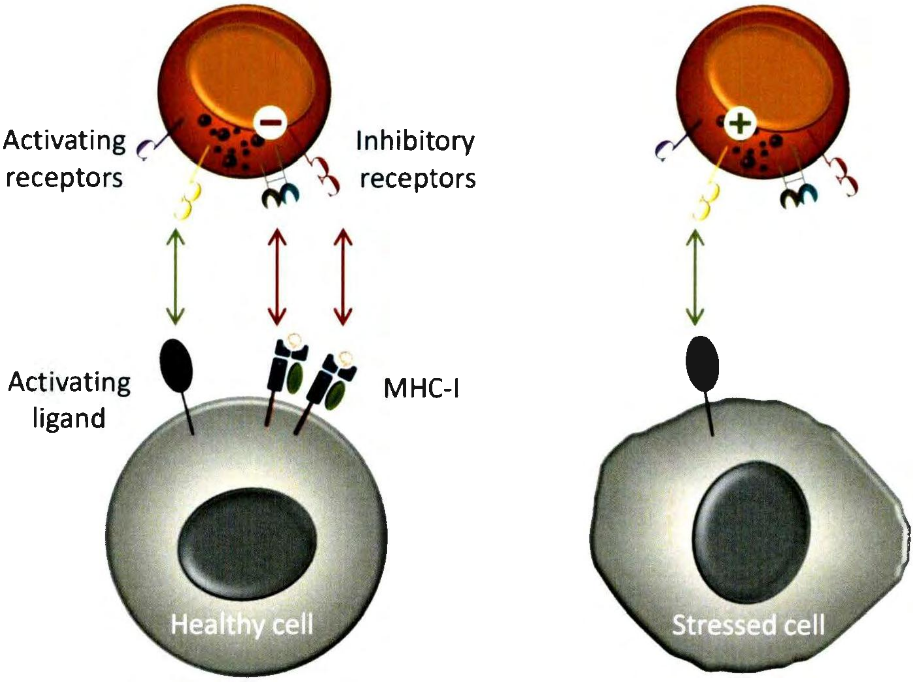
T cell immune responses mediate long-term control of viral infections; however, management of these infections by NK cells prior to the onslaught of the adaptive immune response is crucial. In humans, sensitivity to viral infections is associated with depressed NK cell functions (158). Of particular note, Biron *et* *al.* described the case of an adolescent female patient who had a genetic NK cell deficiency, a complete and specific lack of NK cells, functions, and inducible killer activity, presenting with an extreme sensitivity to herpes virus infections despite normal B and T cell numbers (159). Murine studies have also indicated that NK cell cytotoxicity and IFN-gamma (IFN-γ) production critically contribute to the control of MCMV (160), influenza (161, 162) and herpes simplex-1 (163) infections. Furthermore, not only are NK cells important for the early control of viral infections, but they also play a role in the induction of the adaptive antiviral immune response, through the release of immunomodulatory cytokines and chemokines (164) and through bidirectional interactions with dendritic cells (DC) (reviewed in [165] and [166]). This leads to the induction of type 1 IFNs and subsequent production of T_h1 cytokines driving the T cell response towards the T_h1 adaptive response and ultimately, a reduction in viral replication or clearance of viral infection (167).

NK cells fall into two main functionally distinct subsets. The highly cytotoxic $CD56^{dim}CD16^{bright}$ NK cells are considered more mature, while $CD56^{bright}CD16^{dim}$ immature NK cells predominantly secrete pro-inflammatory cytokines, such as IFN-γ, tumour necrosis factor alpha (TNF-α), and in some cases, the immunoregulatory cytokine interleukin (IL)-10 (13, 168). A recent study showed that $CD56^{dim}$ NK cells also produce large amounts of IFN-γ promptly (2-4 hours) following activation (169). NK cells recognize altered cells and carry out their cytotoxic functions through germ-line encoded receptors. The outcome of engagement of these receptors is determined through the balance of signals and interactions from NK cell inhibitory and activating pathways.

The T cell immunoglobulin mucin-3 (TIM-3) receptor has recently been identified to elicit an inhibitory cytotoxic and cytokine response in NK cells when bound with galectin-9 (Gal-9) or phosphatidylserine (PtdSer) (170-175). Gal-9 is expressed on immune innate and adaptive cells and tissues, such as lymph nodes, thymus, bone marrow and spleen, and is abundant in liver tissues (174, 176). Gal-9 is a soluble protein released from cells that cross-links cell-surface β-galactoside carbohydrate moieties to modulate cell signaling, adhesion, and survival (177, 178). Since Gal-9 recognizes certain carbohydrates, and NK cell receptors can vary in the glycosylation of their ectodomains, the extent of ligand recognition and binding may vary, thereby providing another level of control over NK cell functions (179-181). The phospholipid component of PtdSer is usually expressed on the cytosolic side of cell membranes, however, when a cell is induced to undergo apoptosis, PtdSer becomes expressed on the external surface of the cell membrane and can be recognized by TIM-3 and other receptors (175, 182, 183).

Other inhibitory receptors, such as some KIR receptors or lectin-like NKG2A/CD94 heterodimers predominantly recognize unaltered 'self' cells through their expression of MHC-I complexes, which are expressed on nearly every cell *in vivo*, thereby preventing cytolysis of healthy autologous cells (143). As per the 'missing self' hypothesis, healthy cells expressing optimal amounts of MHC-I complexes are less sensitive to lysis by NK cells than transformed or virus-infected cells with decreased MHC-I expression (184) (Figure 1.5). Tumour and virus-infected cells expressing adequate amounts of MHC-I molecules are actively inhibited from directed cell lysis, and this is one mechanism by which pathogens escape NK cell surveillance (Section 1.1).

Intriguingly though, interaction of certain NK cell inhibitory receptors with MHC-I ligands during NK cell ontogeny influences NK cell responses during ensuing receptor-ligand interaction. This process has been referred to as NK cell 'licensing' and is crucial for NK cell maturation into fully functional licensed effector NK cells (185-187). Thus, the greater number of inhibitory receptors that recognize MHC-I complexes NK cells have, the more responsive these cells will be towards cells lacking MHC-I molecules. The enhancement of NK cell responsiveness following recognition of MHC-I complexes by NK inhibitory receptors indicates the role these inhibitory receptors have is not straightforward. The acquisition of NK cell licensing via interaction of MHC-I complexes with inhibitory receptors requires signaling through associated inhibitory motifs. NK inhibitory receptors can possess intracytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs), distinguished by a conserved cytoplasmic sequence of six amino acids (typically I/L/S/VxYxxL/V, where x represents any amino acid),

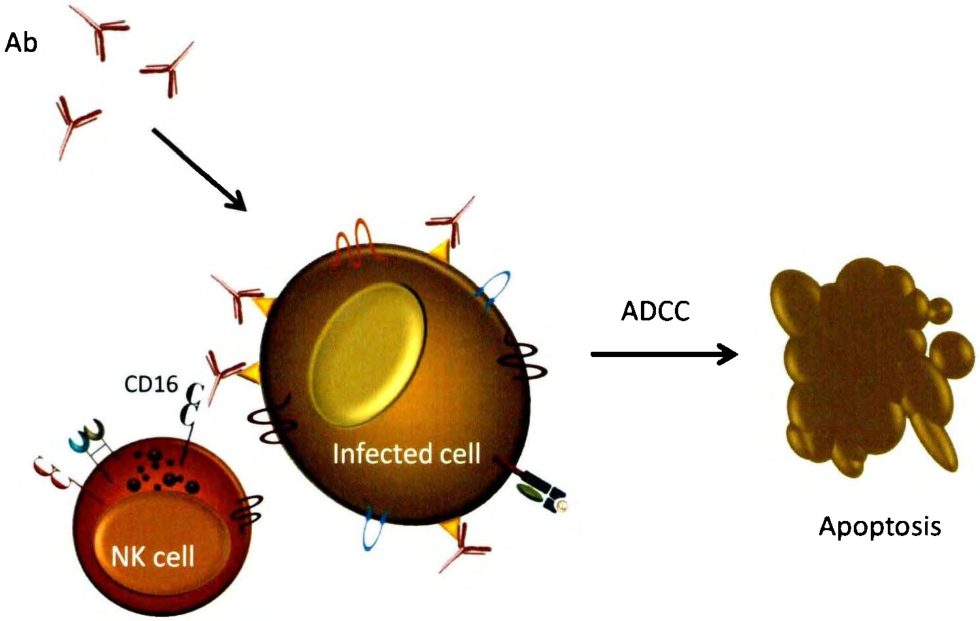

Activating
receptors
Inhibitory
receptors
Activating
ligand
MHC-I
Healthy cell
Stressed cell

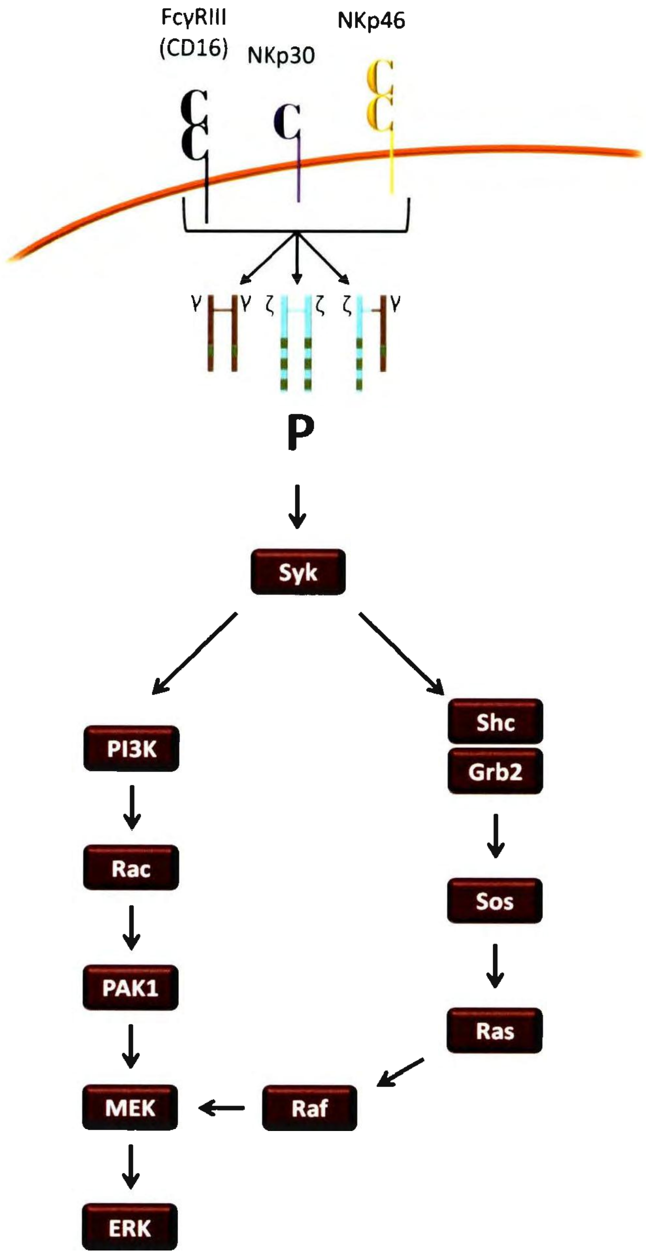
which become phosphorylated at the tyrosine residue, engaging Src homology 2 (SH2) domain-containing protein phosphatases, SHP-1 and SHP-2 (188, 189).

Stress and infection often result in reduced surface MHC-I expression, thereby lessening inhibition and allowing NK cells to lyse altered target cells through stimulation of activating receptors, such as the natural cytotoxicity receptors (NCRs) NKp46, NKp44, NKp30 or NKG2D, and non-classical MHC-I HLA-E-specific NCRs CD94/NKG2C and CD94/NKG2E (143, 190-195). The NCR NKp44 is predominantly expressed on activated NK cells, whereas NKp30 and NKp46 expression appears to be constitutive, with NKp46 being the only specific marker for NK cells (192, 196). A number of ligands have been identified for NK activating and inhibitory receptors, however, many are left to be identified.

For instance, NCR NKp46 is thought to play a role in recognition of *Mycobacterium tuberculosis* (197) and NKp44 is implicated in binding components of bacterial cell walls (198). Although it is believed that NKp46 contributes to the NK cell response against tumour cells (199, 200), tumour ligands for both NKp44 and NKp46 remain elusive. Known ligands for NCR NKp30 include transmembrane protein B7-H6, which is expressed on transformed cells, and nuclear factor HLA-B-associated transcript 3 (BAT3), both of which activate NK cells (201, 202). Other known ligands, such as viral hemagglutinin (HA) and HCMV tegument protein pp65, prevent target cell lysis through NCR NKp30 (203, 204). The best characterized activating NK cell receptor is the low-affinity receptor for immunoglobulin G (IgG), CD16 (FcγRIII), which recognizes antibody-coated cells and is responsible for antibody-dependent cell-mediated cytotoxicity (ADCC) (Figure 1.6) (143).

Although some NK cell activating receptors, such as CD160, associate with membrane-bound proteins (reviewed in [205]), most activating receptors contain adaptor molecules that associate non-covalently with the cytoplasmic receptor domain to initiate signaling cascades. Most adaptor proteins, such as DNAX-activating protein of 10 or 12 kDa (DAP10, DAP12), FcγR, and the ζ chain contain at least one cytoplasmic immunoreceptor tyrosine-based activating motifs (ITAMs). CD16 and the NCRs NKp30 and NKp46 can associate with either FcγR or ζ chain homo- or hetero-dimers to mediate cell signaling, introducing an even greater level of NK cell functional complexity (206) (Figure 1.7).


Ab
ADCC
CD16
Infected cell
NK cell
Apoptosis


FcγRIII
(CD16)
NKp30
NKp46
γ
γ
ζ
ζ
ζ
γ
P
Syk
PI3K
Rac
PAK1
MEK
ERK
Shc
Grb2
Sos
Ras
Raf

1.4 Natural Killer Cells in Hepatitis C Virus Infection

Epidemiological studies suggest NK cells play a role in determining the outcome of HCV infection (19, 207). Some biomedical studies indicate that although NK cell numbers are reduced in chronically infected individuals, NK activating receptor and cytolytic functions increase (18, 208). Conversely, other studies reported decreases in NK cell activating receptor expression, IFN-γ production and cytotoxic activity (140, 209, 210). Nattermann *et al.* showed that *in vitro* activated NK cells derived from HCV RNA-positive patients exhibited decreased cell surface NCR NKp30 and NKp46 expression (210). Mechanistic studies are also inconclusive; some have shown that cross-linking NK CD81 receptors with recombinant HCV E2 protein or immobilized HCV particles directly inhibits NK cytotoxicity and cytokine production (211, 212), while others reported that NK cell functions remained intact when exposed to infectious HCVcc particles (213). In the absence of an immunocompetent small animal model of HCV infection, *in vitro* methods of investigating how NK cells behave in the context of HCV infection are required. As most previous systems used peripheral blood NK cells stimulated *in vitro* with recombinant IL-2 (rIL-2), rIL-12 or IFN-α combined with extended co-culture with HCV-infected cells, the *in vivo* relevance of the reported effects is uncertain (210-212, 214, 215).

Certain inhibitory receptor variants and corresponding ligands may provide protective effects against HCV, resulting in spontaneous clearance of HCV infections. In order to maintain the functional recognition of rapidly evolving antigens in complex with MHC-I, the NK cell KIR receptor genes must also evolve under pathogen-mediated pressure (216, 217). KIR genotyping of individuals exposed to HCV illustrated that if a person expressed genes encoding both NK cell receptor KIR2DL3 and its cognate HLA C group 1 (HLA-C1) ligand, they were more likely to spontaneously resolve infection or make a SVR when treated (19, 20). Interaction of KIR2DL3 and HLA-C1 results in a weaker inhibitory signal than other KIR:ligand interactions allowing a greater functional response from NK cells (21, 22). KIR3DL1 may also be involved in the regulation of chronic HCV infection, as expression of this NK cell receptor decreases in HCV-infected individuals (18).

Both NK cell receptor or ligand expression can impact NK cell functions. Golden-Mason *et al.* demonstrated that circulating levels of the Gal-9 ligand for NK TIM-3 receptors are significantly increased in HCV-positive individuals compared to uninfected controls (218). As this interaction mediates an inhibitory response, this is one way in which HCV can establish and maintain chronic infection. Although there have been many studies examining the suppressive effect TIM-3 has in the context of effector T cell functions and HCV (219, 220), the impact TIM-3 has on NK cells with respect to HCV infection has not been illustrated.

Natural killer cells have an important role in host responses to HCV infection, yet impaired NK cell activity is thought to contribute to the progression of chronic infection. The direct and indirect effects of HCV on NK cell activity is not well understood, therefore, we have investigated this utilizing an *in vitro* experimental system. In the absence of a suitable animal model, the HCV cell culture system allows *in vitro* analysis of the effect HCV has on innate immunity, in particular NK cells. Adaptation of the HCV cell culture system to a microtitre format enables *in vitro* study of the immediate effects HCV-infected human hepatoma (Huh-7.5) cells have on freshly isolated NK cells. We used this platform to explore how HCV-infected cells affect unstimulated *ex vivo* NK cells in short-term assays. Through the assessment of NK cell capability to lyse labeled target cells in the presence and absence of HCV-infected Huh-7.5 cells or titred HCVcc particles. Once we developed this *in vitro* system, we were then able to disturb potential molecular interactions between NK cells and HCV-infected Huh-7.5 cells to gain mechanistic insights on the impact HCV-infected cells have on NK cells.

2 Materials and Methods

2.1 Tissue Culture and Cell Line Maintenance

Human hepatoma Huh-7.5 cells (a kind gift from Charles Rice, Rockefeller University) were maintained in complete Dulbecco's modified Eagle's medium (DMEM) consisting of 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S; all from Invitrogen). At confluency, Huh-7.5 cells were treated 6 minutes with 0.25% trypsin (Invitrogen) and removed from the plate, pelleted by centrifugation for 5 minutes at 290*g,* resuspended in 10 ml of complete DMEM, and either counted and plated for experiments, or re-seeded for maintenance.

Human erythromyeloblastoid leukemia K562 cell line (American Type Culture Collection [ATCC]® #CCL 243™), C1R-B27, an Epstein-Barr virus-transformed human B lymphoblastoid cell line (BLCL) transfected with HLA-B27 (a kind gift from Kelly McDonald, University of Toronto), and murine B lymphocyte hybridoma LS102.9 cell line (ATCC® #HB-97™) were propagated in lymphocyte medium consisting of Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FCS, 200 IU/ml P/S, 1% 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% L-glutamine (all from Invitrogen) and 5.5 x 10^{-5} M 2-mercaptoethanol (Sigma-Aldrich). All cells were cultured at 37°C with 5% CO_2.

2.2 Transformation of Competent Bacteria

$JFH1_T$ (amino acid mutations N417S, N765D, Q1012R, where amino acid numbering begins with the first residue in the complete polyprotein) was obtained from S.U. Emerson and R.H. Purcell (Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, USA) (221). Pre-existing $JFH1_T$ or $JFH1_{D1}$ (amino acid mutations N765D, Q1012R) DNA clones were diluted to a concentration of ~0.1 µg/µl in deionised molecular biology grade water (dH_2O). Aliquots of 40 µl MAX Efficiency® DH5α™ Competent *Escherichia coli* (*E.coli*) cells (Invitrogen) were thawed on ice. An amount of 1 µl diluted DNA was added to a 40 µl aliquot of *E.coli* cells and incubated on ice for 30 minutes. For transformation, competent *E.coli* cells were heat shocked for 45 seconds at 42°C, then incubated on ice for 2 minutes, after which time 150 µl of super optimal broth with catabolite repression (S.O.C.; Invitrogen) was added to each aliquot and incubated for 1 hour at 37°C with constant shaking at 225 rpm to allow expression of the ampicillin (*amp*) gene. After 1 hour incubation, 800 µl of S.O.C. was added and 20 µl and 100 µl plated on lysogeny broth (LB) agar plates inoculated with *amp* (Section 2.4) and incubated at 37°C overnight (16 hours).

2.3 Large-Scale DNA Generation (Maxiprep)

To generate viral plasmid DNA, a transformed *E. coli* bacteria colony (Section 2.2) was used to inoculate 150 ml of LB broth containing 0.1% *amp* (Section 2.5) at 37°C with shaking at 225 rpm overnight. Inoculated LB broth was transferred to a 250 ml nalgene centrifuge tube and pelleted by centrifugation in a Sorvall RC 6 Plus Thermo Scientific centrifuge at 6,000*g* for 15 minutes at 4°C. The DNA pellet was purified using the QIAGEN Plasmid Maxi Kit. To lyse bacteria, the pellet was resuspended in 10 ml buffer P1 (resuspension buffer) followed by adding 10 ml of buffer P2 (lysis buffer). Contents were vigorously mixed by inversion and incubated for 5 minutes at room temperature. To precipitate DNA, 10 ml chilled buffer P3 was added and contents were immediately mixed by inversion and incubated for 20 minutes on ice. Cellular debris was pelleted by centrifugation at 20,000*g* for 10 minutes at 4°C and supernatant was gravity filtered through an equilibrated QIAGEN-tip 500 column then washed two times each with 25 ml of buffer QC (wash buffer) by gravity flow. Purified DNA was eluted with 15 ml of buffer QF (elution buffer), eluate was collected in a 50 ml nalgene centrifuge tube (Thermo Scientific), precipitated with 10.5 ml isopropanol, mixed by inversion, and immediately pelleted by centrifugation at 15,000*g* for 15 minutes at 4°C. Supernatant was carefully decanted and the DNA pellet washed with 5 ml of 70% ethanol and centrifuged at 15,000*g* for 10 minutes, after which time the supernatant was carefully decanted.

The DNA pellet was left to air-dry ~5 minutes, then redissolved in 300 µl dH_2O. DNA concentrations were determined using a Beckman Coulter DU 530 UV/Vis Spectrophotometer.

2.4 LB Broth and Agar

Bacteria growth medium was made by dissolving 20 g of LB broth (10 g tryptone, 5 g NaCl, 5 g yeast extract; Fisher Scientific) into 1 litre dH_2O and sterilizing in an autoclave. Before use, LB broth was treated with 100 µg *amp*/ml broth. LB agar plates were made by dissolving 40 g of LB agar (10 g tryptone, 10 g NaCl, 5 g yeast extract, 15 g agar; Fisher Scientific) into 1 litre dH_2O and sterilizing by autoclave. While warm, LB agar was treated with 100 µg *amp*/ml agar and poured into 10 cm dishes to cool. LB agar/*amp* plates were stored at 4°C until use.

2.5 DNA Linearization

Purified $JFH1_T$ or $JFH1_{D1}$ plasmid DNA was linearized with restriction enzyme *Xba*I (Invitrogen) for RNA synthesis and transfection. 25 µg of plasmid DNA was digested with 75 units (5 µl) *Xba*I, 5 µl 10x buffer M (100 mM Tris-HCl at pH=7.5, 100 mM $MgCl_2$, 10 mM dithiothreitol, 500 mM NaCl), 5 µl 0.1% bovine serum albumin (BSA; all from Invitrogen) in a total reaction volume of 50 µl for 2 hours at 37°C. Linearized DNA was extracted using phenol/chloroform (Section 2.6).

2.6 Phenol/Chloroform Extraction

Linearized DNA (Section 2.5) was treated with phenol/chloroform/isoamyl alcohol, 25:24:1 v/v, (Invitrogen) for purification at a 1:1 ratio. To the linearized DNA, 50 µl of dH_20 was added to make the reaction mixture up to 100 µl, then 100 µl of phenol/chloroform/isoamyl alcohol was combined, vortexed and then centrifuged at 14,000*g* for 3 minutes. 100 µl of the upper aqueous layer (containing nucleic acids) was transferred to a clean microcentrifuge tube and precipitated with 100% ethanol (Section 2.7).

2.7 Ethanol Precipitation

Linearized DNA extracted by phenol/chloroform/isoamyl alcohol was precipitated with NaCl (Sigma-Aldrich) and 100% ethanol. A 5 M solution of NaCl was added to the aqueous layer in Section 2.6 to a final concentration of 250 µM (3 µl), followed by addition of 2.5 volumes of 100% ethanol (230 µl) and stored at -20°C for at least 30 minutes. Following a 10 minute centrifugation at 14,000*g*, the supernatant was decanted and the pellet was washed in 300 µl of 70% ethanol then centrifuged for an additional 5 minutes at 14,000*g*. Supernatant was decanted and the extracted, linearized DNA was resuspended in 30 µl dH_2O. Linearized DNA concentrations were determined as in Section 2.3.

2.8 *In Vitro* RNA Transcription

Linearized viral DNA was *in vitro* transcribed into RNA using the T7 RiboMAX Express large-scale RNA production system (Promega). For every 1 µg of linearized DNA, 5 µl RiboMAX™ Express T7 2x Buffer, 2 µl T7 Express enzyme mix (T7 RNA polymerase, recombinant RNasin® ribonuclease inhibitor, recombinant inorganic pyrophosphatase) and nuclease-free dH_20 were combined in a 10 µl reaction mixture and incubated at 37°C for 30 minutes as per the manufacturer's specifications. The Express T7 2x buffer contains a precipitate that must be dissolved by warming the buffer at 37°C and mixing well prior to use.

2.9 *In Vitro* RNA Transfection

To generate infectious virus stock, Huh-7.5 cells were transfected with linearized genomic RNA derived from $JFH1_T$ or $JFH1_{D1}$ plasmid DNA (Sections 2.5-2.8). Huh-7.5 cells (1.25×10^6) were seeded in 10 cm tissue culture-treated dishes, and then 24 hours later transfected with $JFH1_T$ or $JFH1_{D1}$ RNA using DMRIE-C transfection reagent (Invitrogen) (82). 4 µl of transcribed RNA was combined with 500 µl serum-free (SF) DMEM and 50 µl DMRIE-C, and then added to cells plated with 2 ml SF DMEM. After 4 hours incubation, SF DMEM was replaced with fresh complete DMEM. Seventy-two hours post-infection, supernatants were collected and filtered using MillexHV 45 µm filters (Millipore).

2.10 Infectious HCV Titre Determination

To determine the infectious virus titre of HCVcc in supernatant, 5 x 10^4 Huh-7.5 cells were seeded/well in 8-well chamber slides (Lab-Tek). Twenty-four hours later, infectious supernatant was 10-fold serially diluted in complete DMEM and 100 µl of each dilution added to Huh-7.5 cells in 8-well chamber slides (performed in triplicate). Infectious supernatant was removed after a 4 hour incubation and replaced with complete DMEM. Seventy-two hours post-infection, Huh-7.5 cells were fixed in reagent grade acetone and anti-HCV core protein expression was assessed (Section 2.12.1) to determine focus forming units per ml (ffu/ml) of supernatant by indirect immunofluorescence.

2.11 Generation of Infectious HCVcc Stock

To generate infectious HCVcc stock, 1.25 x 10^6 Huh-7.5 cells seeded in 10 cm culture-treated dishes were subsequently infected for 4 hours with titred HCVcc supernatant (Section 2.9, 2.10) at a multiplicity of infection (MOI) of 0.5. The infectious supernatant was removed and replaced with 6 ml of complete DMEM. Seventy-two hours post-infection, supernatants were removed, filtered and titred as in Sections 2.10, 2.12.1. Transfection supernatants were passaged twice to dilute input RNA from transfection. The final infectious virus titre, in ffu/ml of HCVcc supernatant, was used for all subsequent assays.

2.12 Indirect Immunofluorescence

2.12.1 Determination of Infectious HCV Titre

Huh-7.5 cells, seeded and infected in 8-well chamber slides (Section 2.10), were washed with phosphate-buffered saline (PBS) and fixed for 2 minutes with reagent grade acetone. Slides were incubated with murine monoclonal anti-HCV core (B2; Anogen) at a final concentration of 0.2 µg/µl diluted in 5% BSA/PBS (Sigma-Aldrich) for 20 minutes at room temperature. Chamber slides were washed once for 10 minutes with PBS, then incubated with a 1:1000 dilution of secondary antibody goat anti-murine IgG conjugated with AlexaFluor®488 (Invitrogen) in PBS for 20 minutes at room temperature. Cells washed with PBS were then fixed and mounted using Vectasheild with 4',6-diamidino-2-phenylindole (DAPI) and mounting medium (Vector Labs) (221). Images were visualized at x10, x20 or x40 magnification on a Zeiss Axio Imager.M2 IF microscope.

2.12.2 Intra- and Extracellular HCV E2

For HCV E2 protein visualization, 10^5 Huh-7.5 cells were plated in 2-well chamber slides (Lab-Tek® II Chamber Slide™ Fisher Scientific). Twenty-four hours later, cells were infected with JFH1_{D1} HCVcc at a MOI of 1.25. Seventy-two hours post-infection, cells were washed 3 times with PBS and both unfixed cells or cells fixed for 20 minutes with 2% formaldehyde (FA, paraformaldehyde in PBS; Sigma-Aldrich) were stained with a final concentration of 0.2 µg/µl of anti-HCV E2 (AP33; Genentech) or IgG_1 isotype control (eBioscience) diluted in 5% BSA/PBS for 20 minutes. Cells were washed with PBS, then incubated with a 1:1000 dilution of secondary antibody goat anti-murine IgG conjugated with AlexaFluor®488 in PBS for 20 minutes. Cells were washed with PBS, then fixed and mounted using DAPI with mounting medium. For a positive control, cells fixed with 2% FA were permeabilized with 0.1% Tween® 20 (Sigma-Aldrich) in 5% BSA/PBS for 10 minutes, then stained and washed as above with the exception that diluents and wash consisted of 0.01% Tween® 20 in 0.5% BSA/PBS. Images were visualized at x10 or x40 magnification on a Zeiss Axio Imager.M2 IF microscope.

2.13 Isolation of PBMCs and NK Cell Purification

PBMCs were freshly isolated from whole blood of consenting healthy donors by density gradient centrifugation using Ficoll-Paque lymphocyte isolation solution (VWR) on the day of the assay and suspended at 5 x 10^6 cells/ml in lymphocyte medium. PBMCs were used in all assays unless otherwise stated. For assays using purified NK cells, cells were enriched from isolated PBMCs by negative selection using EasySep™ Human NK Cell Enrichment Cocktail (STEMCELL Technologies), as per the manufacturer's specifications, and re-suspended at 10^6 cells/ml in lymphocyte medium (Section 2.1). This study received ethical approval for the collection of human blood samples from the Health Research Ethics Authority of Newfoundland and Labrador, Canada.

2.14 ^{51}Chromium Release Assays

2.14.1 Natural Cytotoxicity and ADCC ^{51}Chromium Release

Five hour ^{51}Chromium release assays were used to assess the effect of HCV-infected Huh-7.5 cells on NK cell cytotoxicity. Huh-7.5 cells (5×10^3) were seeded/well in a volume of 100 µl in 96-well round bottom tissue culture-treated plates (Falcon) then 24 hours later, infected at various MOIs with titred infectious JFH1$_T$ HCVcc stocks (Sections 2.11 and 2.12.1). Seventy-two hours later, K562 or C1R-B27 cells were labeled for 90 minutes with 100 µCi $Na_2{}^{51}CrO_4$ (^{51}Cr; MP Biomedicals) then washed 4 times with PBS containing 1% FCS. C1R-B27 cells were subsequently incubated for 30 minutes with 2 ml of W6/32 (mouse monoclonal IgG$_{2a}$ anti-human MHC-I) supernatant (ATCC® #HB-95™) to sensitize cells to ADCC (222). PBMC isolated from healthy donors served as effector cells. Immediately prior to the addition of effector and target cells, Huh-7.5 supernatant was removed and replaced with fresh complete DMEM. Huh-7.5 cells were incubated for 5 hours with freshly isolated PBMC and 5×10^3 ^{51}Cr-labeled K562 or C1R-B27 target cells at various effector to target (E:T) ratios in a final reaction volume of 295 µl/well. Supernatant (125 µl) was then removed from each well, transferred to glass Kimble tubes (Fisher) and ^{51}Cr release measured on a Wallac 1480 Wizard gamma counter. Percent specific lysis was calculated by (experimental ^{51}Cr release - spontaneous ^{51}Cr release) / (maximum ^{51}Cr release – spontaneous ^{51}Cr release) X 100. Replicates were performed as indicated in each experiment. Spontaneous ^{51}Cr release was less than 10% or 15% of maximum ^{51}Cr release in all assays using K562 and C1R-B27 as target cells, respectively. Immediately post-assay, the microtitre plate was washed 4 times with PBS to remove non-adherent cells and radioactivity. The remaining adherent Huh-7.5 cells were fixed in acetone as described, and a cell-based enzyme-linked immunosorbent assay (CELISA) performed as in Section 2.15.

2.14.2 Cell-Free ^{51}Chromium Release

To determine whether cell-free virus affected NK cell functions, freshly isolated PBMC and ^{51}Cr-labeled K562 target cells were incubated with or without varying amounts of titred infectious JFH1$_T$ HCVcc stock. An amount of ~50,000 ffu/well were incubated with PBMC and ^{51}Cr-labeled K562 target cells for 5 hours, after which time ^{51}Cr release was measured as in Section 2.14.1.

2.14.3 Inert Huh-7.5 ^{51}Chromium Release

For fixed Huh-7.5 cell ^{51}Cr release assays, Huh-7.5 cells were infected as in Section 2.14.1. On the day of the assay, cells were washed with PBS, fixed for

20 minutes in 2% FA, washed 4 times with PBS, then effector and ^{51}Cr-labeled K562 target cells incubated for 5 hours and ^{51}Cr release measured.

2.14.4 Z-VAD-FMK-Treated Huh-7.5 ^{51}Chromium Release

To block apoptosis, cells were treated with the pan caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK; Tocris Bioscience). Huh-7.5 cells were seeded and infected with JFH1$_T$ HCVcc as indicated and treated with 20 µM Z-VAD-FMK 24 hours prior to cytotoxicity assays (223).

2.14.5 LS102.9 ^{51}Chromium Release

To determine whether apoptotic cells affected NK cell cytotoxicity, LS102.9 cells were untreated or pre-treated for 3 hours with 1 µg hamster anti-murine Fas antibody per 10^6 cells (Jo-2; BD Pharmingen) to induce apoptosis (224). Untreated and anti-Fas-treated LS102.9 cells were incubated in a 5 hour ^{51}Cr release assay with PBMC and ^{51}Cr-labeled K562 cells at an E:T of 60:1. Viability of untreated and anti-Fas-treated LS102.9 cells was measured at the completion of the assay (8 hours post-treatment) by trypan blue staining (Section 2.20).

2.14.6 Trans-Well Co-Culture Assay

To determine whether HCV-infected cells or cell-free HCV affected NK cell cytotoxicity, Huh-7.5 cells were seeded at 7.5 x 10^3 cells/well and infected with

JFH1$_T$ HCVcc (Section 2.14.1) in a HTS Transwell®-96 well receiver microplate (Corning). Seventy-two hours post-infection, ^{51}Cr-labeled K562 target cells and freshly isolated PBMCs were incubated for 5 hours in 3.0 µm membrane inserts placed in receiver plates with uninfected or HCV-infected Huh-7.5 cells and ^{51}Cr release measured.

2.15 Cell-Based Enzyme-Linked Immunosorbent Assay (CELISA)

To measure relative HCV protein levels, CELISA assays were performed. ^{51}Cr release assay plates, containing fixed HCV-infected Huh-7.5 cells (Section 2.14.1), were washed 3 times with PBS containing 1% Tween® 20 (wash buffer; Sigma-Aldrich). Human plasma from an HCV-infected subject (Donor 153) was diluted 1:200 in wash buffer containing 1% FCS (diluent) and 100 µl of diluted serum added to each well of the 96-well plate for 90 minutes at room temperature. Following 4 washes with wash buffer, 100 µl of 1:5,000 diluted alkaline phosphatase (AP)-conjugated goat anti-human IgG (Jackson Laboratories) was added to each well for 60 minutes at room temperature. After 4 washes with wash buffer, 100 µl of substrate (1 p-nitrophenol tablet [Sigma-Aldrich] dissolved in 12 ml of AP substrate buffer [10% diethanolamine {BDH}, 0.02% sodium azide and 0.01% magnesium chloride {both from Sigma-Aldrich} pH=9.8]) was incubated in each well for 30 minutes at room temperature in the dark. Absorbance was read at 405 nm on a Synergy™ HT BioTek microplate reader. Measured absorbance at MOI 0 was considered to be background and subtracted from all values.

2.16 Huh-7.5 Cell Viability Assay

To assess HCV cytopathic effects, Huh-7.5 cells were seeded in 96-well plates and infected with titred JFH1$_T$ HCVcc (Sections 2.11 and 2.12.1) at various MOIs as in Section 2.14.1. Seventy-two hours post-infection, supernatant was removed and cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT; Sigma-Aldrich) diluted 1:10 in complete DMEM for 4 hours. The MTT medium was then removed, and cells were dissolved in dimethyl sulfoxide (DMSO). Absorbance was immediately read at 540 nm on a Synergy™ HT BioTek microplate reader.

2.17 Flow Cytometry

Huh-7.5 cells were seeded at 6.5 x 10^4 cells/well in a 12-well plate and infected with JFH1$_T$ HCVcc (Sections 2.11 and 2.12.1) at a MOI of 3.2 or 4.8. Seventy-two hours later, Huh-7.5 cells were stained for HLA-E expression (1 µg/10^6 cells, clone 3D12; Biolegend) and measured as below, or were incubated with freshly isolated PBMCs with or without K562 cells at an E:T of 5:1 for 1 hour at 37°C, after which 10 ug/ml Brefeldin A (Sigma-Aldrich) was added for an additional 4 hours. After a 5 hour incubation, PBMCs were removed, washed with flow cytometry buffer (0.2% sodium azide, 0.5% FCS, 5 mM EDTA in PBS), and incubated for 20 minutes with 0.4 µg/10^6 cells peridinyll-chlorophyll protein (PerCP)-conjugated murine anti-human CD3 (Biolegend), 1 µg/10^6 cells fluorescein isothiocyanate (FITC)-conjugated murine anti-human CD56 (eBioscience), 1 µg/10^6 cells of phycoerythrin (PE)-conjugated murine anti-human CD107a (Biolegend), anti-NKp30-APC, anti-NKp46-PE, or anti-NKG2D-PE (Biolegend) for NK cell surface markers. Cells were fixed and permeabilized with IntraStain kits (Dako) then incubated 20 minutes with 0.1 µg/10^6 cells allophycocyanin (APC)-conjugated murine anti-human IFN-γ (eBioscience), or 1 µg/10^6 cells APC-conjugated anti-human TNF-α (MAb11; Biolegend) or 1 µg/10^6 cells PE-conjugated anti-human IL-4 (BD Pharmingen™) or 1 µg/10^6 cells PE-conjugated anti-human IL-10 (BD Pharmingen™), washed then fixed with 1% FA.

For fusion protein experiments, uninfected or HCV-infected Huh-7.5 cells were incubated 30 minutes at 4°C with NKp30-IgG_1 Fc_γ chimera or control KIR3DS1-IgG_1 Fc_γ chimera (1 µg/10^6 cells), and stained with APC-conjugated AffiniPure goat anti-human IgG_1 Fc_γ (Jackson Laboratories). Fresh PBMCs were stained with 1 µg primary antibody LEAF™ murine anti-human TIM-3 (F38-2E2; Biolegend), washed then incubated with 0.02 µg secondary antibody FITC-conjugated goat anti-murine IgG Fc (Jackson Laboratories) for analysis of TIM-3 expression. Cells were analyzed on a Becton Dickinson FACS Calibur flow cytometer.

2.18 Propidium Iodide Staining to Detect Huh-7.5 Cell Apoptosis

The efficacy of caspase inhibitor Z-VAD-FMK to prevent apoptosis of Huh-7.5 cells was measured by propidium iodide (PI) binding and subsequent labeling of cellular DNA content, and assessed by flow cytometry. Huh-7.5 cells were seeded at 6.5 x 10^6 cells/well in a 12-well plate and uninfected or Huh-7.5 cells infected with $JFH1_T$ HCV at MOI 4.8 were treated for 24 hours with or without 20 µM Z-VAD-FMK in DMEM. Twenty-four hours post-treatment, Huh-7.5 cells were removed from the 12-well plates using a cell scraper and PBS, transferred to a 15 ml tube and pelleted at 200*g* for 5 minutes. Supernatant was decanted and the pellet re-suspended in 500 µl of PBS. Cells were fixed by adding 4.5 ml of ice-cold 70% ethanol to the re-suspended pellet and stored at -20°C for at least 24 hours (up to several weeks).

Cells were centrifuged at 400*g* for 5 minutes, the ethanol was decanted, and cells were washed with 5 ml of PBS and pelleted for 5 minutes at 400*g*. Cells were then resuspended in 500 µl of PBS and 500 µl of DNA extraction buffer (consisting of 192 ml of 0.2 M Na_2HPO_4 [Sigma-Aldrich] and 8 ml of 0.1% Triton X-100 [Sigma-Aldrich] at pH=7.8). After 5 minutes of incubation at room temperature, cells were pelleted at 400*g* for 5 minutes and the supernatant decanted. Cells were resuspended in 1 ml of freshly-prepared DNA staining solution (200 µg of PI [Sigma-Aldrich] dissolved in 10 ml of PBS and 2 mg of DNase-free RNase [Sigma-Aldrich]), and incubated in the dark at room temperature for at least 30 minutes. Cells were analyzed as described in Section 2.17 by flow cytometry using a 488 nm laser for excitation (225).

2.19 Antibody-Mediated Receptor/Ligand-Masking

To block receptor:ligand interactions, freshly isolated PBMCs were pre-treated for 30 minutes at 4°C with either 1 μg/10^6 cells of isotype control (IC; Ag8, a kind gift from Chris Ford, Memorial University of Newfoundland), murine anti-human CD81 (JS-81; BD Biosciences) or murine anti-human LEAF™ TIM-3 monoclonal antibodies. PBMCs were washed once, resuspended in lymphocyte medium (Section 2.1) then incubated in a ^{51}Cr release assay as in Section 2.14.1 with uninfected or HCV-infected Huh-7.5 (PBMCs pre-treated with anti-TIM-3 or anti-CD81) or LS102.9 (PBMCs pre-treated with anti-TIM-3) cells. Alternatively, 96 hours post-infection with JFH1$_{D1}$ HCVcc at a MOI of 1.3 (Section 2.11), Huh-7.5 cells were pre-incubated for 30 minutes with either 1 μg/well anti-human HCV E2 or IC antibodies diluted in complete DMEM, and then incubated with target and effector cells in a ^{51}Cr release assay as in Section 2.14.1.

2.20 LS102.9 Cell Viability Assay

Trypan blue (0.4% trypan blue in 0.85% NaCl [both Sigma-Aldrich]) staining was used to asses LS102.9 cell viability. Untreated and anti-Fas-treated LS102.9 cells were diluted 1:1 in trypan blue and a live to dead cell ratio was determined by counting cells using a hemocytometer.

2.21 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. Effects were tested using a two-tailed Student's paired *t* test (Gaussian distribution) or a Mann-Whitney *U* test (non-parametric distribution) and are indicated. A $p \leq 0.05$ was considered to be statistically significant.

3 Modulation of Natural Killer Cell Functions by HCV

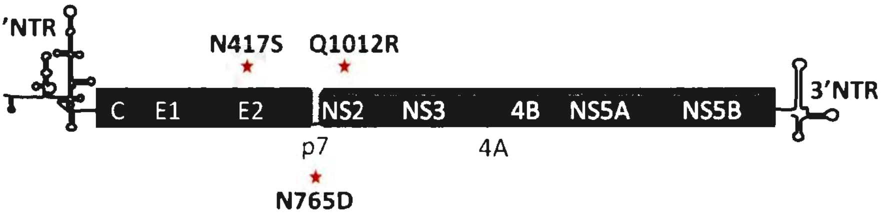
3.1 Rationale

HCV successfully evades the immune system and establishes chronic infection in approximately 80% of cases. As successful immune evasion is key for the persistence of any pathogen, one strategy that HCV employs to establish persistence may involve modulating NK cell functions. Therefore, we developed a short term assay to assess immediate effects of HCV-infected cells on *ex vivo* NK cell cytotoxicity and cytokine production.

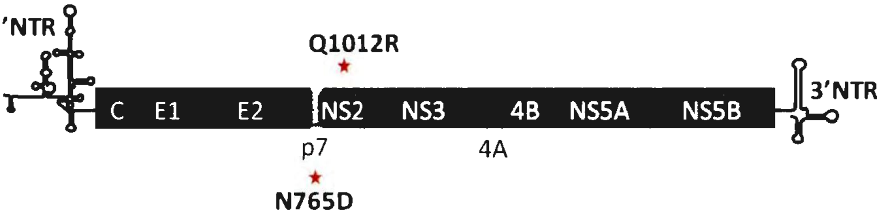
3.2 Development of a Novel Assay Platform to Detect the Effects of HCV on NK Cells

To study the effects HCV-infected Huh-7.5 cells have on NK cell functions, we developed a microtitre assay by infecting Huh-7.5 cells in 96-well microtitre plates with $JFH1_T$ (221) or $JFH1_{D1}$ HCVcc (Figure 3.1). Virus stocks were generated from passaged transfection supernatant and titred to determine the levels of infectious virus titres. Viral titres used were greater than 4×10^5 ffu/ml. Huh-7.5 cells were infected for 72 hours at a MOI of 0, 0.2, 0.4, 0.8, 1.6, 3.2, 4.8 then a CELISA was used to assess the extent of infection. As MOI increased, we observed a 6-fold increase in relative HCV protein levels compared to MOI 0.2, detectable by antibodies present in HCV positive patient plasma (Figure 3.2 A).

$\mathrm{JFH1}_{\mathrm{T}}$


5'NTR
N417S
Q1012R
C
E1
E2
NS2
NS3
4B
NS5A
NS5B
3'NTR
p7
4A
N765D

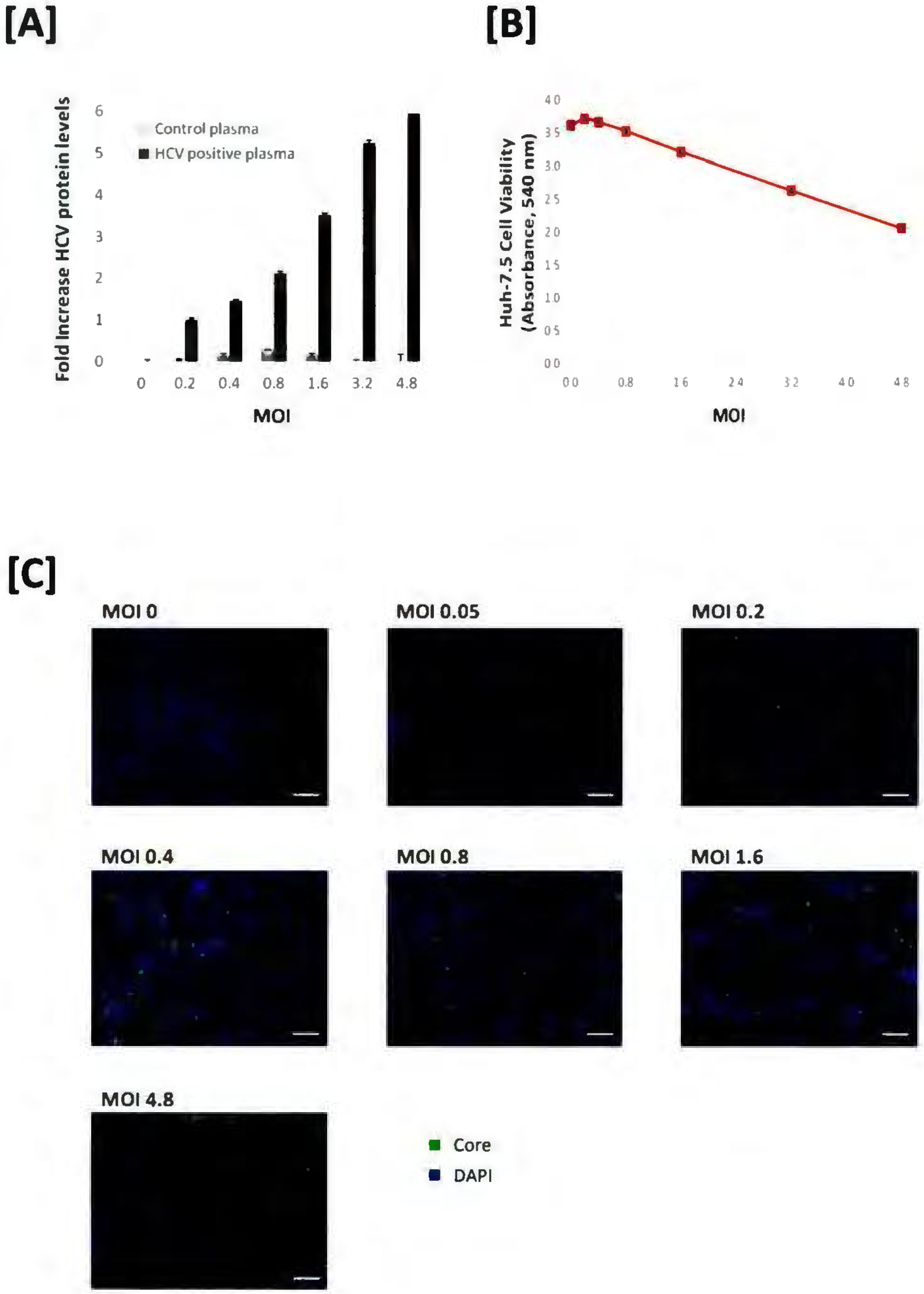
$\mathrm{JFH1}_{\mathrm{D1}}$


5'NTR
Q1012R
C
E1
E2
NS2
NS3
4B
NS5A
NS5B
3'NTR
p7
4A
N765D

MTT assays were performed in parallel with CELISA to assess Huh-7.5 cell viability following infection. As the extent of HCV infection increased, cell viability decreased, falling as much as 40% at high MOIs (Figure 3.2 B). The extent of HCV infection in Huh-7.5 cells was also visualized by IF microscopy with greater degrees of infection noted at higher MOIs compared to the lower MOIs (Figure 3.2 C). As 80-90% of the Huh-7.5 cells stained positive for HCV core proteins, a MOI of 3.2 or 4.8 was chosen for subsequent experiments to optimize virus infection levels in this system.

3.3 HCV-Infected Huh-7.5 Cells Reduce NK Cell Cytotoxicity

NK cell functions in the presence of HCV-infected Huh-7.5 cells were assessed using a classic 5 hour ^{51}Cr release assay. Target cells and freshly isolated PBMCs from 12 consenting healthy donors were incubated with uninfected or JFH1$_T$ HCV-infected Huh-7.5 cells in microtitre format and NK cell cytotoxicity evaluated. The presence of HCV-infected Huh-7.5 cells reduced NK cell cytotoxicity against K562 cells by a mean of 23 ± 2.2% (standard error of the mean [SEM]; $p < 0.0001$, Student's paired *t* test; $n = 12$) (Figure 3.3 A). To determine whether alternate mechanisms of NK cell cytotoxicity were affected by HCV-infected Huh-7.5 cells, we used anti-HLA class I-coated BLCL as targets in an ADCC ^{51}Cr release assay. The presence of HCV-infected Huh-7.5 cells reduced NK ADCC by a mean of 18 ± 6.0% (SEM; $p = 0.001$, Student's paired *t* test; $n = 6$) compared with NK ADCC in the presence of uninfected Huh-7.5 cells

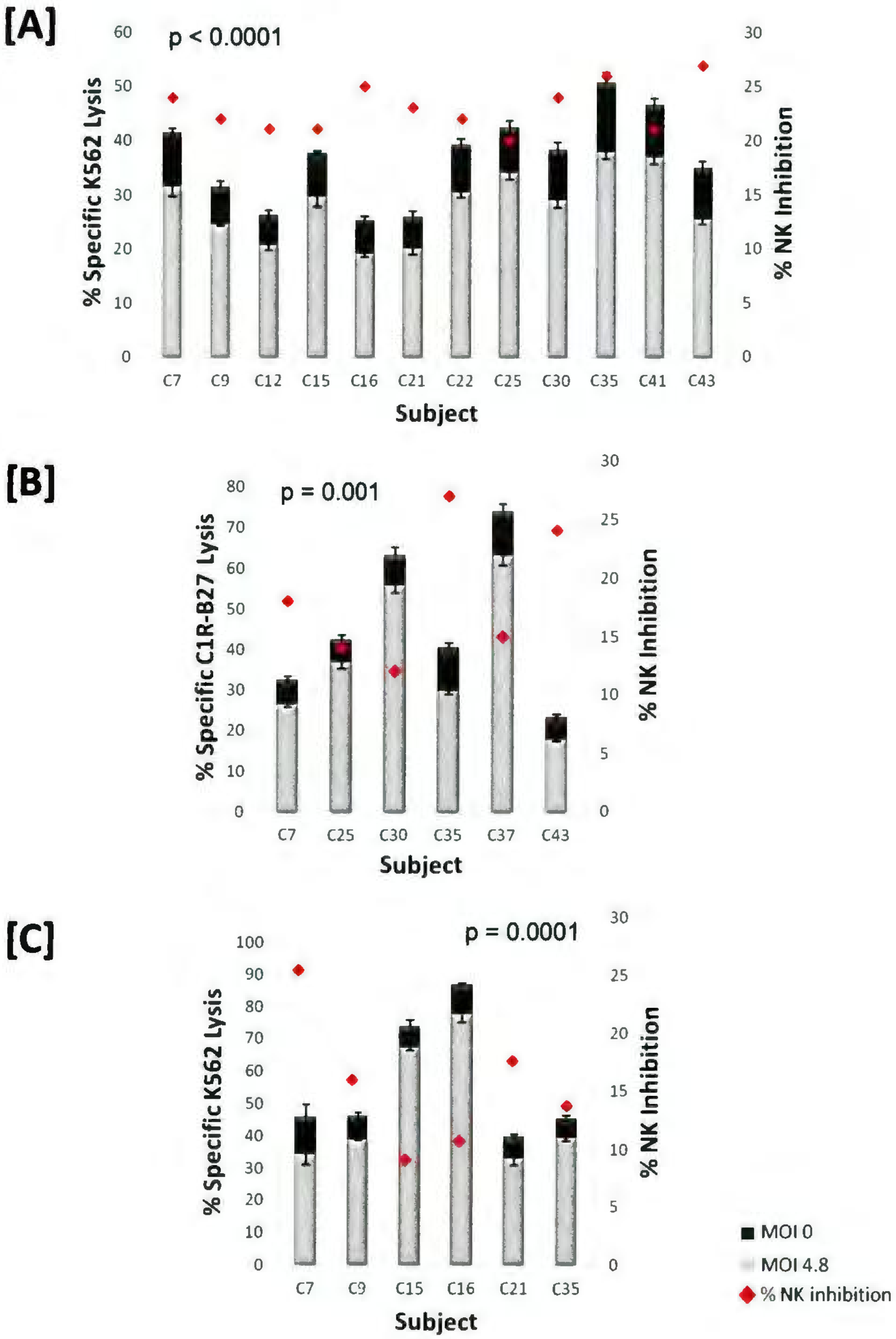


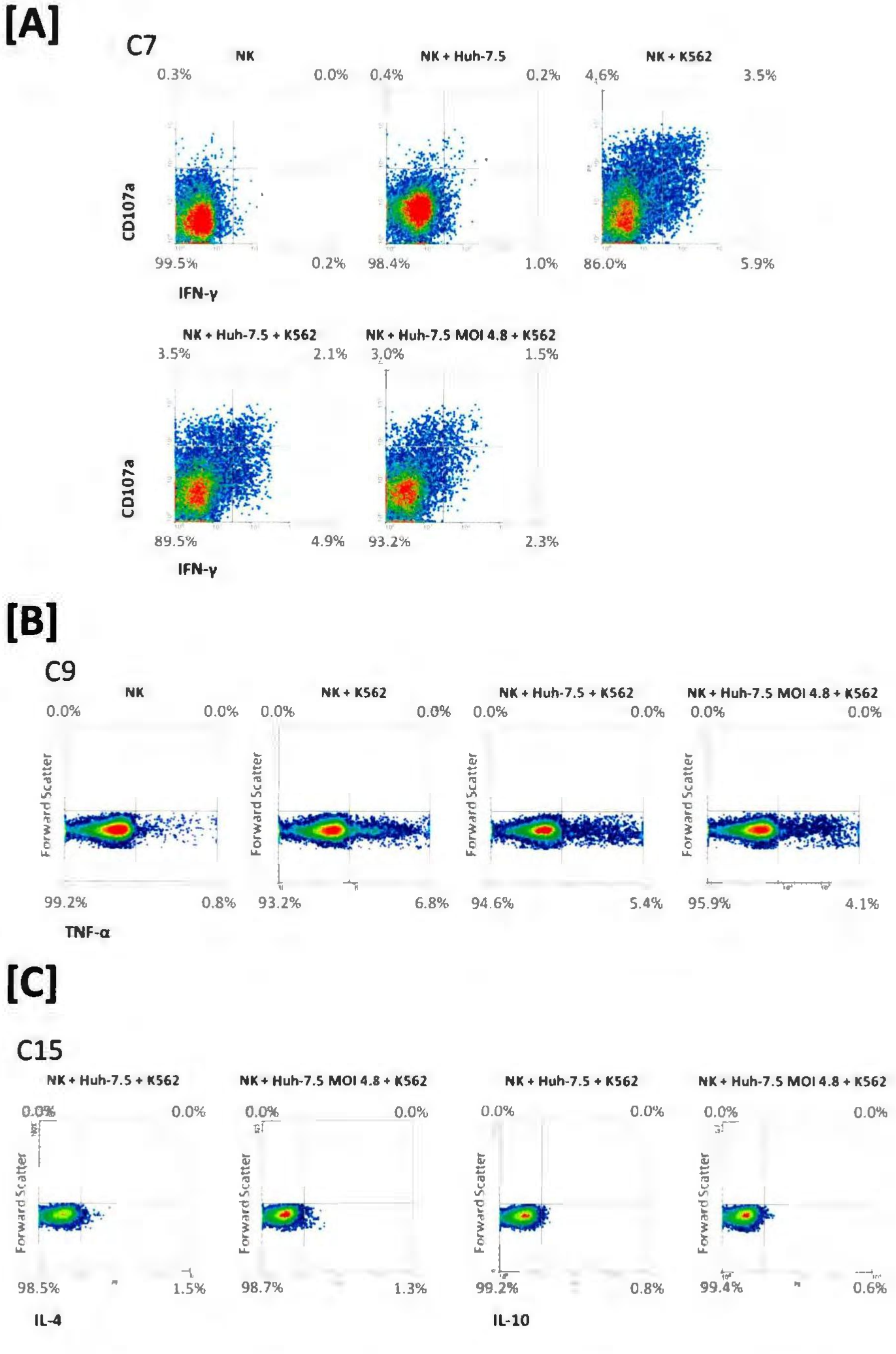
[A]
Fold Increase HCV protein levels
0
1
2
3
4
5
6
Control plasma
HCV positive plasma
0
0.2
0.4
0.8
1.6
3.2
4.8
MOI
[B]
Huh-7.5 Cell Viability (Absorbance, 540 nm)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0.0
0.8
1.6
2.4
3.2
4.0
4.8
MOI
[C]
MOI 0
MOI 0.05
MOI 0.2
MOI 0.4
MOI 0.8
MOI 1.6
MOI 4.8
Core
DAPI

(Figure 3.3 B). Although different donors had varying levels of NK cytotoxicity, the percent decline in cytotoxicity was consistently between 20-27%, and all healthy donors displayed a significant reduction of NK cell cytotoxicity in the presence of HCV-infected cells. To determine if this was a direct effect on NK cells, or mediated through another PBMC subset, we purified NK cells from fresh PBMCs. Purified NK cells (85-95% purity, data not shown) also exhibited 15 ± 2.4% (SEM; $p = 0.0001$, Student's paired *t* test; $n = 6$) lesser cytotoxicity, on average, in the presence of HCV-infected Huh-7.5 cells compared to uninfected Huh-7.5 cells (Figure 3.3 C). Thus, HCV-infected cells have a negative effect on both general NK cell cytotoxicy as well as ADCC functions in this short-term experiment.

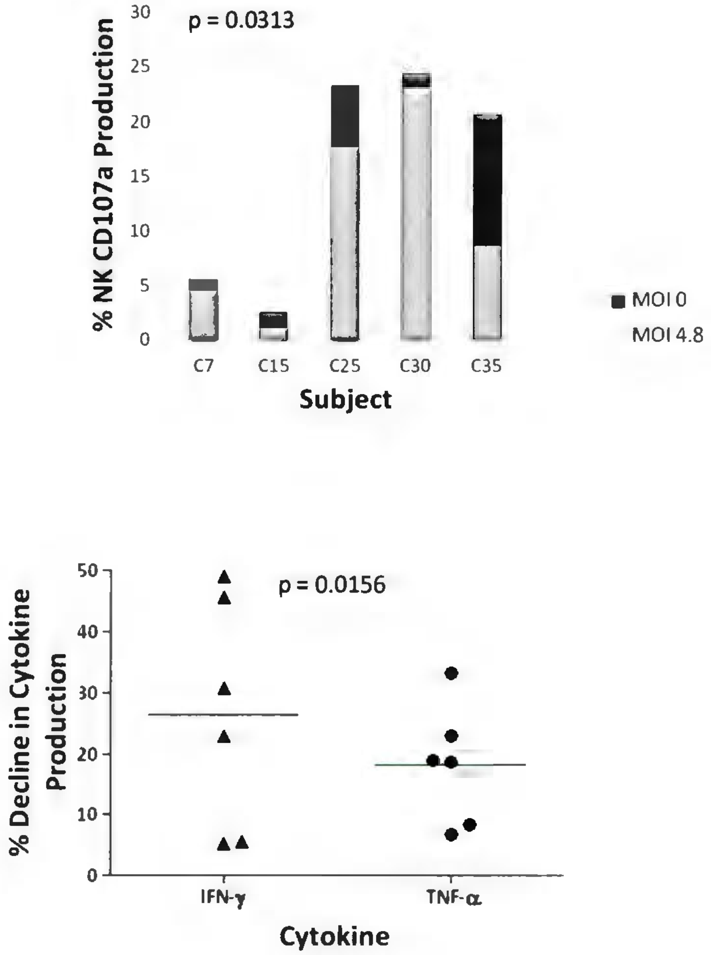
3.4 HCV-Infected Huh-7.5 Cells Reduce NK Cell Degranulation. IFN-γ and TNF-α Production

We next investigated whether HCV-infected cells perturbed NK cell degranulation and cytokine production over a 5 hour incubation period. Flow cytometry of the $CD56^{+}CD3^{-}$ PBMC population revealed reduced NK cell degranulation by a mean of 33% ($p = 0.0313$, one-tailed Wilcoxon signed rank test; $n = 5$), as measured by CD107a expression, in the presence of HCV-infected Huh-7.5 cells compared with uninfected Huh-7.5 cells (Figure 3.4 A). This corroborated the effects observed on NK cell cytotoxicity in the 5 hour K562

[A]
p < 0.0001
% Specific K562 Lysis
% NK Inhibition
C7 C9 C12 C15 C16 C21 C22 C25 C30 C35 C41 C43
Subject
[B]
p = 0.001
% Specific C1R-B27 Lysis
% NK Inhibition
C7 C25 C30 C35 C37 C43
Subject
[C]
p = 0.0001
% Specific K562 Lysis
% NK Inhibition
C7 C9 C15 C16 C21 C35
Subject
MOI 0
MOI 4.8
% NK inhibition


[A]
C7
NK
0.3%
0.0%
99.5%
0.2%
NK + Huh-7.5
0.4%
0.2%
98.4%
1.0%
NK + K562
4.6%
3.5%
86.0%
5.9%
CD107a
IFN-γ
NK + Huh-7.5 + K562
3.5%
2.1%
89.5%
4.9%
NK + Huh-7.5 MOI 4.8 + K562
3.0%
1.5%
93.2%
2.3%
CD107a
IFN-γ
[B]
C9
NK
0.0%
0.0%
99.2%
0.8%
NK + K562
0.0%
0.0%
93.2%
6.8%
NK + Huh-7.5 + K562
0.0%
0.0%
94.6%
5.4%
NK + Huh-7.5 MOI 4.8 + K562
0.0%
0.0%
95.9%
4.1%
Forward Scatter
TNF-α
[C]
C15
NK + Huh-7.5 + K562
0.0%
0.0%
98.5%
1.5%
NK + Huh-7.5 MOI 4.8 + K562
0.0%
0.0%
98.7%
1.3%
NK + Huh-7.5 + K562
0.0%
0.0%
99.2%
0.8%
NK + Huh-7.5 MOI 4.8 + K562
0.0%
0.0%
99.4%
0.6%
Forward Scatter
IL-4
IL-10

[D]


p = 0.0313
% NK CD107a Production
30
25
20
15
10
5
0
C7
C15
C25
C30
C35
Subject
■ MOI 0
MOI 4.8
p = 0.0156
% Decline in Cytokine Production
50
40
30
20
10
0
IFN-γ
TNF-α
Cytokine

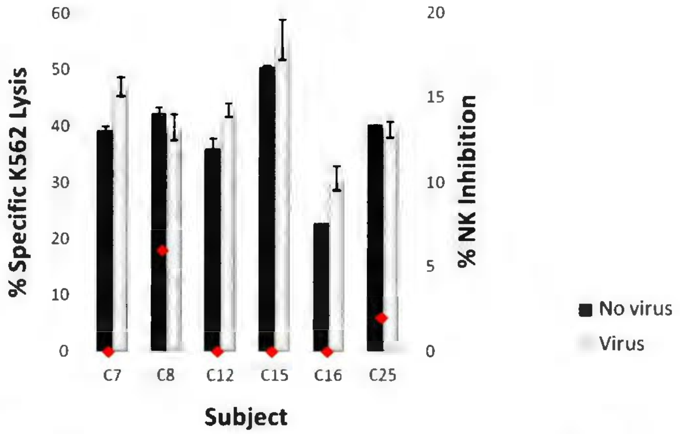
^{51}Cr release assay. Analysis of permeabilized CD56^{+}CD3^{-} PBMCs also indicated that NK cell IFN-γ and TNF-α expression were reduced in the presence of HCV-infected Huh-7.5 cells by a mean of 26% and 18%, respectively ($p = 0.0156$, one-tailed Wilcoxon signed rank test; $n = 6$; Figure 3.4 A,B). We observed no difference in NK IL-4 or IL-10 release in the presence of uninfected versus HCV-infected Huh-7.5 cells (Figure 3.4 C). These data demonstrate a generalized effect that HCV-infected Huh-7.5 cells have on NK cell activation, affecting both cytotoxicity and cytokine production.

3.5 HCV-Infected Cells Inhibit NK Cell Functions by Direct Contact

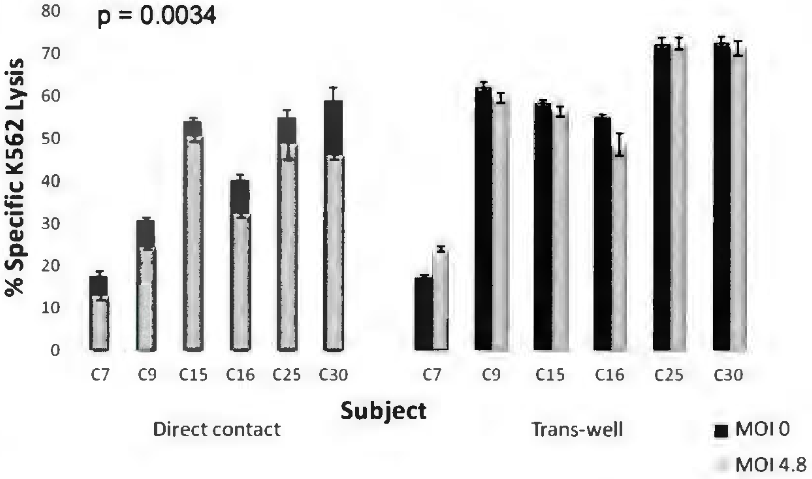
Since infectious virus particles are released from HCV-infected Huh-7.5 cells, we next determined whether cell-free HCV particles, soluble factors released from HCV-infected cells, or the HCV-infected Huh-7.5 cells themselves mediated inhibited NK cell functions. PBMCs were incubated in a 5 hour ^{51}Cr release assay with titred infectious cell-free HCVcc, or complete DMEM as a control. Cell-free virus had no significant effect on NK cell cytotoxicity, as assessed by ^{51}Cr-labeled K562 lysis (Figure 3.5 A).

To evaluate whether either cytokines or other soluble factors were down-regulating NK cell functions, we performed a 5 hour trans-well assay in which both PBMCs and target cells were sequestered from direct contact with uninfected or HCV-infected Huh-7.5 cells. While a 7-27% ($p = 0.0034$, Student's

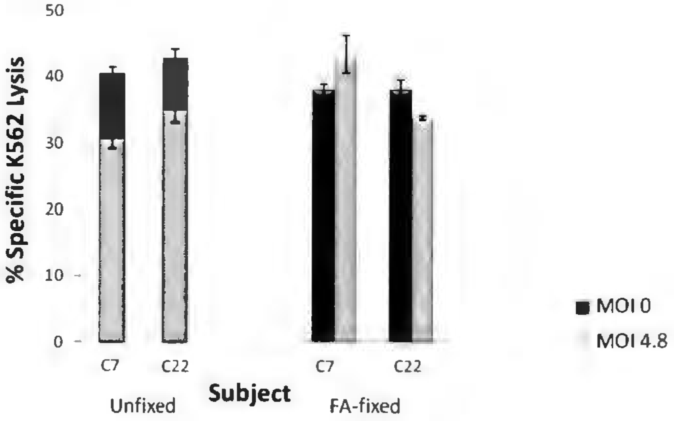
[A]


% Specific K562 Lysis
60
50
40
30
20
10
0
% NK Inhibition
20
15
10
5
0
C7
C8
C12
C15
C16
C25
Subject
■ No virus
Virus

[B]

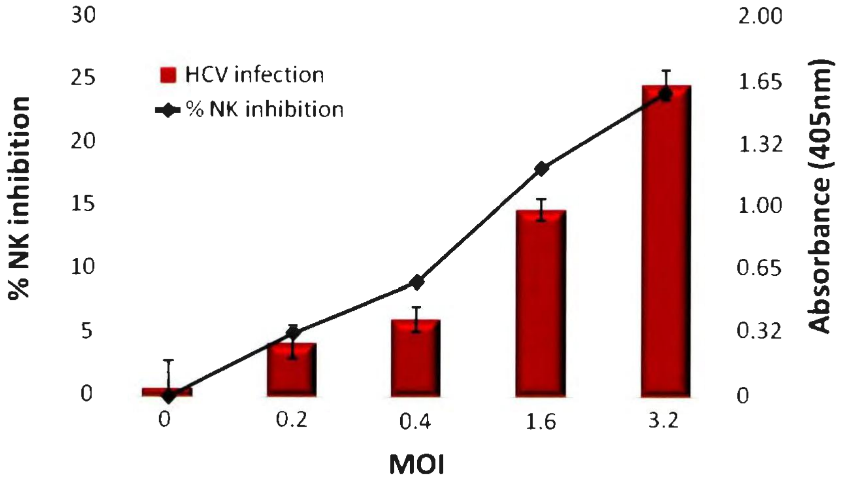

p = 0.0034
% Specific K562 Lysis
80
70
60
50
40
30
20
10
0
C7
C9
C15
C16
C25
C30
C7
C9
C15
C16
C25
C30
Subject
Direct contact
Trans-well
■ MOI 0
MOI 4.8

[C]


% Specific K562 Lysis
50
40
30
20
10
0
C7
C22
C7
C22
Subject
Unfixed
FA-fixed
■ MOI 0
MOI 4.8

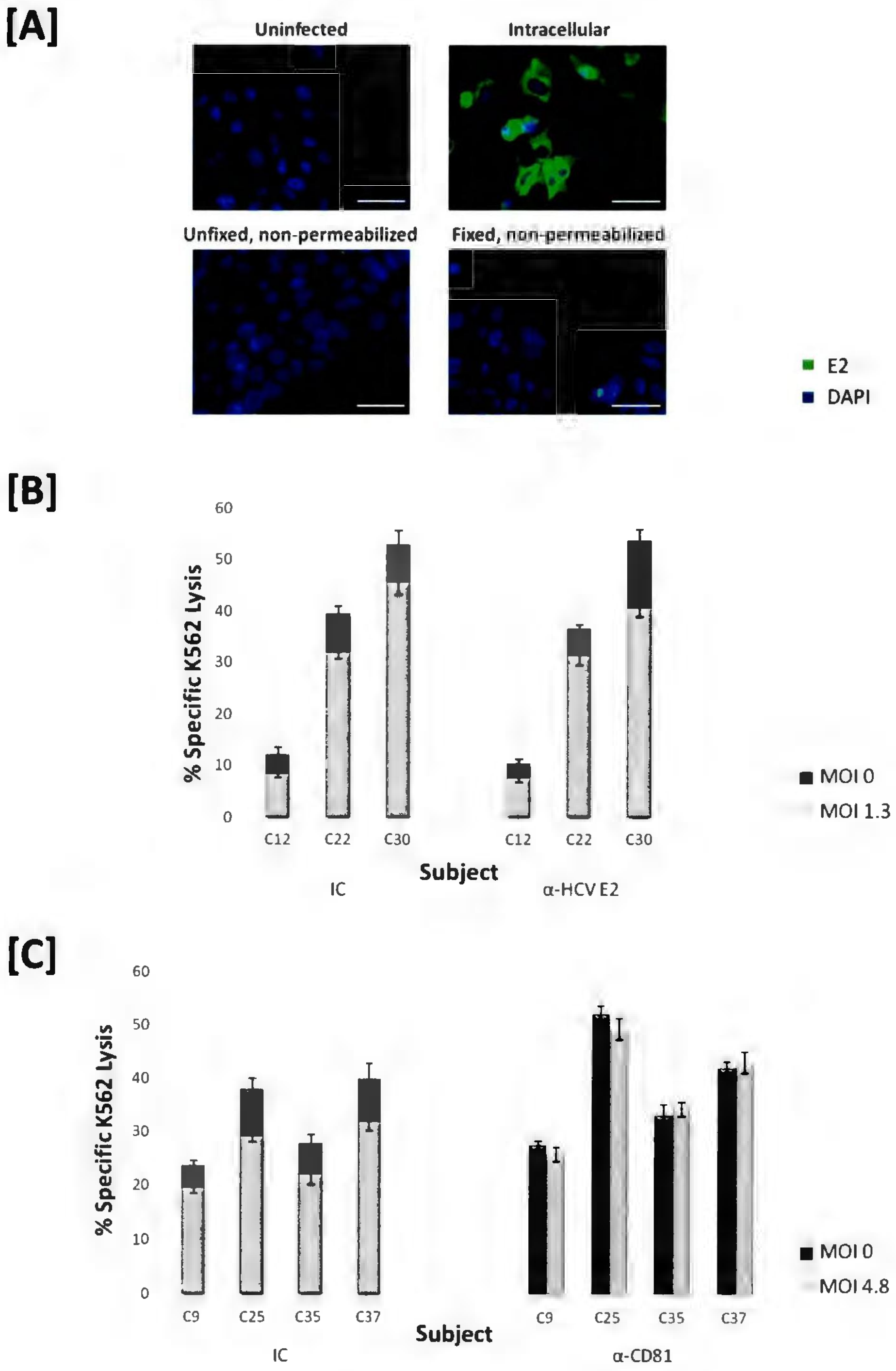
paired *t* test; n = 6) decline in NK cell cytotoxicity was noted when PBMCs were in direct contact with HCV-infected Huh-7.5 cells, we only observed a 0-11% (n = 6) decline in NK cell cytotoxicity when PBMCs were segregated from HCV-infected Huh-7.5 cells (Figure 3.5 B). These results suggest that neither cell-free virus nor soluble factors released from HCV-infected Huh-7.5 cells inhibit NK cytotoxicity. Thus, direct cell-cell contact between NK cells and HCV-infected Huh-7.5 cells is necessary to inhibit NK cell functions. Inhibition of NK cell functions relies on an active interaction with HCV-infected Huh-7.5 cells as FA-fixed Huh-7.5 cells did not support a decline in NK cell cytotoxicity (Figure 3.5 C). Although fixation with FA generally preserves the cellular structure close to its native state (226), it is possible that the absence of NK cell inhibition in the context of FA-fixed HCV-infected Huh-7.5 cells may be caused by the destruction of important epitopes during this fixation process.

We also noted that NK cell inhibition was directly related to HCV infection levels of Huh-7.5 cells. At a MOI of 4.8 and the greatest relative levels of HCV proteins, we observed the most inhibition of NK cell cytotoxicity (Figure 3.6). Therefore, not only is NK cell inhibition dependent on cell-cell contact with HCV-infected Huh-7.5 cells, but our data suggest that this effect is directly related to the extent of HCV infection.

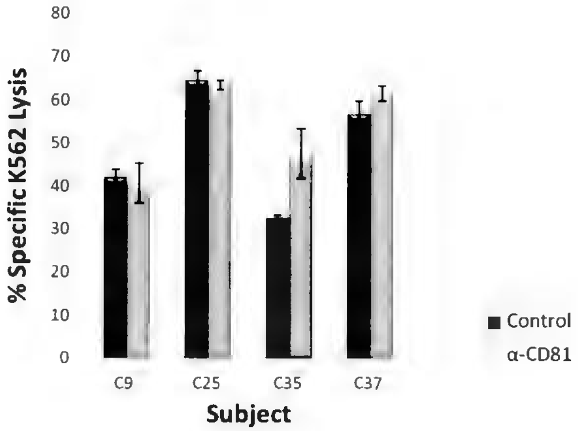

30
25
20
15
10
5
0
% NK inhibition
🟥 HCV infection
◆ % NK inhibition
2.00
1.65
1.32
1.00
0.65
0.32
0
Absorbance (405nm)
0
0.2
0.4
1.6
3.2
MOI

3.6 Blocking HCV E2 Protein Has No Effect on NK Cells, Whereas Engagement of NK CD81 Abrogates Inhibition

Previous studies indicated that HCV E2 protein binding to NK CD81 can affect NK cell function (211, 212). Although we could not detect cell surface HCV E2 expression on HCV-infected Huh-7.5 cells by IF microscopy (Figure 3.7 A), we performed antibody-masking experiments to determine whether an E2:CD81 interaction could down-regulate NK cell functions in our system. JFH1$_T$ has a mutation at amino acid 417 in E2 (N→S) that prevents anti-HCV E2 monoclonal antibody AP33 binding (227), therefore, this antibody could not be used in these experiments. JFH1$_{D1}$ lacks the E2 adaptive mutation but invokes the same effects on NK cell cytotoxicity as JFH1$_T$ (shown in Figure 3.7 B). JFH1$_{D1}$ HCV-infected Huh-7.5 cells were pre-treated with an anti-HCV E2 monoclonal antibody (AP33), which binds E2 at amino acid residues 412-423 and blocks CD81:E2 interactions, then incubated with PBMCs in a 5 hour ^{51}Cr release assay. Pre-treating HCV-infected cells with anti-HCV E2 to block potential HCV E2:NK CD81 interactions did not abrogate NK cell inhibition (Figure 3.7 B). Interestingly, pre-treating fresh PBMCs with a soluble CD81 antibody prior to ^{51}Cr release assays with JFH1$_T$ HCV-infected Huh-7.5 cells negated NK cell cytotoxic inhibition (Figure 3.7 C). The purpose of the experiments involving soluble anti-human CD81 antibody was to not cross-link this receptor on NK cells, but merely block interactions involving this receptor to ascertain whether it was involved in inhibiting NK cell functions. Pre-treatment of PBMCs with soluble anti-CD81 alone had no effect on NK cell cytotoxicity in control assays using ^{51}Cr-labeled K562 cells (Figure 3.7 D). These results imply that it is unlikely that HCV E2 is present on the surface of HCV-infected Huh-7.5 cells at levels sufficient to mediate the observed inhibition, and that the mechanism by which pre-treating PBMCs with soluble anti-CD81 abrogates HCV-infected Huh-7.5 cell inhibition of NK cell cytotoxicity is E2-independent.

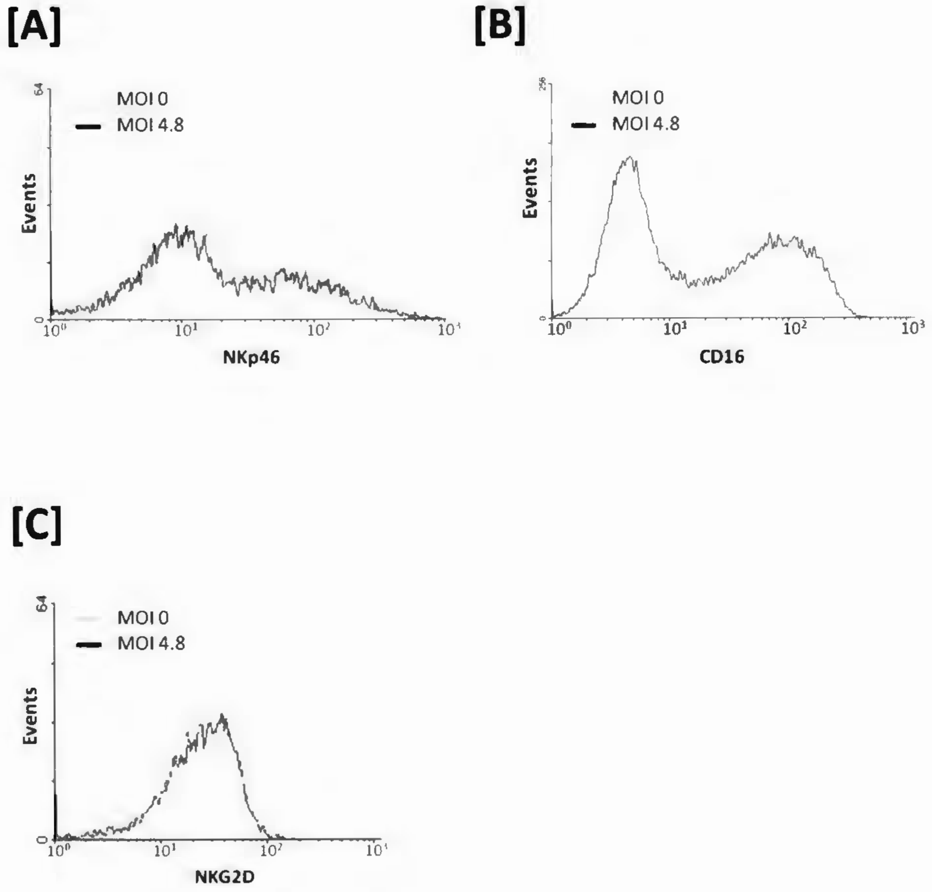

[A]
Uninfected
Intracellular
Unfixed, non-permeabilized
Fixed, non-permeabilized
E2
DAPI
[B]
% Specific K562 Lysis
0
10
20
30
40
50
60
C12
C22
C30
C12
C22
C30
Subject
IC
α-HCV E2
MOI 0
MOI 1.3
[C]
% Specific K562 Lysis
0
10
20
30
40
50
60
C9
C25
C35
C37
C9
C25
C35
C37
Subject
IC
α-CD81
MOI 0
MOI 4.8

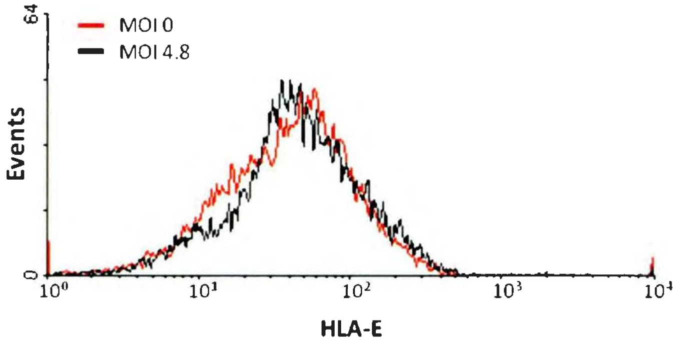
[D]


80
70
60
50
40
30
20
10
0
% Specific K562 Lysis
C9
C25
C35
C37
Subject
■ Control
α-CD81

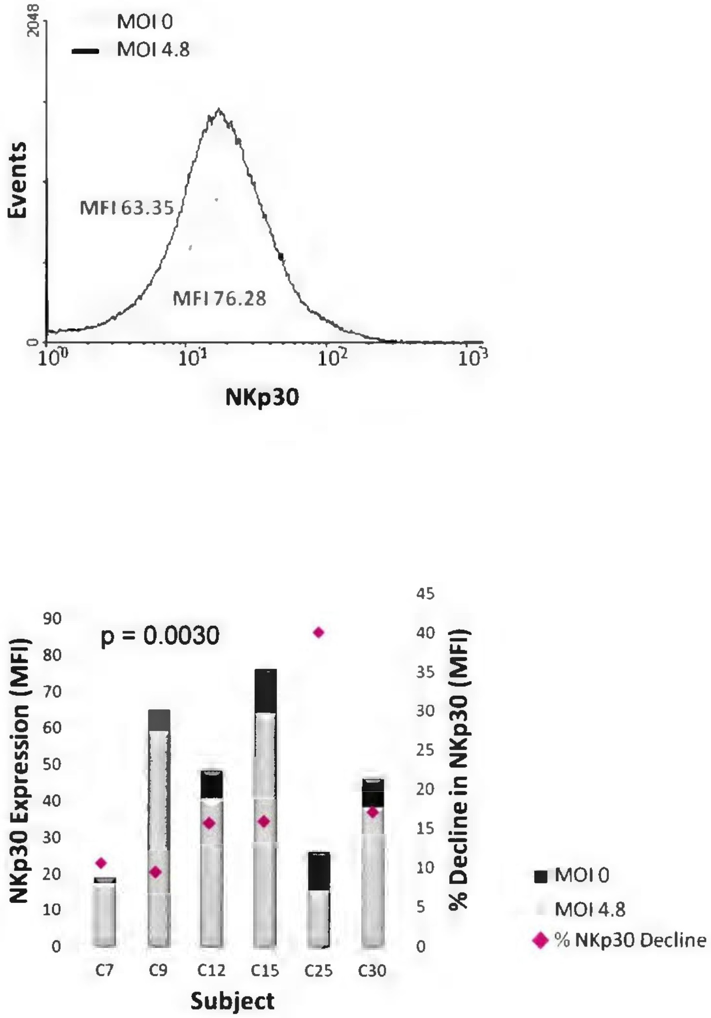
3.7 Direct Interaction with HCV-Infected Cells Down-Regulate NKp30 Expression

To address the mechanism by which HCV-infected Huh-7.5 cells inhibit NK cell functions, we examined surface expression of various NK cell receptors. Flow cytometric data showed that NKp46, CD16 and NKG2D expression levels remain unaltered over a 5 hour incubation of PBMCs with HCV-infected compared to uninfected Huh-7.5 cells (Figures 3.8 A-C). Previous studies indicated that cell surface HLA-E complexes can be stabilized by a peptide derived from HCV core protein and its expression up-regulated upon HCV infection (228). However, we saw no increase of HLA-E expression on Huh-7.5 cells following HCV infection (Figure 3.9). In contrast, we found that NCR NKp30 expression was down-regulated by a mean of 18 ± 4.6% (SEM; $p = 0.0030$, Student's paired *t* test; $n = 6$), assessed by mean fluorescence intensity (MFI) following 5 hour incubation with HCV-infected Huh-7.5 cells (Figure 3.10 A). As NKp30 exhibited selectively reduced expression, and direct cell-cell contact with HCV-infected cells was required to inhibit NK cell functions, we examined whether a putative NKp30 ligand was up-regulated on Huh-7.5 cells following HCV-infection. We observed a ~20% increase in surface binding of an NKp30-IgG_1 Fc_γ fusion protein to HCV-infected Huh-7.5 cells (MOI 4.8) in comparison with uninfected Huh-7.5 cells (Figure 3.10 B). To account for potential non-specific binding of NKp30-IgG_1 Fc_γ fusion proteins to the Huh-7.5 cell surface, we compared binding of KIR3DS1-IgG_1 Fc_γ fusion proteins to uninfected and HCV-infected Huh-7.5 cells. There was no increased binding of the KIR3DS1-IgG_1 Fc_γ fusion protein to HCV-infected compared to uninfected Huh-7.5 cells (Figure 3.10 C). However, there was a 2-fold ($p = 0.0286$, Mann-Whitney *U* test; $n = 4$) increase in NKp30-IgG_1 Fc_γ protein binding to HCV-infected over uninfected Huh-7.5 cells (Figure 3.10 C). This suggests that HCV infection up-regulates an antagonistic ligand that binds the NKp30 receptor, which could impact NK cell cytotoxicity and cytokine production.

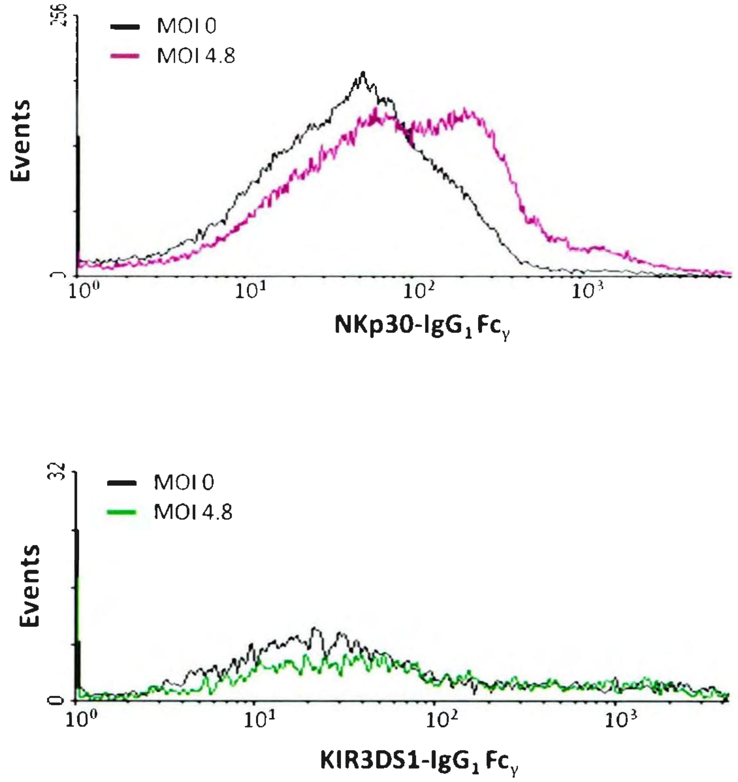

[A]
64
MOI 0
MOI 4.8
Events
0
10⁰
10¹
10²
10³
NKp46
[B]
256
MOI 0
MOI 4.8
Events
10⁰
10¹
10²
10³
CD16
[C]
64
MOI 0
MOI 4.8
Events
0
10⁰
10¹
10²
10³
NKG2D


64
0
Events
MOI 0
MOI 4.8
10⁰
10¹
10²
10³
10⁴
HLA-E

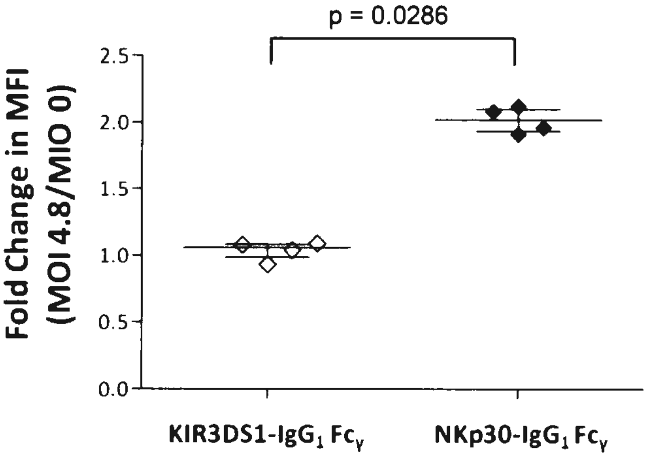
[A]


2048
MOI 0
MOI 4.8
Events
MFI 63.35
MFI 76.28
0
10^0
10^1
10^2
10^3
NKp30
p = 0.0030
NKp30 Expression (MFI)
90
80
70
60
50
40
30
20
10
0
45
40
35
30
25
20
15
10
5
0
% Decline in NKp30 (MFI)
C7
C9
C12
C15
C25
C30
Subject
■ MOI 0
MOI 4.8
◆ % NKp30 Decline

[B]


256
0
Events
— MOI 0
— MOI 4.8
10^0
10^1
10^2
10^3
NKp30-IgG$_1$ Fc$_\gamma$
32
0
Events
— MOI 0
— MOI 4.8
10^0
10^1
10^2
10^3
KIR3DS1-IgG$_1$ Fc$_\gamma$

[C]


p = 0.0286
Fold Change in MFI
(MOI 4.8/MIO 0)
2.5
2.0
1.5
1.0
0.5
0.0
KIR3DS1-IgG$_1$ Fc$_\gamma$
NKp30-IgG$_1$ Fc$_\gamma$

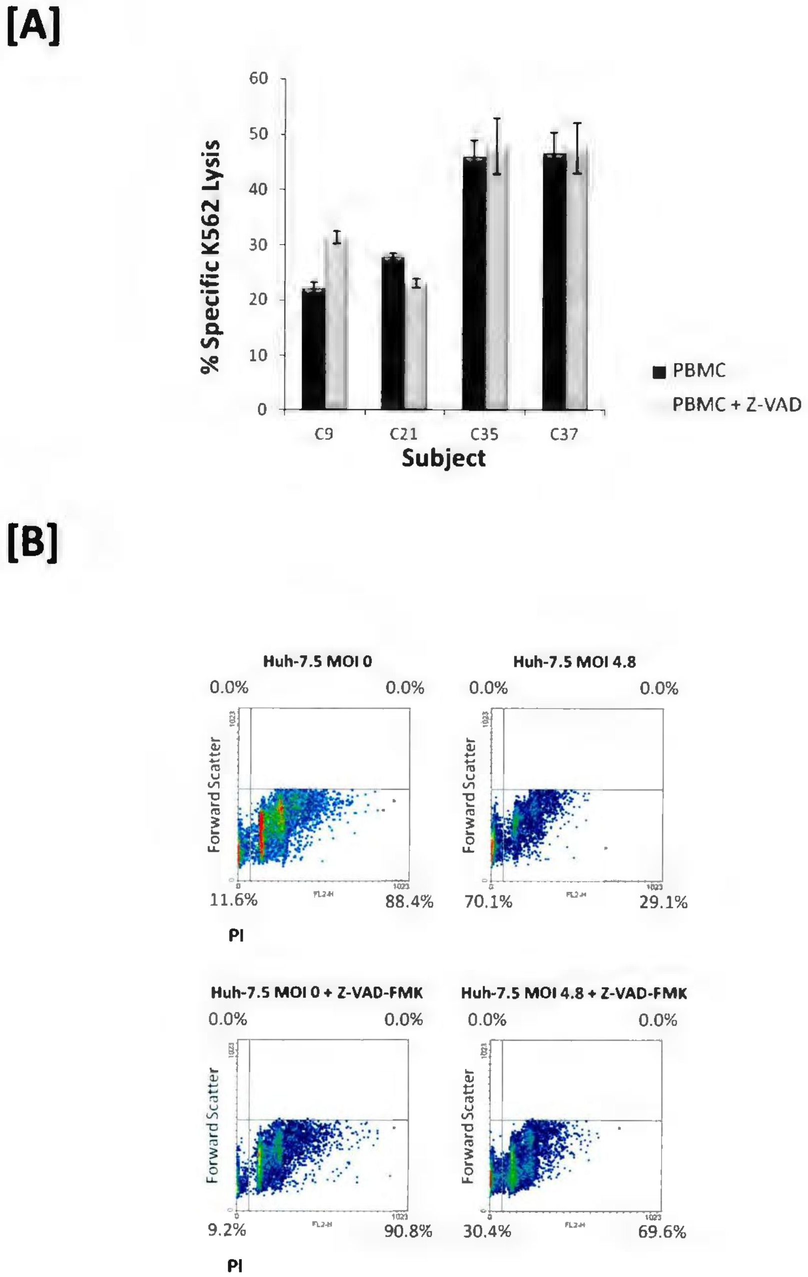
4 The Effect of Apoptotic Cells on NK Cell Functions

4.1 Rationale

Although data suggests that the decline in NK cell functions is likely directly related to HCV infection of Huh-7.5 cells, it was important to address the possibility that cytopathic effects mediated by HCV infection were responsible for inhibiting NK cell functions. This potential mechanism was queried as we noted decreased cell viability at high MOIs with HCV (refer to Figure 3.2 B). Furthermore, preliminary evidence suggests HCV proteins such as NS4B (85) may induce or cause cytopathic effects, leading to cellular apoptosis. A recently discovered inhibitory receptor, TIM-3, is expressed on the surface of NK cells and interacts with PtdSer and Gal-9. Mengshol *et al.* demonstrated high levels of circulating Gal-9 in the serum of individuals chronically infected with HCV (218). Here we use the assay developed in Chapter 3 to assess whether inhibited NK cell functions is caused by HCV-infected Huh-7.5 cells or indirectly a result of apoptotic Huh-7.5 cells.

4.2 Z-VAD-FMK Treatment Has No Effect on NK Cell Cytotoxicity

Since the apoptosis inhibitor Z-VAD-FMK is a pan caspase inhibitor, we wanted to determine whether Z-VAD-FMK treatment would affect NK cell-mediated granzyme-induced caspase-dependent apoptosis of target cells. We assessed NK cell lysis of ^{51}Cr-labeled K562 cells in the presence of Z-VAD-FMK and saw no difference in percent specific lysis of labeled K562 cells in the Z-VAD-FMK-treated assay compared with the untreated assay (Figure 4.1 A). To determine whether Z-VAD-FMK was able to prevent apoptosis in the Huh-7.5 cell line, we compared the extent of cellular apoptosis in uninfected and HCV-infected Huh-7.5 cells through the detection of DNA content by PI binding. As apoptosis is characterized by DNA fragmentation, hypodiploid cells are identified as apoptotic, diploid cells are classified as being in resting phase (Gap 0 and Gap 1) and tetraploid cells are undergoing cell division (Gap 2). Approximately 12% of uninfected Huh-7.5 cells were apoptotic, and 24 hour treatment with Z-VAD-FMK marginally reduced apoptotic cell numbers to ~9% (Figure 4.1 B). Comparably, PI staining indicates that ~70% of the Huh-7.5 cells infected at a MOI of 4.8 are undergoing apoptosis, whereas only ~30% of the infected Huh-7.5 cells are undergoing apoptosis when treated with Z-VAD-FMK for 24 hours (Figure 4.1 B). Kohafi *et al.* (unpublished data) have also shown that Z-VAD-FMK treatment reduced Huh-7.5 apoptosis in HCV-infected Huh-7.5 cells by at least 50% when compared to untreated infected Huh-7.5 cells, as measured by PI staining. Thus, to determine whether inhibition of NK cell functions, as described in Chapter 3, occurs as a consequence of viral infection or from Huh-7.5 cell-induced apoptosis, we used Z-VAD-FMK to inhibit apoptosis and examined whether NK cell cytotoxicity declined, or remained intact.

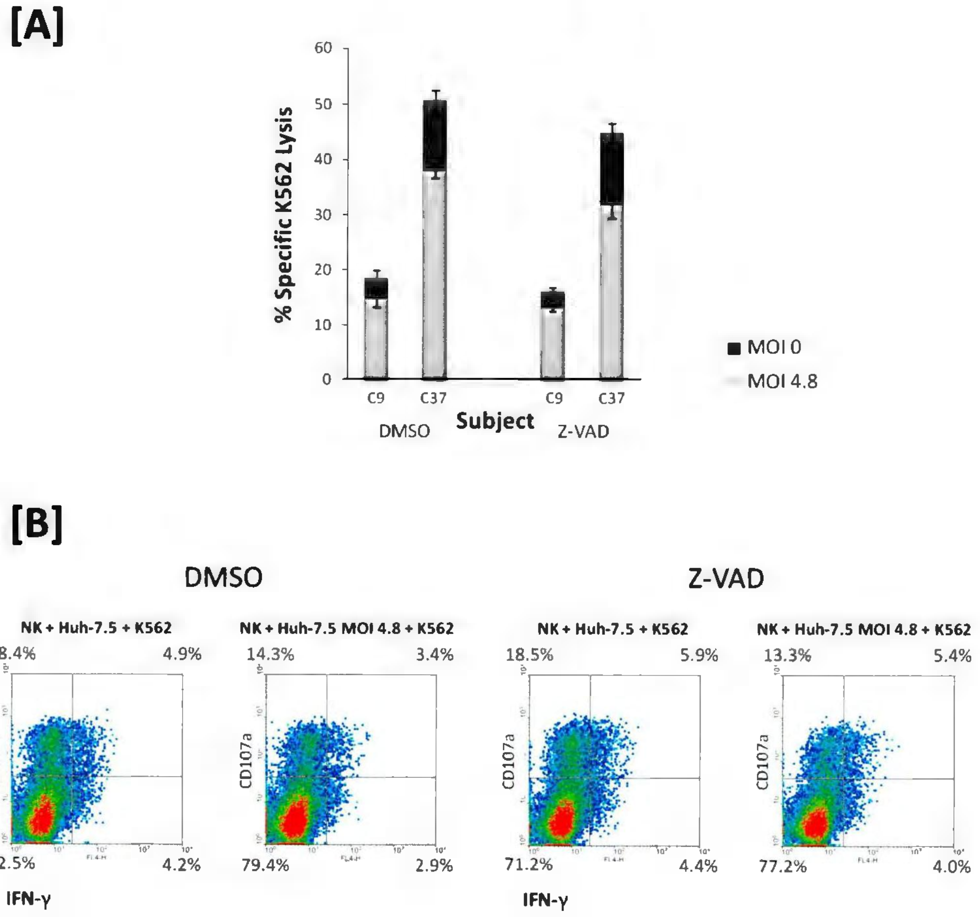

[A]
60
50
40
30
20
10
0
% Specific K562 Lysis
C9
C21
C35
C37
Subject
■ PBMC
PBMC + Z-VAD
[B]
Huh-7.5 MOI 0
0.0%
0.0%
Forward Scatter
11.6%
88.4%
PI
Huh-7.5 MOI 4.8
0.0%
0.0%
Forward Scatter
70.1%
29.1%
Huh-7.5 MOI 0 + Z-VAD-FMK
0.0%
0.0%
Forward Scatter
9.2%
90.8%
PI
Huh-7.5 MOI 4.8 + Z-VAD-FMK
0.0%
0.0%
Forward Scatter
30.4%
69.6%

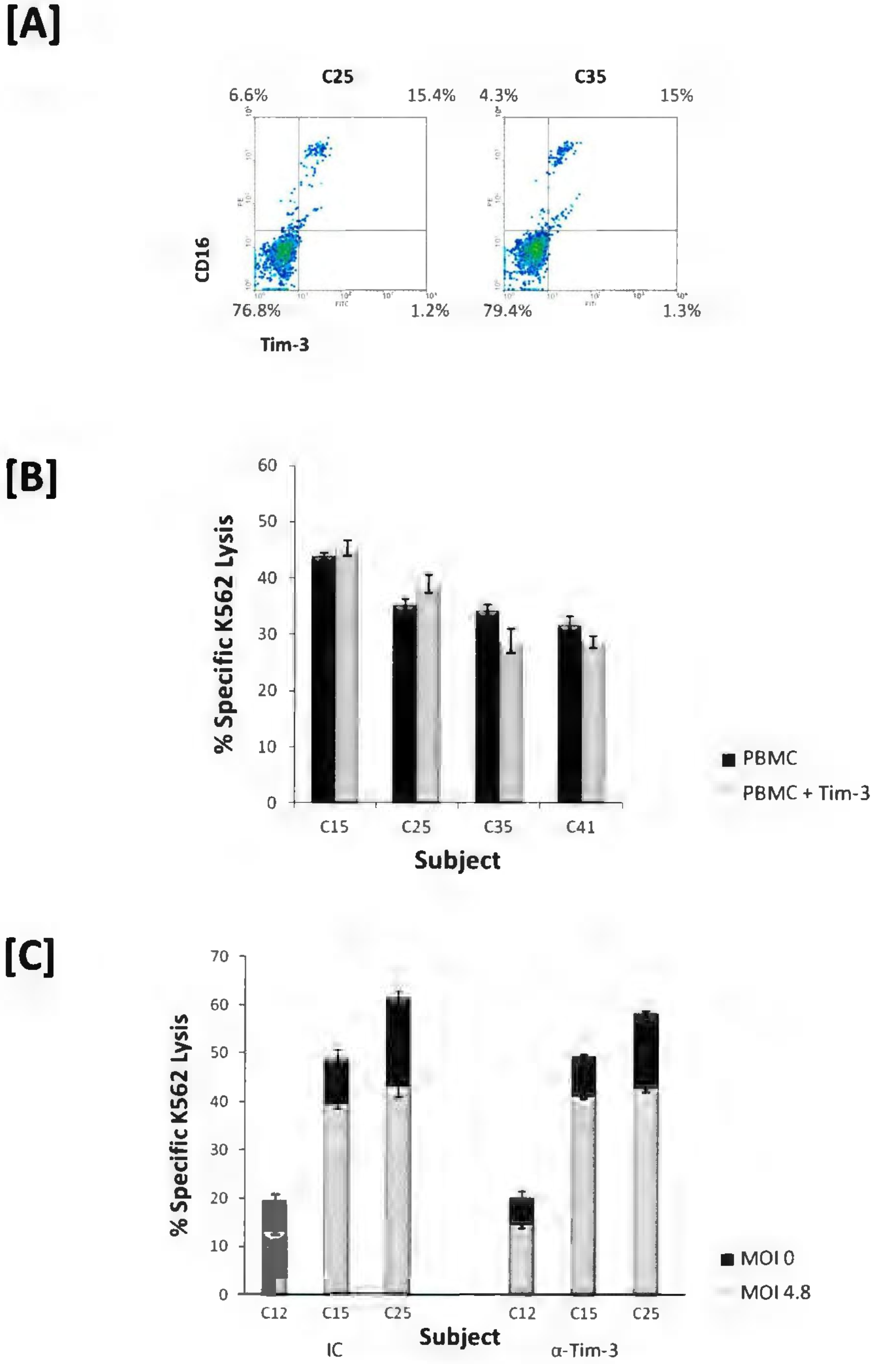
4.3 Preventing Huh-7.5 Cell Apoptosis Does Not Restore NK Cell Cytotoxicity

To assess whether the decline in NK cell cytotoxicity and cytokine production was the result of HCV infection of Huh-7.5 cells or Huh-7.5 cells undergoing apoptosis, uninfected or HCV-infected Huh-7.5 cells were treated for 24 hours with pan caspase inhibitor Z-VAD-FMK to prevent apoptosis. NK cell cytotoxicity remained inhibited in the presence of Z-VAD-FMK (Figure 4.2 A), indicating that apoptotic Huh-7.5 cells were not inhibiting NK cell cytotoxicity. These results were corroborated by the assessment of NK cell degranulation (CD107a production) via flow cytometry where we found no prevention of NK cell inhibition by Z-VAD-FMK treatment (Figure 4.2 B). We did, however, observe rescue of IFN-γ production upon Z-VAD-FMK treatment (Figure 4.2 B). Therefore, NK cell inhibition is a direct result of HCV infection rather than an effect caused by the decline in Huh-7.5 cell viability.

4.4 NK Cell Inhibition Is Not NK TIM-3-Mediated

Recent findings suggest an inhibitory role for NK TIM-3 receptors cross-linked by known ligands, PtdSer and Gal-9, expressed on apoptotic cells (170). We used an available anti-human TIM-3 antibody that had been shown to recognize the TIM-3 Ig variable domain and block TIM-3:Gal-9 interactions (229). We detected cell surface TIM-3 expression on NK cells (Figure 4.3 A), and

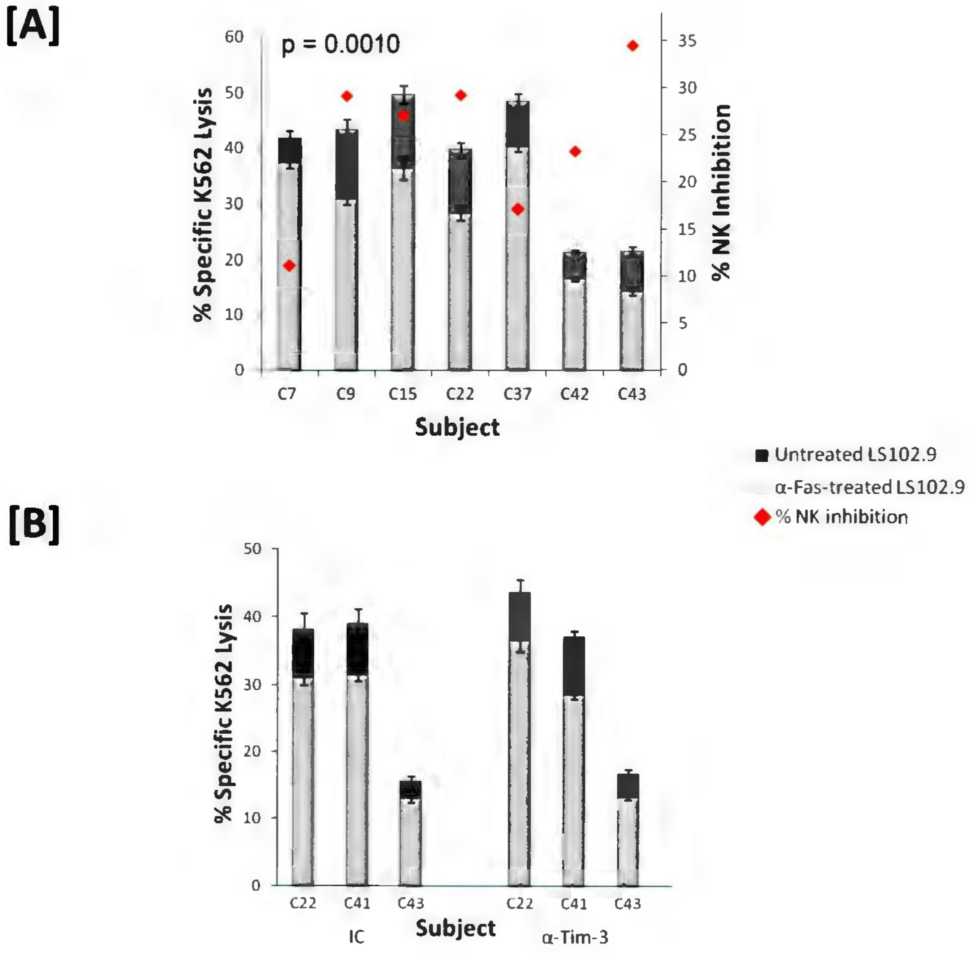

[A]
% Specific K562 Lysis
60
50
40
30
20
10
0
C9
C37
C9
C37
Subject
DMSO
Z-VAD
MOI 0
MOI 4.8
[B]
DMSO
Z-VAD
NK + Huh-7.5 + K562
18.4%
4.9%
72.5%
4.2%
NK + Huh-7.5 MOI 4.8 + K562
14.3%
3.4%
79.4%
2.9%
NK + Huh-7.5 + K562
18.5%
5.9%
71.2%
4.4%
NK + Huh-7.5 MOI 4.8 + K562
13.3%
5.4%
77.2%
4.0%
CD107a
IFN-γ

[A]
C25
6.6%
15.4%
76.8%
1.2%
C35
4.3%
15%
79.4%
1.3%
CD16
Tim-3
[B]
60
50
40
30
20
10
0
% Specific K562 Lysis
C15
C25
C35
C41
Subject
■ PBMC
PBMC + Tim-3
[C]
70
60
50
40
30
20
10
0
% Specific K562 Lysis
C12
C15
C25
C12
C15
C25
IC
Subject
α-Tim-3
■ MOI 0
MOI 4.8

treatment with a soluble anti-human TIM-3 antibody, which would block TIM-3:ligand binding, did not affect NK cell cytotoxicity in a 5 hour ^{51}Cr release assay compared to IC-treated cells (Figure 4.3 B). To determine if NK cell inhibition was TIM-3-mediated, we performed an antibody-mediated receptor/ligand masking experiment. Treating NK cells with anti-TIM-3 did not prevent inhibition of NK cell cytotoxicity in the presence of HCV-infected Huh-7.5 cells compared with uninfected Huh-7.5 cells (Figure 4.3 C). Although TIM-3 is expressed on NK cells and may be capable of eliciting an inhibitory response, in this instance, NK cell inhibition is TIM-3-independent.

4.5 Apoptotic LS102.9 Cells Inhibit NK Cell Cytotoxicity Independent of NK TIM-3

As Ndhlovu *et al.* found that NK cells express TIM-3 and provides an inhibitory signal (170), we decided to examine the possibility that apoptotic cells expressing PtdSer and/or Gal-9 could inhibit NK cell cytotoxicity in a TIM-3-mediated mechanism. We used the anti-Fas (also known as APO-1 or CD95)-sensitive murine LS102.9 cell line to induce cellular apoptosis, then examined if treatment with anti-TIM-3 prevented the ability of NK cells to lyse ^{51}Cr-labeled K562 cells. We found that apoptotic LS102.9 cells (42% dead on average, assessed by trypan blue staining) inhibited NK cell cytotoxicity by a mean of 25 ± 8% (SEM; $p = 0.0010$, Student's paired *t* test; $n = 7$) (Figure 4.4 A). However, TIM-3 treatment did not rescue NK cell cytotoxicity against ^{51}Cr-labeled K562 cells in the presence of apoptotic LS102.9 cells (Figure 4.4 B). This data suggests that the mechanism by which apoptotic cells inhibit NK cells in this assay is TIM-3 independent.


[A]
p = 0.0010
% Specific K562 Lysis
0
10
20
30
40
50
60
% NK Inhibition
0
5
10
15
20
25
30
35
C7
C9
C15
C22
C37
C42
C43
Subject
■ Untreated LS102.9
α-Fas-treated LS102.9
◆ % NK inhibition
[B]
% Specific K562 Lysis
0
10
20
30
40
50
C22
C41
C43
C22
C41
C43
IC
Subject
α-Tim-3

5 Discussion

Through interaction with host innate and adaptive immune responses, HCV has evolved mechanisms to circumvent immune pressures and establish chronic infection. Cytokines produced by NK cells, such as IFN-γ and TNF-α, play a synergistic role in innate and adaptive immune responses (230). Several studies suggest that NK cells play a role in clearance or control of HCV infection or associate NK cell dysfunction with HCV infection (19, 231, 232). Here, we designed an assay platform to study the effects HCV-infected cells have on NK cells and found that direct contact with HCV-infected cells *in vitro* inhibited *ex vivo* NK cell cytotoxicity and cytokine production. We found that inhibition of NK cell cytotoxicity and cytokine production was contact-dependent with HCV-infected cells and appeared proportional to HCV infection levels.

Previous studies addressing the role NK cells play in HCV infection focused on interactions between HCV E2 and CD81 on NK cells as a potential mechanism for NK cell dysfunction (211-213). These studies used recombinant HCV E2, cell-free HCV, or virus attached to a solid support and IL-2-stimulated (212) or purified NK cells (211). In some cases, this resulted in decreased NK cell cytotoxicity and cytokine production via the cross-linking of NK CD81. As a consequence, inhibition of NK cell functions through HCV E2:NK CD81 cross-linking is often proposed as a means to promote HCV persistence (233-236). AP33, a broadly neutralizing antibody against the CD81-binding site, recognizes a linear epitope located between amino acid residues 412 and 423 of HCV E2 (114, 227, 237). We used this antibody to probe for extracellular HCV E2 expression on Huh-7.5 cells, as well as to block HCV E2 and NK CD81 interactions. We found no evidence for surface HCV E2 expression on HCV-infected Huh-7.5 cells and disruption of potential HCV E2:NK CD81 interactions by masking HCV E2 did not prevent the decline in NK cell cytotoxicity mediated by HCV-infected cells.

NK cell functions remained intact when incubated with cell-free JFH1_T HCV, providing additional evidence that the mechanism by which HCV evades NK cell cytotoxicity is not HCV E2 mediated, and requires direct contact with HCV-infected Huh-7.5 cells. Previous studies also indicated that HCV E2 molecules, as displayed on HCV genotype 1a or 2a virions, do not inhibit NK cell functions, even when used at titres exceeding HCV RNA and protein concentrations found in the blood of infected individuals (213). These results suggest that although the configuration of natural HCV E2 may support binding to NK CD81, E2 as part of an intact infectious virus particle does not facilitate the necessary cross-linking of NK CD81 receptors to mediate an inhibitory signal (211, 212). Also, though HCV E2 is able to bind host hepatocyte CD81 to facilitate entry, CLDN1, OCLN, and especially SRBI are important co-receptors for virus infection and are not constituents of the NK cell receptor repertoire. Our results suggest that an HCV E2:NK CD81 interaction is not the primary cause of impaired NK cell functions mediated *in vitro,* and likely *in vivo,* by HCV-infected cells. Also, while HCV infection of Huh-7.5 cells may cause the induction of antagonistic host proteins to interact with NKp30, it is possible that a viral protein other than E2 may cause disrupted NK cell functions. This possibility may be explored through the examination of NK cell functions in the presence of a stable cell line autonomously replicating subgenomic RNA (NS3-NS5B). However, HCV virions assemble and mature through the cell secretory pathway, rather than bud through the cell surface. Thus, it is unlikely that viral proteins are found on the surface of HCV-infected cells and, therefore, are unable to interact with, and inhibit NK cells.

Although blocking HCV E2:NK CD81 interactions did not rescue NK cell function, we did find that treating NK cells with anti-CD81 abrogated NK cell inhibition. Previous studies show that cross-linking NK CD81 receptors by either CD81 antibodies or immobilized HCV E2 blocks NK cell cytotoxicity, cytokine production and proliferation (211, 212). NK CD81 cross-linking has been shown to block the signaling pathway induced by CD16 engagement by reducing the overall level of tyrosine phosphorylation, specifically of the ζ chain and downstream mitogen-activated protein kinase (MAPK) and extracellular signal-related kinase (ERK)-2 molecules (211, 238). As CD16 and NCRs NKp30 and NKp46 share a conserved signaling pathway, and may also signal through ζ chain homo- or heterodimer adaptor proteins, the CD81 tetraspanin may also negatively affect NK cell signaling through the same mechanism of reduced tyrosine phosphorylation of signaling substrates. The HCV-infected cell-mediated decline in NK cell functions could be CD81-dependent through the direct interaction with an antagonistic ligand, with the exception of HCV E2, on HCV-infected Huh-7.5 cells, or regulated indirectly, through a tetraspanin:membrane-associated receptor interaction with an antagonistic ligand up-regulated on HCV-infected Huh-7.5 cells.

One characteristic of the tetraspanin CD81 is its proclivity for interacting with partner proteins embedded in the cell membrane to form signal transduction complexes (239). The effect of CD81 cross-linking varies not only with cell type, but also with which membranous protein it interacts. Lateral interaction of CD81 with an associated protein could either augment or impair positive or negative stimuli depending on the cell type (239). Predominantly, the extracellular domain, LEL, mediates lateral protein-protein interactions or interactions with the few known ligands for CD81 (reviewed in [240]). CD81 forms complexes with CD19 and CD21 (CD19-CD21 co-receptor complex) on B cells and is thought to recruit this co-stimulatory complex to the B cell receptor to augment activation thresholds. It also associates with T cell signaling proteins CD3, CD4 and CD8 (239). Thus, although no associated proteins have yet to be identified on NK cells, it is possible that CD81 laterally interacts with membrane proteins on NK cells to increase or decrease stimuli delivered, for example, by ligands up-regulated following HCV-infection. Blocking NK CD81 with anti-CD81 could prevent these lateral protein-protein associations and in turn modulate negative stimuli and reduce or eliminate the inhibition of NK cell functions.

The extent to which tetraspanins contribute to immune interactions is currently unknown; the paucity of mechanistic information likely stems from the complexity of their associations. The cytoplasmic regions of tetraspanins organize extracellular stimuli through association with intracellular signaling molecules to facilitate signaling cascades (reviewed in [240]). However, there are indications that tetraspanins are also involved in cytoskeleton rearrangement in both T and NK cells. In fact, Crotta *et al.* suggest that inhibition of NK cell cytotoxicity and cytokine production through CD81 is mediated by cytoskeletal rearrangement (238). Coffey *et al.* further show that engaging CD81 leads to the phosphorylation of ezrin (at Y353), a protein which bridges membrane proteins to the actin cytoskeleton to allow membrane reorganization for cellular signaling (241). Our preliminary results could not determine a role for ezrin as a mechanism by which CD81 affects NK cell functions, and propose that the negative signaling cascade stimulated by direct contact with HCV-infected Huh-7.5 cells may be augmented by lateral interactions between CD81 and an as yet unidentified partner receptor. However, further studies regarding the importance of ezrin and cytoskeletal rearrangement for the control of NK cell lytic capabilities may be warranted, based upon studies regarding T cell receptors (TCR) discussed below.

Although we believed that HCV infection of the Huh-7.5 cells is responsible for disrupted NK cell functions, we noted decreased Huh-7.5 cell viability at high levels of infection with HCV. Thus, it was necessary to determine whether cytopathic effects on Huh-7.5 cells from HCV infection impacted NK cell functions. Although treating Huh-7.5 cells with pan caspase inhibitor, Z-VAD-FMK, blocked ~50% of the Huh-7.5 cells from undergoing apoptosis at a high MOI, this treatment did not prevent the decline in NK cell cytotoxicity. Thus, the negative effect that Huh-7.5 cells have on NK cell functions is HCV-dependent while independent of HCV-induced cytopathic effects. To further confirm that cytopathic effects were not eliciting down-regulated NK cell functions, we assessed whether the TIM-3 NK cell receptor was responsible for restrained NK cell cytotoxicity. PtdSer is implicated as a TIM-3 ligand and is expressed at the outer leaflet of the plasma membrane upon initiation of apoptosis (175). TIM-3 is expressed on NK cells and, when engaged and cross-linked, suppresses NK cell cytotoxicity and reduces anti-viral properties in chronic infection (171). The mechanism by which this occurs is unknown, as this receptor lacks any known inhibitory motifs (170-173). Consequently, disruption of NK cell TIM-3:ligand interactions should perturb any inhibitory effect TIM-3 may have on NK cells in the context of HCV-infected Huh-7.5 cells. We did not observe abrogation of the inhibition of NK cell cytotoxicity upon blockade of TIM-3 receptors. However, further exploration into this mechanism of NK cell inhibition is necessary to eliminate this possibility. Of note, high levels of circulating Gal-9 occurs in the serum of chronically HCV-infected individuals (218) and expression levels of neither Gal-9 nor PtdSer were explored in the context of uninfected and HCV-infected Huh-7.5 cells. Additionally, incubation of *ex vivo* NK cells with uninfected or HCV-infected Huh-7.5 cells in the presence of recombinant soluble TIM-3 glycoprotein, which competes for TIM-3 ligands, can be used to address the possibility that the TIM-3 antibody used in this study did not mask the epitope recognized by either Gal-9 or PtdSer.

On the basis that apoptotic Huh-7.5 cells could affect NK cell cytotoxicity, we also examined whether other apoptotic cells had any influence on NK cell function. We determined that Fas receptor (FasR)-mediated induction of apoptosis in the murine LS102.9 cell line reduced NK cell cytotoxicity. However, down-regulated NK cell cytotoxicity was independent of TIM-3, as blocking TIM-3 receptors did not rescue NK cell cytotoxicity. Additional experiments, including those outlined above, are needed to effectively eliminate TIM-3 signaling as a means by which NK cell inhibition, in the presence of apoptotic cells, occurs. An alternative means by which apoptotic cells could influence NK cell cytotoxicity is through the PBMC cytokine microenvironment *in vitro*. Monocytes release IL-10 and also immunosuppressive cytokine, transforming growth factor (TGF)-β, upon exposure to apoptotic cells (reviewed in [242]). Although IL-10 has stimulatory effects on NK cells (reviewed in [243]), especially in the presence of IL-12 or IL-18 (244), TGF-β attenuates immune responses to eliminate self-reactivity, including NK cell responses (245, 246). *In vitro* studies showed that TGF-β decreased expression of activating receptors and their respective adaptor proteins on NK cells, corresponding with reduced NK cell functions, however, NK cell activity could be restored by treatment with anti-TGF-β antibody (247, 248). Thus, further examination to assess whether apoptotic cells may affect monocytes, T cells or B cells in the PBMC microenvironment, which, in turn impact NK cell cytotoxicity, would be beneficial.

In order to determine the manner in which NK cell cytotoxicity is down-regulated in the presence of HCV-infected Huh-7.5 cells, we probed for differences in NK cell receptor expression when incubated with HCV-infected, compared to uninfected, Huh-7.5 cells and probed HCV-infected Huh7.5 cells for NK cell ligands. Nattermann *et al.* demonstrated up-regulated HLA-E expression on hepatocytes during chronic infection, as HLA-E can be stabilized and up-regulated by HCV core peptide (aa35-44) (228). Although HLA-E is known to be a ligand for inhibitory NK receptor NKG2A/CD94, we and others did not observe increased expression of HLA-E on HCV-infected Huh-7.5 cells (215). Despite inhibition of ADCC, CD16 expression levels were unaltered on NK cells exposed to HCV-infected cells. A previous study by Yoon *et al.* showed that NK cell cytotoxicity and IFN-γ production were reduced following 18 hour co-culture with HCV-infected Huh-7.5 cells, and also noted a decline in NKp30 and NKG2D cell surface expression on NK cells (215). However, we did not observe a reduction in NKG2D expression on NK cells after 5 hours of incubation with infected cells. NKp30 was the only NK receptor we examined with decreased cell surface expression. These *in vitro* data corroborate the *ex vivo* observation of reduced circulating NKp30-expressing NK cells in individuals with chronic HCV infection (210). Interestingly, CD16 and NKp30 can associate with the same ζ chain adapter molecule, which mediates intracellular signaling and ultimately leads to NK cell cytokine secretion, cytotoxicity and ADCC through CD16 (249).

The ζ chain is composed of a short, extracellular domain consisting of nine amino acids and an intracellular domain containing ITAMs. Upon ITAM phosphorylation by lymphocyte-specific protein tyrosine kinase (Lck), the SYK family kinases, spleen tyrosine kinase (Syk), and ζ chain associated protein kinase of 70 kDa (ZAP70) are recruited. This recruitment ultimately leads to ERK phosphorylation and subsequent NK cell cytotoxicity and cytokine production (249-252) (Figure 1.7). Although the cytoskeleton is essential for the structural support of a cell, it is also an important physical support for the enzymatic processes governing cellular signaling, linking the extracytoplasmic stimulus to the intracellular signaling cascade (253, 254). Studies of ζ chain involvement with TCRs have shown that the ζ chain links approximately one third of expressed TCRs to the actin cytoskeleton and may also stabilize extracellular TCR expression (253, 254). Based on these findings that the ζ chain links the actin cytoskeleton in T cells, and the role CD81 has in actin redistribution via ezrin phosphorylation in order to activate, or inhibit T and NK cells, respectively (241), we can speculate that an altered pattern of actin distribution is an aspect of the underlying mechanism which inhibits NK cell functions through direct contact with HCV-infected Huh-7.5 cells.

Our data show impairment of NK cell cytotoxicity and cytokine production in the presence of HCV-infected Huh-7.5 cells. This corresponds with down-regulation of the NCR NKp30, and increased binding of an NKp30-IgG_1 Fc_γ fusion protein to the surface of HCV-infected cells. An antagonistic NKp30 ligand induced on HCV-infected cells could inhibit NK cell-mediated immune mechanisms and favour progression to chronic HCV infection in a manner similar to that observed for HCMV. Arnon *et al.* demonstrated a direct antagonistic interaction between HCMV tegument protein pp65 and NK cell activating receptor NKp30 that reduced NK cell cytotoxicity through disassociation of the ζ chain adaptor protein from NKp30 (204).

Antagonism of the ζ chain associated with the NKp30 receptor, through an interaction with a putative NKp30 ligand, can abrogate activating signal transduction through the Lck→ERK signaling pathway (249). Further studies to determine this putative antagonistic ligand for the NKp30 receptor, as well as its binding site on NKp30 are necessary. Recently, the extracellular domain of NKp30 was crystallized and its structure determined (255). Although many ligands for NKp30 have been identified, the physiological significance of these ligand:NKp30 receptor interactions remain to be determined (201, 204, 256-259). Interestingly, NKp30 exhibits structural homology to programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and CD28 receptors, which all recognize the N-terminal region of their respective B7 family ligands (260-262). Thus, based on structural homology with the CD28 family of receptors (CD28, CTLA-4, PD-1), NKp30 may recognize other as yet unidentified homologues of the B7-H6 ligand (201, 202, 255). It should be noted, however, that these structural studies only considered the extracellular binding domain of NKp30 and did not take into account the remaining domains comprising NKp30.

NKp30 possesses an extracellular ligand binding domain (LBD), a transmembrane domain (TMD) to which adaptor molecules (i.e. ζ homodimers) bind, and a short, 33 amino acid cytoplasmic tail (193). Recently, Hartmann *et al.* discovered that the flexible stalk domain that connects the LBD to the TMD is N-linked glycosylated. The extent to which this domain is glycosylated not only impacts the affinity of ligand:receptor binding, but may also regulate NK cell cytoxicity via ζ chain signaling (179). This study goes on to show that the binding of B7-H6 to the ligand binding pocket of NKp30 is dependent on N-linked glycosylation at amino acid 42 (N42), and efficient intracellular signaling is contingent upon glycosylation of both N42 and N68 (179). These layers of complexity surrounding NKp30 and the transduction of extracellular stimulus to intracellular signals should be considered for the elucidation of an antagonistic ligand for NKp30 on HCV-infected Huh-7.5 cells.

Although we note increased expression of the putative NKp30 ligand on the surface of HCV-infected Huh-7.5 cells *in vitro*, the Huh-7.5 cell line is derived from transformed primary human hepatoma cells, thus, further *in vivo* or *ex vivo* study of primary hepatocytes to determine whether this is a genuine physiological effect are warranted. For instance, human cancers express high levels of B7-H1 protein, whereas human tumour cell lines have lowered B7-H1 expression, perhaps caused by the lack of tumour microenvironment or altered molecular profiles (reviewed in [263]). Based upon the structural homology of the NKp30 receptor with the CD28 receptor family, which recognize the B7-family of proteins, it is possible that a B7-family protein could be interacting with, and inhibiting, NK cell functions through NKp30 in our *in vitro* system (201, 202, 255). As both B7-H1 [also referred to as programmed cell death ligand 1 (PD-L1) or CD274] and B7-H4 (also referred to as B7S1 or B7x) ligands provide inhibitory signals through their interacting receptors, these ligands will be discussed in brief with respect to HCV infection and their expression on tumour cells (reviewed in [263]).

Several studies demonstrated high levels of inhibitory B7-family molecules, B7-H1 and B7-H4, which are known to contribute to T cell immune evasion, on human cancer cell lines (reviewed in [264, 265]). Increased expression of inhibitory B7-family molecules on the human hepatoma Huh-7.5 cell line could potentially explain low levels of direct Huh-7.5 cell lysis by NK cells *in vitro* (data not shown), contingent on an inhibitory B7-family molecule:cognate NK cell receptor interaction. Primary hepatocytes constitutively express B7-H1 at low levels, however, B7-H1 expression is enhanced upon viral infection (266). Also, it has been shown that high levels of inhibitory B7-family ligand expression (B7-H1 and B7-DC) correlated with chronic liver inflammation in HCV-infected individuals (267). Thus, infection of human hepatoma Huh-7.5 cells with HCV could have a compounding effect on the expression of stress-induced inhibitory B7-family ligand expression and may provide an antagonistic ligand for NKp30. Not only would this perturb immune modulation of HCV-infected (or cancerous) cells by T cells through PD-1, but could also play a dual role in the modulation of cytotoxic NK cell functions; the latter remains to be determined. Recently, Zhao *et al.* characterized a human endogenous retrovirus long terminal repeat-associating protein 2 (HHLA2) as a member of the B7 family of proteins that binds to a putative CD4^{+} and CD8^{+} T cell receptor, inhibiting cytokine production and cellular proliferation (268). Determining an antagonistic NKp30 ligand of host or viral origin on HCV-infected cells could provide new insights as to the pathogenesis of this viral infection.

There are many mechanistic possibilities by which HCV-infected Huh-7.5 cells inhibit NK cell cytotoxicity and cytokine production. Based upon published literature and our own observations, we reason that inhibited NK cell functions stems from the interaction of NKp30 with an inhibitory B7 family protein, which is induced on Huh-7.5 cells upon HCV infection. This ligand may either interact directly with the NKp30 receptor, or interact with and disrupt the ζ chain adaptor protein in a manner similar to HCMV pp65. Ultimately, downstream NK cell signaling is affected resulting in inhibited cytotoxicity and cytokine production. Interaction of an inhibitory B7 family ligand with NKp30 can also account for disrupted ADCC noted through CD16, as these receptors can have a common adaptor molecule (i.e. ζ chain homo or heterodimers) and share a concerted signaling pathway. CD81 could contribute to NK cell signaling in the same manner as it does with B and T cell co-receptors, through its lateral interaction with NKp30. Finally, as the actin cytoskeleton rearranges to allow release of cytotoxic granules, engagement of NKp30 with a putative antagonistic ligand and its potential interaction with CD81 can ultimately lead to an alteration of actin cytoskeleton, thereby depressing granzyme release.

Evidence is emerging that indicates NK cells play an important role in host defenses against virus infection and tumour progression. Although our understanding of T cells has led to the development of therapies to suppress or enhance T cell functions, NK cell-based immunotherapy has been delayed, as the mechanisms involving NK cell activation and inhibition as well as knowledge of the cellular distribution of NK cells and *in vivo* homing to tissues remained undefined until the late 1990s (269-271). Before we can determine if NK cells can be modified for viral therapy, further insight into their complete role in HCV infection must be achieved. It is important to establish why the combination of inhibitory KIR2DL3 with HLA-C group 1 alleles is beneficial for the spontaneous clearance of HCV infection (19, 20, 272). In the context of HIV infection, Alter *et al.* describe attenuation of HIV-1 replication in the presence of KIR2DL2$^+$ NK cells, however, HIV-1 evades this mechanism of NK cell recognition by selecting viral variants with amino acid polymorphisms that augment the binding of inhibitory KIRs to infected CD4$^+$ T cells (17). Thus, these data convey that enhanced NK cell activity contributes to the control of viral infection, and that viruses can evade this immune response through the rapid emergence of variants, which can escape stimulatory, and enhance inhibitory, KIR recognition. Inhibitory KIR genes can affect NK cell responses to viral infections through KIR education (273), a phenomenon that gives plasticity to a once-rigid theory for NK cell responsiveness. Our data adds to the increasing evidence that NK cells play a role in the initial control of viral infections and provides insights into the basic biology of NK cells, since HCV-mediated attenuation of activating receptor

(NKp30) expression can impact NK cell responsiveness. Future antiviral or cancer therapy may follow a more extensive understanding of functionally distinct NK cell subsets.

6 Future Directions

As discussed in Chapter 5, the initial focus of future research in this area should involve elucidating the antagonistic NKp30 ligand up-regulated on HCV-infected Huh-7.5 cells. In addition, determining the NKp30 ligand-binding domain or the association of the putative NKp30 ligand with an adaptor molecule associated with NKp30 would be beneficial. Further, the impact this ligand binding to NKp30 on downstream signaling cascades or cytoskeletal redistribution should be probed. Although ADCC was inhibited, it would be interesting to determine the link between down-regulated NKp30 expression and CD16 dysfunction. Finally, this established assay could further be used to probe activity of NK cells from HCV positive individuals compared to healthy controls. The data obtained from clinical samples could determine whether altered NKp30 expression is an immediate or lasting effect in the context of HCV infection. Furthermore, this assay platform could also be beneficial for the study of NK cell functions in the context of other adherent tissue culture models of virus infection, such as HCMV.

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