Critical roles of cytomegalovirus-induced Natural Killer cells in chronic hepatitis C virus infection and rituximab-mediated cancer therapy

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ABSTRACT

Natural Killer (NK) cells, members of the innate lymphoid cells (ILCs), are known to play an important role in the defense against foreign cells and abnormal host cells that have arisen due to viral infection or cancer inducing mutations. The typical immune response of NK cells involves the release of cytotoxic granules containing perforin and granzyme, and the secretion of immune-regulatory cytokines such as interferon gamma (IFN-γ). Unlike the adaptive lymphocytes such as T cells and B cells, NK cells do not require prior sensitization, enabling them to initiate an immune response much faster. This unique feature of NK cells is made possible by the utilization of an array of germline encoded receptors; but on the other hand, it limits NK cells ability to respond against rapidly evolving pathogens. NK cells overcome this shortcoming with an antibody-assisted process called antibody dependent cellular cytotoxicity (ADCC).

A novel subset of human NK cells, which displays potent and broad antiviral responsiveness in concert with virus-specific antibodies, was recently discovered in cytomegalovirus positive (CMV+) individuals. This NK cell subset, called g-NK cell, was characterized by a deficiency in the expression of FcεRIγ, an adaptor protein that associates with CD16 which enables ADCC. Surprisingly, despite this deficiency, g-NK cells displayed an enhanced ADCC as compared to their conventional counterparts. Furthermore, having a long-lasting memory-like NK-cell phenotype suggests a role for g-NK cells in chronic infections.

This study investigates the importance of g-NK-cells in clinical settings, first by investigating whether the presence of g-NK cells is associated with the magnitude of liver disease during chronic hepatitis C virus (HCV) infection. Analysis of g-NK cell proportions and function in the peripheral blood mononuclear cells (PBMCs) of healthy controls and chronic HCV subjects showed that chronic HCV subjects had slightly lower proportions of g-NK cells, while having

similarly enhanced ADCC responses compared to conventional NK cells. Notably, among CMV+ chronic HCV patients, lower levels of liver enzymes and fibrosis were found in those possessing g-NK cells. g-NK cells were predominant among the $CD56^{neg}$ NK cell population often found in chronic HCV patients, suggesting their involvement in the immune response against HCV.

Rituximab is a chimeric anti-CD20 antibody used to treat B cell lymphoma patients; and studies have suggested that its efficacy is associated with the ADCC potency and CD16 affinity. Since g-NK cells are characterized by their superior ADCC compared to their conventional counterpart, I decided to investigate whether the presence of g-NK cells can improve the effectiveness of rituximab against malignant B cells in the context of lymphoma and leukemia. The analysis of g-NK cells' ADCC response against rituximab-coated lymphoma cell lines and B cells from a CLL patient indicated a superior ADCC by g-NK cells compared to their conventional NK cell counterparts.

Taken together, for the first time, my findings indicate that the presence of g-NK cells in CMV+ individuals is associated with milder liver disease in chronic HCV infection. In addition, an enhanced ADCC response by g-NK cells upon encountering rituximab coated target cells suggests the beneficial roles of g-NK cells, and opens an avenue for novel therapeutic approaches where g-NK cells can be utilized to treat persistent diseases such as chronic viral infection and cancer.

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Contributions:

Jun S. Oh: Performed all experiments. Contributed to the writing and editing of the manuscript.

Alaa K. Ali: Assisted with the blood process, surface and intracellular staining, and ELISA. Contributed to the editing of the manuscript.

Sungjin Kim: Provided guidance regarding g-NK cells.

Daniel J. Corsi: Provided guidance in regard to statistical analysis.

Curtis L. Cooper: A clinician and collaborator from The Ottawa Hospital who provided guidance as well as patient data and samples.

Seung-Hwan Lee: Supervised, designed and participated in the execution of experiments. Contributed to the writing and editing of the manuscript.

Table of Contents

LIST OF ABBREVIATIONS

^{o}C	Celsius (temperature)
ADCC	Antibody Dependent Cellular Cytotoxicity
ALL	Acute Lymphoblastic Leukemia
ALT	Alanine Transaminase
AML	Acute Myeloid Leukemia
AST	Aspartate Transaminase
CCL	Chemokine C-C Motif Ligand
CD	Cluster of Differentiation
CLL	Chronic Lymphocytic Leukemia
CMV	Cytomegalovirus
DAA	Direct acting antiviral
DAB2	Disabled homolog 2
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EAT-2	Ewing's sarcoma-associated transcript 2
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FcεRIγ	Fragment Crystallizable Epsilon Receptor I gamma
FcγRIIIα	CD16a
FcR	Fc Receptor
g-NK	Gamma-chain negative Natural Killer
GGT	Gamma Glutamyl Transpeptidase
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HBV	Hepatitis B virus
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV	Human Immunodeficiency Virus
HL	Hodgkin Lymphoma
HSV	Herpes Simplex Virus
IFN	Interferon
IgG	Immunoglobulin G
ILCs	Innate Lymphoid Cells
iNOS	Inducible Nitric Oxide Synthase
ITAM	Immuno-receptor Tyrosine-based Activation Motifs
kb	kilo base
LAMP	Lysosome Associated Membrane Glycoprotein
mAb	Monoclonal Antibody
METAVIR	Meta-analysis of Histological Data in Viral Hepatitis
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
µg	microgram
µL	microlitre
NHL	Non-Hodgkin Lymphoma
NK	Natural Killer
NKG2	Natural Killer group 2
NO	Nitric Oxide
PAMPs	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PLZF	Promyelocytic Leukaemia Zinc Finger
pp65	Phosphoprotein 65
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
RCF	Relative centrifugal force
RNA	Ribonucleic Acid

RPMI 1640	Roswell Park Memorial Institute 1640 medium
SVR	Sustained virologic response
SYK	Spleen Tyrosine Kinase
TNF	Tumor Necrosis Factor
TRAIL	TNF Related Apoptosis Inducing Ligand
ZAP70	Zeta-chain Associated Protein Kinase 70kDa (kilo Dalton)

LIST OF FIGURES AND TABLES

1. INTRODUCTION

1.1 Natural Killer (NK) cell

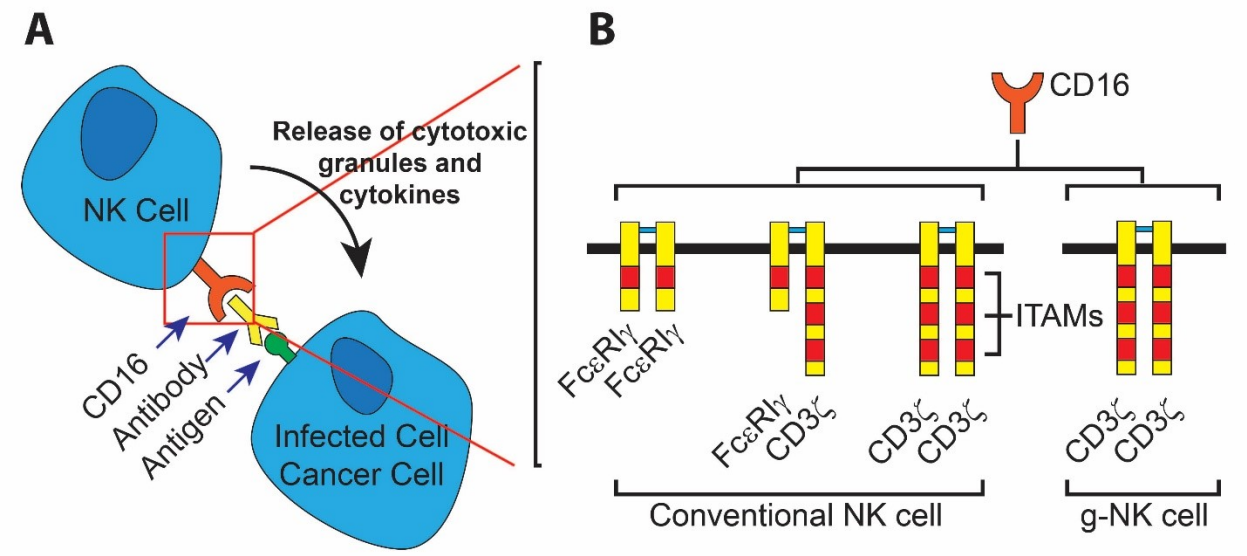
The Natural Killer (NK) cell, a large cell featuring a distinctive granular cytoplasm, is part of the innate lymphoid cells (ILCs) that arise from common lymphoid progenitor cells found in the bone marrow. The resulting NK precursor cell then traffics through the lymph nodes, spleen and peripheral blood for further maturation and differentiation (Huntington et al., 2007). NK cells are specialized in the rapid recognition and killing of abnormal target cells, such as virus-infected cells and cancer cells, without prior sensitization (Biron et al., 1999; Lee et al., 2007). This unique ability is achieved by utilizing a repertoire of germline-encoded natural cytotoxicity receptors (NCR), as well as activating and inhibitory receptors that are specifically designed to detect stress ligands, self-ligands, Pathogen-Associated Molecular Patterns (PAMPs), and antibodies, exclusively Immunoglobulin G (IgG). Upon activation, NK cells induce the apoptosis of abnormal cells through the release of cytotoxic granules containing perforin and granzyme. Alternatively, NK cells can induce cell lysis through Fas/FasL interactions, Tumor necrosis factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL), or TNF-α pathways (Biron et al., 1999; Lee et al., 2007; Vivier et al., 2008; Warren and Smyth, 1999). NK cells also participate in the immune regulation through their surface receptors and production of pro-inflammatory and regulatory cytokines such as Interferon gamma (IFN-γ), TNF, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Macrophage Inflammatory Proteins (MIP-1α/CCL3), MIP-1β/CCL4, and Regulated on Activation Normal T cell Expressed and Secreted (RANTES/CCL5) (Biron et al., 1999). Human NK cells in healthy individuals are commonly identified by using CD16 (Fc receptor) and CD56 (neural cell adhesion molecule) markers and divided into two

separate populations: $CD16^{-}CD56^{bright}$, and $CD16^{+}CD56^{dim}$. The $CD16^{-}CD56^{bright}$ NK cell population, which is found at low proportions in the blood, is highly immune-regulatory; whereas the $CD16^{+}CD56^{dim}$ NK cell population is highly cytotoxic and makes up the majority of the NK cell population in the blood of healthy individuals (Caligiuri, 2008). Adaptive immune cells such as T cells and B cells utilize gene rearrangement mechanisms to generate a near limitless repertoire of antigen specific receptors. This mechanism involves the rearrangement of variable (V), diversity (D), joining (J) gene segments, and constant (C) genes located in TCRα locus (chromosome 14) and TCRβ locus (chromosome 7) for T cells, and the immunoglobulin heavy, κ, and λ loci (located in chromosome 14, 2 and 22 respectively) for B cells. This feature, although time consuming, enables them to recognize any known or unknown highly evolving foreign target and mount an immune response such as antibody production. Unlike these adaptive immune cells, NK cells lack such mechanism that would allow them to recognize rapidly evolving pathogens. In order to overcome this limitation, NK cells utilize the Fc receptor CD16, an activating receptor, to expand the range of targets that can be detected. The CD16 molecule interacts with IgG antibody coated target cells; and plays a crucial role in a mechanism known as antibody dependent cellular cytotoxicity (ADCC) (Vivier et al., 2008). This allows NK cells to enjoy the benefit of a faster response, and in addition, a limitless target recognition capability driven by antibody.

Several immune cells possessing Fc receptors, such as NK cells, monocytes, macrophages, and eosinophils can utilize ADCC. This critical mechanism is important for NK cells as it allows them, with the help of target specific antibodies, to recognize highly evolving abnormal cells that have managed to develop ways to evade direct recognition by NK cells through germ-line encoded receptors. In NK cells, the process of ADCC begins from NK cells' Fc receptor CD16

(FcγRIIIα) that binds to the Fc fragment of IgG (preferably to IgG1 and IgG3) bound to the target cell (Takai, 2002). Upon binding, activated CD16 covalently interacts with hetero- or homodimers of membrane-bound signalling adaptors such as FcεRIγ (γ chain of high affinity tetrameric IgE receptor that can also interact with other Fc receptors) and CD3ζ that possess Immunoreceptor Tyrosine-based Activation Motif (ITAM), which acts as signalling transducers (**Figure 1**) (Hwang et al., 2012). The phosphorylation of ITAM leads to the recruitment of Spleen Tyrosine Kinase (SYK) and Zeta-chain Associated Protein kinase 70kDa (ZAP70) which subsequently activate Linker for Activation of T cells (LAT) (Maghazachi, 2005). The activation of LAT leads to a change in the intracellular calcium ion concentration via Phospholipases C gamma 1 and 2 (PLC-gamma 1 and 2) activation (Ting et al., 1992). The change in calcium ion concentration activates calcineurin which is responsible for dephosphorylating Nuclear Factor of Activated T-cell Cytoplasmic Calcineurin-dependent 2 (NFATC2). Dephosphorylated NFATC2 then travels to the nucleus in order to initiate the transcription of various cytokines, such as IFN-γ, for NK cell (Sica et al., 1997). NK cells activated by CD16 rapidly internalize and/or shed the receptor, leading to reduced CD16 expression on their surface (Masilamani et al., 2009).

Figure 1: The representative diagram of signaling molecules involved in ADCC. **(A)** Upon encounter with antibody coated target cell, the Fc receptor, CD16, of NK cells binds to the Fc region of target cell bound IgG antibody and releases cytotoxic granules and anti-viral immune-regulatory cytokines. **(B)** Upon activation, CD16 associates with a heterodimer or homodimer of intracellular adaptor proteins, FcεRIγ and CD3ζ, in order to transmit signals further downstream via immunoreceptor tyrosine-based activation motif (ITAM). CMV associated NK cell subset, g-NK cells, lost the FcεRIγ chain, hence it only has CD3ζ. It is also important to note that the FcεRIγ chain contains a single (ITAM), whereas the CD3ζ chain contains three ITAMs, implying that enhanced CD16-mediated signaling may be due to CD3ζ chain.


A
NK Cell
Release of cytotoxic granules and cytokines
CD16
Antibody
Antigen
Infected Cell
Cancer Cell
B
CD16
ITAMs
FcεRIγ
FcεRIγ
FcεRIγ
CD3ζ
CD3ζ
CD3ζ
CD3ζ
CD3ζ
CD3ζ
Conventional NK cell
g-NK cell

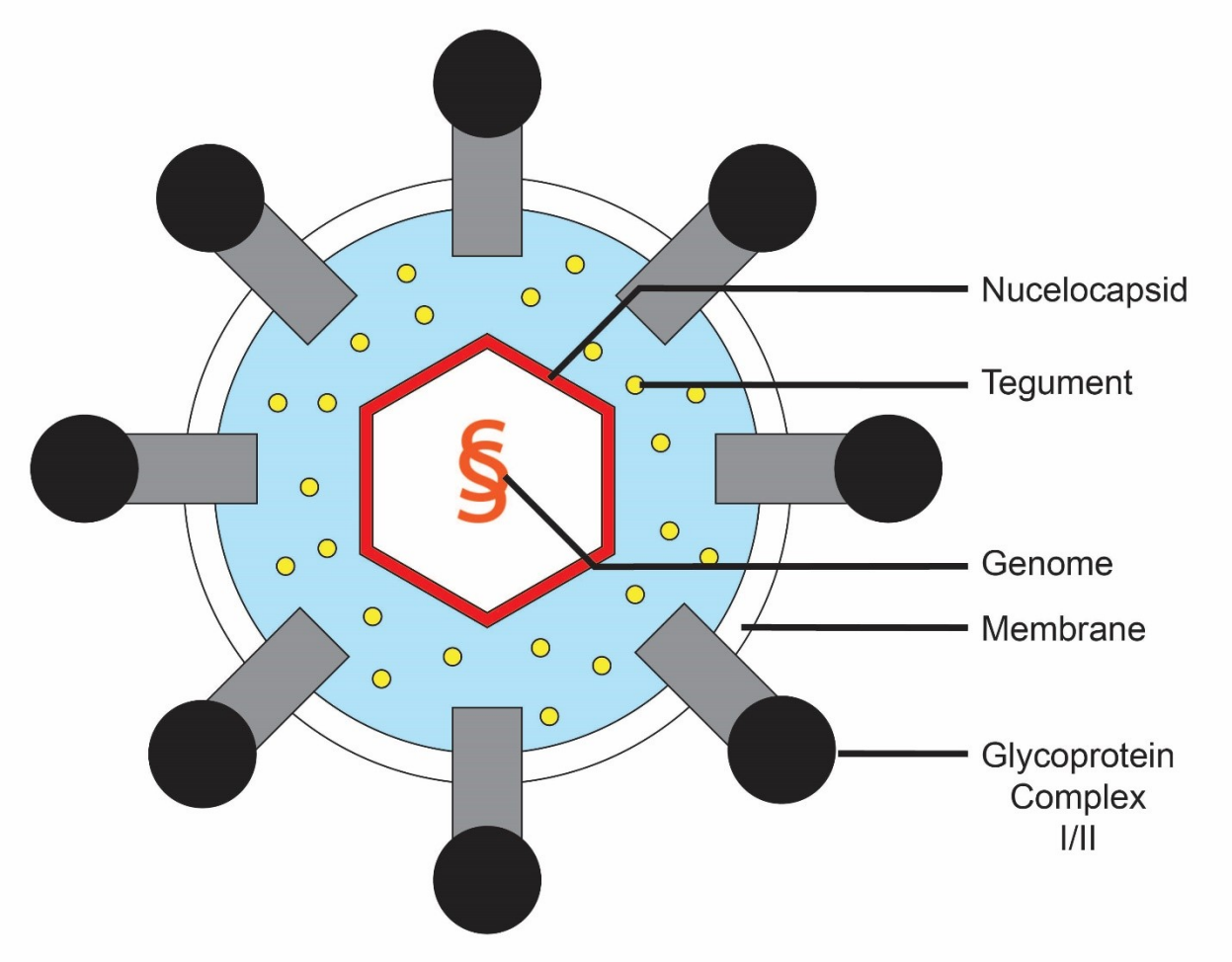
1.2 Human Cytomegalovirus (HCMV)

The human cytomegalovirus (HCMV) belongs to the *Herpesviridae* family under the *Betaherpesvirinae* subfamily. This virus shares common structural characteristics with other herpesviruses. Its viral genome, which consists of 235 kilobase pair (kb) of double stranded DNA, is contained within an icosahedral nucleocapsid. The nucleocapsid is surrounded by amorphous tegument proteins within the outer lipid membrane that contains glycoprotein complex I and II which facilitate membrane fusion with the host cell, thereby these glycoproteins are critical in viral entry (Griffiths et al., 2015). Upon fusion of both viral and host cell membranes, the nucleocapsid and tegument proteins are released into the cytoplasm of the host cell. Upon entry, the expression of viral immediate-early (IE) genes produces IE proteins that are necessary to replicate the viral DNA genome. The expression of late genes begins after DNA replication and leads to the production of late proteins which mostly consist of structural proteins necessary for the assembly and release of progeny virus. The tegument mainly consists of the pp65 protein, which is also referred to as ppUL83 or Lower matrix protein (**Figure 2**). Although pp65 is not critical for viral replication, this protein is critical in the formation of noninfectious extracellular viral particle, so called dense bodies that lack a nucleocapsid. The pp65 protein is also known to play a role in immune evasion (Kalejta, 2008). Firstly, pp65 interacts with NKp30, which is an activating receptor on NK cells, to inhibit innate NK cell mediated cytotoxicity. Secondly, pp65 can help HCMV evade the adaptive immune response by either preventing the presentation of viral IE proteins derived peptides via major histocompatibility complex (MHC, also known as HLA in human) class I molecules, or by causing the degradation of the alpha chain in the MHC class II cell surface receptor HLA-DR. Thirdly, pp65 is also known to down-modulate the expression of MHC class II, interferon, and chemokines. These properties indicate

that pp65 plays a significant role in immune evasion against NK cells and T cells (Kalejta, 2008; Tomtishen, 2012). A study has found that pp65 is a major epitope that is commonly recognized by CD4 and CD8 T cells (Sylwester et al., 2005); hence making it an ideal antigen to test individuals' prior HCMV encounter.

NK cells are known to play a critical role in the immune response against herpesvirus infections. Deficiencies in NK cell population or function have been associated with an increased susceptibility to herpesviruses, including cytomegalovirus (CMV), EBV, and varicella zoster virus (Orange, 2013). Among herpesviruses, CMV is highly prevalent, with a 50-100% seroprevalence rate depending on the geographical location and socioeconomic factors. On average, the prevalence of CMV infection is low in modernized countries located in Europe, Australia, and North America, while it is relatively high in developing countries located in Africa and Southeast Asia. The prevalence of CMV infection also varies within the same country (Ho, 1990). HCMV infects people of all ages, gender, and races. This virus transmits via close contact with infected bodily fluids such as saliva, blood, urine, breast milk, cervical and vaginal excretions, and semen. In addition, it is also commonly known to be transmitted *in utero*, leading to birth defects in infants (Gaytant et al., 2002). Once it enters a host, it can infect many cell types, such as fibroblasts, smooth muscle cells, tissue macrophages, epithelial cells and endothelial cells (Sinzger et al., 1995). The CMV infection is generally asymptomatic in an immunocompetent host, while establishing a lifelong latent infection. However, reactivation from a latent infection can occur in immunocompromised individuals including transplant recipients, neonates, or human immunodeficiency virus (HIV) infected individuals, leading to morbidity and mortality (Crough and Khanna, 2009; Dowd et al., 2009).

Figure 2. The representative diagram of CMV structure. HCMV, a member of the herpesviridae family, consists of an icosahedral nucleopcapsid containing a double stranded DNA viral genome. The nucleocapsid is surrounded by the tegument, and an outer lipid membrane that contains glycoprotein complex I and II.


Nucelocapsid
Tegument
Genome
Membrane
Glycoprotein
Complex
I/II

1.3 NK cell subset: g-NK cell

Interestingly, CMV infection was shown to induce the expansion of a unique subset of human NK cells that highly express the CD94-NKG2C activating receptor which can interact with HLA, and are sustained for many years (Lopez-Verges et al., 2011). This finding was surprising because NK cells were initially thought of as short-lived cells, lasting about 10 days (Prlic et al., 2003; Ranson et al., 2003). Interestingly, this unique NK cell subset can be activated upon subsequent encounters with other viruses, including HIV-1, hantavirus, chikungunya virus, hepatitis B virus (HBV), and HCV (Beziat et al., 2012; Bjorkstrom et al., 2011; Brunetta et al., 2010; Guma et al., 2006; Petitdemange et al., 2011), suggesting the importance of the subset in a broader range of infections. The NK cell subset was also marked by the expression of the cell surface receptor CD57, a maturation marker (Lopez-Verges et al., 2011).

Recently, an NK cell subset that is highly associated with prior CMV infection and lacking the FcεRIγ adaptor protein (hence called g-NK or gamma-negative NK cells) was identified. CD57 and NKG2C were largely coexpressed on the g-NK cells, suggesting that g-NK cells are highly related to CD57+NKG2C+ NK cells (Hwang et al., 2012; Zhang et al., 2013). Upon binding of CD16 with the Fc portion of a cell bound antibody, either a homodimer or heterodimer of adaptor proteins, FcεRIγ and CD3ζ, will physically associate with CD16 in conventional NK cells. This interaction induces ADCC by transmitting biochemical signals through ITAMs that are present on the adaptor proteins (**Figure 1**) (Lanier, 2008). Upon phosphorylation, the ITAM-bearing subunits bind the tyrosine kinases SYK and ZAP-70 that activate the functions of NK cells such as cytokine secretion and cytotoxicity. Surprisingly, similar to the loss of FcεRIγ expression, SYK downmodulation also occurred in g-NK cells (Hwang et al., 2012; Schlums et al., 2015). In addition, despite the lack of both FcεRIγ and SYK proteins, which are critical in

transmitting biochemical signals, g-NK cells exhibited enhanced ADCC compared to conventional NK cells. The expression of other critical proteins, CD3ζ adaptor and ZAP-70, were intact in g-NK cells, suggesting that the CD3ζ-ZAP70 pathway might be predominantly responsible for the signal transduction emanated from CD16 in g-NK cells (Hwang et al., 2012; Lee et al., 2015; Zhang et al., 2013). Moreover, the downregulation of several signaling molecules including EAT-2, PLZF, and DAB2 was found in varied manners, suggesting the complex nature of g-NK cells (Lee et al., 2015; Schlums et al., 2015).

Currently, g-NK cells are known to be highly associated with prior CMV infection; but their ability to respond to infection is not strictly limited to CMV infection. Interestingly, in addition to an enhanced response against CMV infected cells, g-NK cells were also able to exhibit enhanced ADCC capability against HSV and influenza infected cells in the presence of plasma from infected individuals (Lee et al., 2015; Zhang et al., 2013). Therefore, it would be interesting to determine the importance of g-NK cells in other chronic virus infections such as HBV, HCV, and HIV.

1.4 Hepatitis C Virus (HCV) infection

Hepatitis C virus, a member of the *Flaviviridae* family, contains a positive sense, single stranded RNA as its genome. Human is the only natural host for this virus and it is transmitted mainly via contaminated blood, and unsafe usage of needles. Upon entering the host, HCV gains access to the liver via the hepatic artery and the portal vein, where it primarily infects hepatocytes (Heim, 2013). HCV replicates very fast as it is capable to produce about 10 trillion viral particles per day. Despite this rapid replication, it is suggested that the virus itself does not destroy the infected

hepatocytes, but instead it is the host's own immune cells, such as cytotoxic T cells, that are actually causing damage to the liver (Guidotti and Chisari, 2006). Destroyed hepatocytes will release various enzymes such as alanine transaminase (ALT), aspartate transaminase (AST), and gamma-glutamyl transpeptidase (GGT) into the blood; hence the measurement of these enzymes in the serum is used to evaluate the liver function and allows the detection of liver injury. The METAVIR stage, which indicates the level of liver fibrosis, is also used to assess the liver condition (Gowda et al., 2009; Theise, 2007).

Currently, over 130-150 million people worldwide are infected with hepatitis C virus (HCV) (World Health Organization). This corresponds to approximately 3% of the world's population. HCV is a major cause of chronic viral hepatitis that can lead to cirrhosis and eventually to hepatocellular carcinoma (HCC). Not all HCV infections lead to chronic viral hepatitis as spontaneous resolution can occur in a minority of cases (about 25%), with the majority (about 75%) progressing to chronic infection that can culminate in liver cirrhosis, liver failure (about 20%), and HCC (about 2% per year) (Post et al., 2009). A natural resistance to HCV infection has been associated with a strong, multifaceted cellular immune response, which includes a rapid and strong NK cell response early on during infection (Holder et al., 2014; Lloyd et al., 2007; Rehermann, 2013). As IFNs are known to have immune-regulatory and antiviral features, IFN based therapy, in combination with ribavirin, has been used in the past to treat HCV infection. A strong NK cell-mediated cytotoxicity induced by the IFN treatment was highly correlated with the virologic response in HCV infection (Ahlenstiel et al., 2011). Moreover, an association of IFN-induced expression of TRAIL on NK cells with the control of HCV infection has been reported (Stegmann et al., 2010). The recent development of direct acting antiviral (DAA) treatment, such as Sofosbuvir that directly inhibits viral RNA synthesis via disabling the viral

RNA polymerase, has led to more success in achieving sustained virologic response (SVR) (Gentile et al., 2015), where the SVR status is defined by absence of detectable HCV in the blood 24 weeks after completing the therapy. This surprising benefit comes without any IFN associated side effects, such as fever, headache, fatigue, arthralgia, and myalgia; making the DAA treatment superior to the IFN based HCV treatment. Even though NK cells are known to be important for the early immune control of HCV, it is unclear whether the NK cell response also contributes to the protection from liver disease in chronic HCV infection (Park and Rehermann, 2014).

1.5 Lymphoma and leukemia

Lymphoma is a type of cancer that begins from lymphocytes in the lymphatic system; whereas leukemia is a type of cancer that begins from blood forming cells in the bone marrow. Lymphoma is classified into either Hodgkin Lymphoma (HL) or Non-Hodgkin Lymphoma (NHL) depending on the presence or absence of Reed-Sternberg cells (a large, abnormal multinucleated cell derived from B cells) respectively (American Cancer Society). On the other hand, leukemia is classified into four types depending on the type of blood forming cells that are affected. The four types are Acute Lymphoblastic Leukemia (ALL), Acute Myeloid Leukemia (AML), Chronic Lymphocytic Leukemia (CLL), and Chronic Myeloid Leukemia (CML). Although CLL is type of leukemia, it is also considered as a NHL due to its shared disease characteristics and treatment regime with one of the NHL subtype known as Small Lymphocytic Lymphoma (SLL) (American Cancer Society). Among the various types of leukemia, CLL is the most frequent form found in Western countries and appears more commonly in men aged on average in the 60s (Rozman and Montserrat, 1995). It is characterized by the proliferation and

accumulation of abnormal B cells that expresses CD5, CD19, CD20, and CD23, but with reduced levels of surface-membrane immunoglobulins, such as IgM, IgD, and CD79b. These abnormal cells accumulate in the blood, bone marrow, lymph nodes, and spleen and are commonly diagnosed via a blood smear test. The course of the disease varies between individuals. While some patients can live many years without therapy, others rapidly progress into a fatal outcome and die within five years after detection despite the intense therapy (Chiorazzi et al., 2005; Rozman and Montserrat, 1995). Although the cause of disease remains unknown, the severity of the disease was found to be determined by factors such as the presence of V gene mutations, as well as CD38 and ZAP70 expression. For example, patients with clones lacking V gene mutations or having many B cells expressing CD38 or ZAP70 were found to carry an aggressive form of CLL; while patients with mutated clones or having few B cells expressing CD38 or ZAP70 were found to have an indolent form of CLL (Chiorazzi et al., 2005).

1.6 Monoclonal antibody-mediated cancer therapy

Over the last few years, therapeutic monoclonal antibodies (mAbs) that recognize tumor-specific antigens on the surface of tumor cells have been used successfully as cancer therapy (Scott et al., 2012; Wang et al., 2015). These therapeutic mAbs include humanized anti-GD2 antibody for the treatment of melanoma and neuroblastoma cells, trastuzumab (humanized anti-HER2 Ab) for breast carcinoma, and cetuximab (humanized anti-EGFR Ab) for metastatic colorectal cancer and head and neck cancer. In addition, humanized anti-CD20 antibody (rituximab) is being used to treat B cell lymphoproliferative malignancies, particularly NHL (Alderson and Sondel, 2011; Iannello and Ahmad, 2005). Various mechanisms have been suggested to explain the beneficial effect of monoclonal antibodies, including blocking proliferation signals on tumor cells,

complement-dependent cytotoxicity, and ADCC by FcR+ cells (Wang et al., 2015; Weiner, 2010). Notably, the anti-tumor effect of rituximab was greatly reduced in FcR-deficient mice in xenograft mouse models of B cell lymphoma (Clynes et al., 1998; Clynes et al., 2000), suggesting that ADCC is a principal mechanism for the anti-tumor response. Approaches to enhance ADCC appear to clinically translate to improved antitumor activity. For example, patients with NHL harboring a high-affinity CD16 polymorphism have been shown to respond more favorably to rituximab with better remission and overall survival rates (Bowles and Weiner, 2005; Cartron et al., 2002; Kim et al., 2006). As g-NK cells are known for their superior ADCC capability, g-NK cells may have a great potential to enhance monoclonal-mediated cancer therapy.

1.7 Hypothesis and statement of objectives

Considering the long lasting and enhanced ADCC characteristics that endow g-NK cells with a broad range of immune surveillance capabilities, I reasoned that maintaining g-NK cells is advantageous to fight against an array of chronic infections and cancer. Therefore, I investigated the impact of g-NK cells in chronic HCV infection and in monoclonal antibody-mediated immunotherapy against cancer (rituximab coated lymphoma cell lines and B cells from CLL patients).

AIM 1: Investigate whether the maintenance of g-NK cells is advantageous to resist against chronic viral infection.

I analyzed blood samples from chronic HCV patients to answer the following main questions:

1) Are g-NK cells also present in chronic HCV patients who are CMV seropositive?
2) Do g-NK cells in chronic HCV patients retain their superior ADCC compared to their conventional counterparts?
3) Is the presence of g-NK cells in chronic HCV patients associated with a positive clinical outcome?

AIM 2: Investigate the effectiveness of g-NK cells during monoclonal antibody-mediated therapy against cancer such as lymphoma and CLL.

I analyzed lymphoma cell lines (Daudi, Raji, and RAMOS) and PBMC of CLL patient to answer the following main questions:

1) Can g-NK cells exhibit superior ADCC upon encountering rituximab coated target cells?

2. MATERIALS AND METHODS

2.1 Study subjects

Blood samples from 34 healthy volunteers with an average age of 36 years and 47 chronically infected HCV+ individuals (i.e. >6 months HCV RNA positive) with an average age of 49 years who were antiviral naive were collected at The Ottawa Hospital in collaboration with Dr. Curtis Cooper (**Table 1**). Heparin treated green capped tubes (Vacutainer; BD, Franklin Lakes, NJ, USA) were used for the collection and transportation of the blood samples. The patients information, such as age, sex, ethnicity, alcohol consumption status, HCV viral load level, ALT, AST, GGT, and METAVIR, were provided by the hospital. A written informed consent was obtained from all participants, and the study was approved by The Ottawa Health Science Network Research Ethics Board (Ottawa Health Science Network REB 2012-0009, REB attached in appendix).

Table 1. Patient Characteristics. This study consists of 34 healthy volunteers with an average age of 36 years, and 47 chronic HCV patients (i.e. >6 months HCV RNA positive) with an average age of 49 years.

	Healthy (n = 34)		HCV (n = 47)		CLL (n = 1)
Age	36±13		49±9		72
Sex	M = 15; F = 19		M = 32; F = 15		M = 1
Ethnicity	Asian	4	Black	1	Not given
	Black	1	Caucasian	44	
	Caucasian	25	Methis	2	
	Hispanic	2			
	Mid East/Arab	2			
EtOH consumption	N/A		Yes	29	N/A
			No	15	
			Unknown	3	
CMV serostatus (+/-)	15/19		27/20		Not given
METAVIR F0	ND		1		ND
METAVIR F1	ND		15		ND
METAVIR F2	ND		7		ND
METAVIR F3	ND		6		ND
METAVIR F4	ND		5		ND
ALT	ND		39		ND
AST	ND		39		ND
GGT	ND		37		ND

Age values are represented as mean±SD.
ND = Not determined.

2.2 Extraction and storage of peripheral blood mononuclear cells and plasma from blood samples

Plasma was isolated by centrifuging patient blood samples supplemented with heparin (Fisher Scientific, Waltham, MA, USA). PBMCs were isolated by Ficoll gradient density centrifugation (Ficoll-Paque PLUS; GE Healthcare, Mississauga, ON, Canada). This process was done by carefully overlaying blood on Ficoll and centrifuging at 515 relative centrifugal force (RCF) for 35 minutes with the lowest acceleration and without any brakes. The collected PBMCs were then aliquoted in freezing media consisting of 10% DMSO (Fisher BioReagents, Fisher Scientific) and 90% FBS (Gibco; Life Technologies, Burlington, Ontario, Canada), and stored at -80^{o}C for later use. The cells were thawed and incubated in complete RP-10 media, consisting of RPMI 1640 (HyClone; GE Healthcare) supplemented with 10% FBS, 2 mM L-glutamine (HyClone), 10 mM HEPES buffer (HyClone), 1% penicillin/streptomycin (HyClone), and 55 μM 2-mercaptoethanol (Gibco), overnight before assessing cell surface and intracellular proteins expression and cell response upon stimulation.

2.3 CMV serology and peptide stimulation

Plasma samples were screened for CMV serological status using human CMV (HCMV) specific ELISA kits (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. For the detection of CMV specific T cell response, PBMCs were incubated overnight in complete RP-10 media. Subsequently, PBMCs were stimulated in sterile 96-well V-bottom plates at 2 x 10^5 cells/well with HCMV-specific pp65 peptide (NIH AIDS reagent program, Germantown, MD, USA) at 0.5 μg/mL, for 1 hour followed by an additional 6 hours stimulation in the presence of 5 μg/mL of Brefeldin A (Sigma-Aldrich, Oakville, Ontario, Canada). Positive

controls contained control CEF peptide pool, which is a pool of peptides containing MHC class I restricted T cell epitopes from human CMV, Epstein Barr, and influenza virus (NIH AIDS reagent program). Prior CMV infection was primarily determined by HCMV specific ELISA. Subjects showing uncertain ELISA data, where the absorbance reading was very close to the threshold value calculated in accordance with the kit, were further analyzed by measuring their HCMV specific T cell response.

2.4 Flow cytometry analysis of NK cell phenotype and function

Multicolor flow cytometry was performed using the CyAn ADP 9 analyzer (Beckman Coulter, Indianapolis, IN, USA) to analyze NK cell subpopulations and phenotypes. A complete list of antibodies used for staining is available in **Table 2**. The gating strategy for the identification of NK cells and the definition of g-NK cells is shown in **Figure 5A**. In order to determine g-NK cells, $CD14^{-}CD19^{-}$ live cells were utilized to set a gate for FcεRIγ. Then, the same gate was applied to the $CD56^{dim}CD16^{+}$ NK cell population. If two distinctive peaks representing FcεRIγ- and FcεRIγ+ NK cells were observed, the FcεRIγ- population was considered as g-NK cells. In situations where two distinctive peaks were not observed due to overlap, the subject was considered to have g-NK cells only if the proportion of NK cells within the FcεRIγ- gate was above the threshold of 14.5%. To evaluate the ADCC function, a sterile ELISA plate was first coated for at least 90 minutes with 2 μg/mL of anti-human CD16 antibody in PBS. After the removal of unbound antibody by thorough rinses, NK cells were stimulated in complete RP-10 medium on the plate-bound anti-human CD16 antibody (BD Pharmingen, BD Bioscience, San Jose, CA, USA) for 1 hour followed by an additional 6 hours stimulation in the presence of Brefeldin A at a final concentration of 5μg/mL. NK cells were analyzed for their IFN-γ

intracellular staining via flow cytometry. LIVE/DEAD fixable dead cell stain kit (Invitrogen; Life Technologies) was used to exclude dead cells. The frequency and phenotype of NK cells were analyzed using Kaluza 1.2 software (Beckman Coulter).

Table 2. List of antibodies used in flow cytometry. For NK cell analysis, CD3, CD14, and CD19 were utilized to exclude T cells, monocytes and B cells from PBMC. CD16 and CD56 were used to define NK cells population. NKG2C, FcεRIγ, SYK were used for NK cell phenotype study, and IFN-γ and CD107a were used for NK cell function. CD4 and CD8a were used to examine T cells. Lastly, CD20 was used to examine CD20 expression on lymphoma cell lines and B cells of a CLL patient.

Antigen	Reacts to	Conjugated	Supplier	Cat. No
FcεRIγ	Human	FITC	Millipore	FCABS400F
NKG2C	Human	PE	R&D Systems	FAB138P-100
NKG2C	Human	APC	R&D Systems	FAB138A-100
CD16	Human	PE-Cy7	BioLegend	302016
CD56	Human	APC	Miltenyi Biotec	130-090-843
CD56	Human	PE	BioLegend	318306
CD56	Human	Brilliant Violet 421	BioLegend	318327
CD56	Human	eFluor 450	eBioscience	48-0566-42
CD3	Human	Brilliant Violet 510	BioLegend	317332
CD3	Human	eFluor 450	eBioscience	48-0038-82
SYK	Human	APC	eBioscience	17-6696-42
CD14	Human	APC-Cy7	BioLegend	325620
CD19	Human	APC-Cy7	eBioscience	47-0199-42
CD19	Human	Brilliant Violet 786	BD	563325
IFN-γ	Human	PE	BioLegend	506507
CD4	Human	PE-Cy	eBioscience	25-0049-42
CD8a	Human	eFluor 450	eBioscience	48-0086-42
CD107a	Human	APC	BD	560664
CD20	Human	PE	BD	560961
Live/Dead	Human	Near-IR	Life Technologies	L10119

2.5 METAVIR stage system

HCV patients develop fibrosis on their liver, which is a formation of scar tissue resulting from the constant damage and healing process of the liver. This leads to a gradual loss of liver function and eventually a complete loss of liver function, known as cirrhosis, as the majority of the functioning hepatocytes are replaced with scar tissue. The fibrosis level of the liver were determined by transient elastography (FibroScan; Echosens) and/or liver biopsy, and grouped in accordance with the METAVIR stages, a scoring system used to assess the degree of scarring. The METAVIR stages are divided into five groups as follows (Dhingra et al., 2016): F0 represents no fibrosis; F1 represents minimal fibrosis; F2 represents spreading of fibrosis to other areas of the liver including blood vessels; F3 represents spreading and presence of a fibrosis network in the liver; and lastly F4 which represents cirrhosis, the total loss of liver function. The fibrosis evaluation was performed at The Ottawa Hospital Viral Hepatitis Clinic.

2.6 Analysis of NK cell response against rituximab coated target cells

Prior to the co-culture of NK cells with target cells, NK cells were enriched from PBMCs of healthy individuals using the human NK cell enrichment kit via negative selection (affymetrix eBioscience, San Diego, CA, USA); and $1x10^6$ cells/mL of lymphoma cell lines or PBMC of CLL patients were treated with final concentration of 10 μg/mL of either IgG antibody (Hizentra; CSL Behring, Ottawa, ON, Canada) or rituximab (Rituxan; Roche, Mississauga, ON, Canada) diluted in RP-10 media for 1 hour at room temperature. The target cells were washed thoroughly with RP-10 after the antibody treatment in order to remove any unbound antibodies. To examine the ADCC response of NK cells against antibody coated B cells, enriched NK cells from healthy individuals, who possesses g-NK cells, were co-cultured with antibody treated B cell lymphoma

cell lines (Daudi, Raji and RAMOS, which are originated from patients with Burkitt lymphoma, a type of NHL) or antibody treated PMBC from a CLL patient at a 1 to 1 cell number ratio on flat-bottom 96 well plate for 1 hour, followed by an additional 6 hours stimulation in the presence of Brefeldin A at a final concentration of 5μg/mL, and CD107a antibody. Upon completion, cells were stained and the proportion of NK cells expressing IFN-γ and the degranulation marker, CD107a, were measured using flow cytometry.

2.7 Statistical analysis

One-way ANOVA followed by unpaired parametric Student's t-test with Welch's correction was used for NK cell phenotype analysis between defined groups. Ratio paired parametric t-test was used to compare between conventional NK cell and g-NK cell in terms of phenotype and function. Pearson correlation was applied for the statistical analysis of correlational studies. Differences were considered significant when $p \leq 0.05$ (GraphPad Prism version 6.05).

3. RESULTS

3.1 Characterization of g-NK cells in healthy controls

CMV infection is known to induce the expansion of NK cells expressing NKG2C that are sustained for many years. These cells are also marked by CD57 expression (Lopez-Verges et al., 2011). Furthermore, other studies have found that g-NK cells, one of the NK cell subsets that are highly associated with prior CMV infection and lacking FcεRIγ adaptor, also co-express CD57 and NKG2C while lacking SYK expression. However, the expression of CD3ζ and ZAP-70 in g-NK cells remained intact (Hwang et al., 2012; Zhang et al., 2013). Therefore, these surface and intracellular markers, NKG2C, CD57, FcεRIγ, SYK, CD3ζ and ZAP-70 provide a good starting point to confirm the identification of g-NK cells prior to the examination of actual samples from chronic HCV patients. I first examined these phenotypic markers on NK cells from a small group of healthy individuals.

Upon examination, the presence of NKG2C+ CD57+ NK cell population (indicated by an arrow) was observed in a healthy individual who had prior exposure to CMV. However, no such distinct NK cell population was observed in a healthy individual who is CMV seronegative (**Figure 3A, left panels**). In addition, NK cells lacking FcεRIγ (g-NK cells as indicated by arrows) are present in the individual with CMV; and those g-NK cells express NKG2C, yet again, no such population was observed in the CMV- individual (**Figure 3A, middle panels**). An NK cell population lacking Syk was only found among g-NK cells in the CMV+ individual (**Figure 3A, right panels**).

The expression of membrane-bound signaling adaptor proteins (FcεRIγ and CD3ζ), and their immediate downstream signaling proteins (Syk and ZAP70) on NK cells, gated using CD16 and

CD56, were also examined via flow cytometry. Consistent with **Figure 3A**, NK cells lacking FcεRIγ and Syk were only present in the individual with prior CMV infection. However, CMV infection had no effect on the expression of CD3ζ and ZAP-70 (**Figure 3B**). These results are consistent with the literature where differences in NKG2C and Syk expressions were observed in g-NK cells (Hwang et al., 2012; Zhang et al., 2013). These findings are also consistent with a report associating CMV infection with CD57 expression in NK cells (Lopez-Verges et al., 2011). These preliminary results suggest that NKG2C, CD57, FcεRIγ, SYK are the ideal markers to further examine in my study. Hence, in order to ensure that these patterns are consistent among a variety of individuals, I expanded my sample pool and examined these markers in multiple healthy individuals.

The group of healthy individuals who were examined consisted of two CMV- and four CMV+ individuals. It was evident that a similar trend could be seen in a variety of individuals. As shown in **Figure 4**, none of the two individuals who are CMV- (Control 4 and 11) have an NK cell population lacking FcεRIγ, clearly indicating that these individuals do not have g-NK cells. Furthermore, it was evident that their NK cell population neither expressed NKG2C nor lacked Syk (although there seems to be a very tiny population of NK cells lacking Syk in control 11). In regard to CD57 expression, their NK cell population could be divided into two subsets, one that expresses CD57, and another that lacks CD57. Previously, CD57 has been used as a maturation marker (Nielsen et al., 2013). This indicates that CD57 is not an ideal marker to distinguish between conventional NK cells and g-NK cells.

When four individuals who are CMV seropositive (Control 3, 7, 18, and 19) were examined, the results were somewhat diverse. In terms of FcεRIγ, two out of four individuals have a clear g-NK cell population that lacks the adaptor protein, indicating that not all CMV infection gives rise

to a g-NK cell population. Among CMV+ individuals, a distinct NK cell population that lacks Syk was only found in individuals who have g-NK cells (Control 7 and 19, indicated by arrows). In addition, a portion of the g-NK cell population in control 19 also expresses Syk. A pattern similar to that of Syk was observed in regard to NKG2C expression. An NK cell population that expresses NKG2C was only found in CMV+ individuals who possess g-NK cells (Control 7 and 19, indicated by arrows). In addition, there is a portion of the g-NK cell population in control 19 that does not express NKG2C. In regard to CD57 expression, NK cells, including g-NK cells, of all CMV+ individuals consist of a population that expresses CD57 and another population that lacks CD57.

In summary, I have looked at NKG2C, CD57, FcεRIγ, SYK, CD3ζ and ZAP-70 expressions in NK cells of healthy individuals in order to determine ideal markers to investigate g-NK cells. Consistent with the literature, my preliminary data showed that an NK cell population that lacks FcεRIγ, representing g-NK cells, was only found among CMV+ individuals. The NK cell population in all individuals consists of a subset that expresses CD57 and another subset that does not express CD57 regardless of CMV infection; indicating that CD57 is not an ideal marker to study g-NK cells. Despite the heterogeneous expression of Syk and NKG2C that was observed in g-NK cells, the NK cell population that lacks Syk and expresses NKG2C was specific to only g-NK cells. These results confirm that FcεRIγ, NKG2C and SYK are the ideal markers to investigate the phenotype of g-NK cells, providing us with a solid background to investigate g-NK cells in chronic HCV patients.

Figure 3. Characterization of g-NK cells in healthy control individuals. PBMCs of representative healthy individuals who are CMV- (n = 1) or CMV+ (n = 1) were analyzed using flow cytometry. Live cells that do not express CD3, CD14, and CD19 were further gated for cells expressing CD56 and CD16. **(A)** Expression of CD57, NKG2C, FcεRIγ, and SYK were examined and presented on plots. Arrows indicate the NK cell population of interest. **(B)** Expression of FcεRIγ and CD3ζ adaptor proteins, and SYK and ZAP70 downstream signaling molecules were examined and presented on histograms. The red dash lines indicate a positive peak.

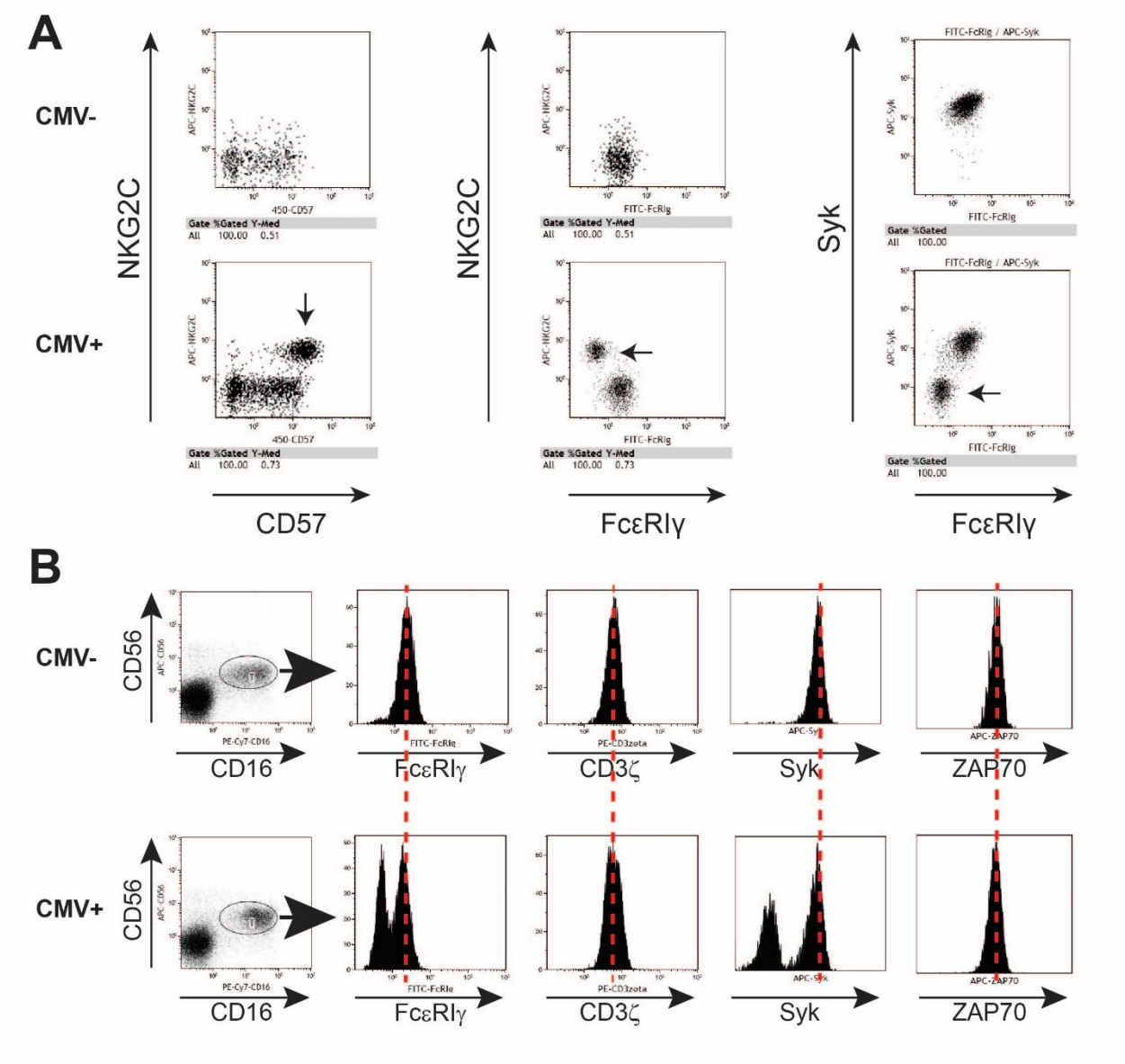
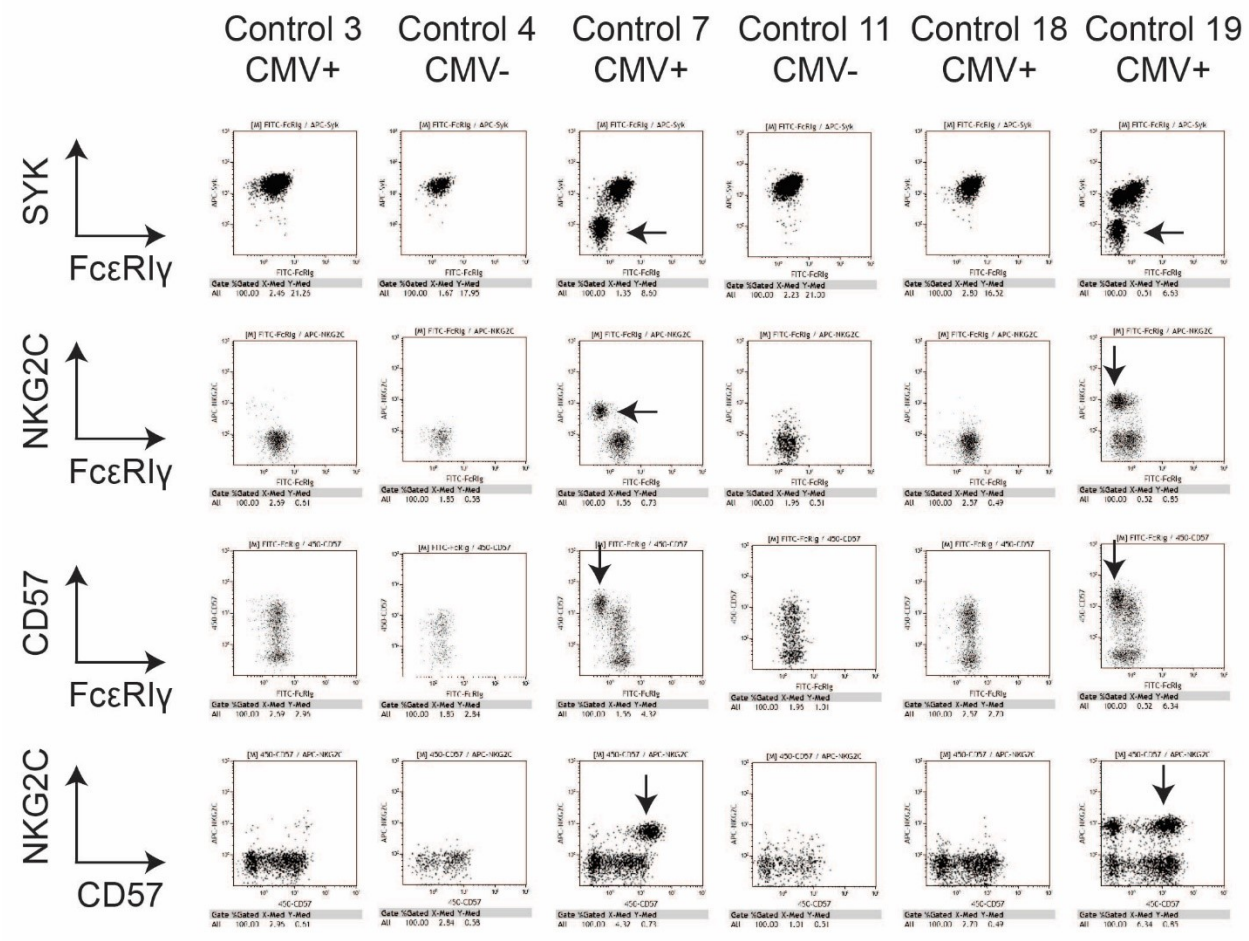

A
CMV-
CMV+
NKG2C
CD57
FcεRIγ
Syk
B
CD56
CD16
FcεRIγ
CD3ζ
Syk
ZAP70

Figure 4. Heterogeneity of g-NK cell population in six healthy individuals. PBMCs of multiple healthy individuals who are CMV- (total two individuals) or CMV+ (total four individuals) were analyzed using flow cytometry. Live cells that do not express CD3, CD14, and CD19 were further gated for cells expressing CD56 and CD16. Expression of CD57, NKG2C, FcεRIγ, and SYK were examined and presented on histograms. Arrows indicate a unique NK cell population that is highly associated with prior CMV infection.


Control 3
CMV+
Control 4
CMV-
Control 7
CMV+
Control 11
CMV-
Control 18
CMV+
Control 19
CMV+
SYK
FcεRIγ
NKG2C
FcεRIγ
CD57
FcεRIγ
NKG2C
CD57

3.2 A strong association of g-NK cells with prior CMV infection is also found in chronic HCV patients

The strong association of the g-NK cell subset with prior CMV infection has been shown in independent studies involving healthy individuals (Beziat et al., 2012; Lee et al., 2015; Lopez-Verges et al., 2011; Schlums et al., 2015; Zhang et al., 2013). Due to the stable existence of g-NK cell population after CMV infection and their possible roles in a broad range of infections, I decided to investigate the phenotype and function of the g-NK cell subset in individuals chronically infected with HCV. Since g-NK cells were reported to be restricted to the $CD56^{dim}$ population (Hwang et al., 2012; Lee et al., 2015; Schlums et al., 2015; Zhang et al., 2013), the expression of FcεRIγ, NKG2C and SYK were comparatively analyzed on the $CD56^{dim}CD16^{+}$ NK cell population of chronically HCV-infected and healthy individuals. The characteristics of the study subjects are summarized in **Table 1**. Using multicolor flow cytometry, g-NK cells were identified as FcεRIγ-negative cells among $CD56^{dim}CD16^{+}$ NK cells after gating on lymphocytes based on their characteristics of cell size and granularity, followed by the exclusion of $CD14^{+}$ monocytes, $CD19^{+}$ B cells, dead cells, and $CD3^{+}$ T cells. Upon separation of g-NK cells and conventional NK cell population based on FcεRIγ expression, each NK cell population was analyzed and compared for their NKG2C and Syk expression (**Figure 5A**). As previously demonstrated with the small pool of samples from the healthy individuals, g-NK cells were largely overlapped with cells expressing NKG2C and lacking SYK expression, with heterogeneous patterns in both CMV+ healthy and chronic HCV patients (**Figure 5B,** control 29 and HCV13) (Hwang et al., 2012; Lee et al., 2015; Lopez-Verges et al., 2011; Schlums et al., 2015). Furthermore, none of their conventional NK cells had a clear distinct population expressing NKG2C or lacking Syk (**Figure 5B,** control 11 and HCV41).

Consistent with other reports, the association between prior CMV infection and the presence of g-NK cells was observed among health individuals (**Figure 5C**) (Zhang et al., 2013). But among the group of chronic HCV patients, the comparison of g-NK cell frequencies between CMV- and CMV+ individuals failed to show a statistical significance (**Figure 5C**). However, this analysis was influenced by an outlying data from a lone CMV-seronegative individual, HCV37 (indicated with an arrow), with a high proportion of the g-NK cell subset. As the presence of g-NK cells is known to be associated with prior CMV infection, this high proportion of g-NK cells in HCV37, who is CMV seronegative, was unusual. Measuring this individual's CMV-specific T cell response (based on the IFN-γ producing T cells) upon stimulation with CMV peptide pp65, a major epitope that is commonly recognized by CD4 and CD8 T cells, resulted in the absence of a CMV-specific T cell population in this specific individual compared to others who are CMV seropositive, further supporting that individual HCV37 was never subjected to CMV infection (**Figure 6**). The exclusion of this outlying individual, justified by his/her possession of non-CMV associated g-NK cells, resulted in a significant association ($p = 0.003$). Interestingly, when the proportions of g-NK cells in CMV+ individuals are compared between healthy and chronic HCV patients, there is a trend where the proportions of g-NK cells are found lower in the HCV+ group than the control group (**Figure 5C**), even though the difference was not statistically significant. The average age of the HCV+ group was higher than that of the control group (averaging 47 years old and 34 years old respectively) (**Table 1**), raising the possibility that the reduced proportion of g-NK cells in the HCV+ group is age-related. To determine whether this difference in the proportions of the g-NK cell subset is due to the age difference between the two groups, I looked for a correlation between the age and NK cell proportions in each group.

However, I could not find any correlation between g-NK cell proportions and the subjects' ages in both groups (**Figure 7**).

Earlier, I have observed a heterogeneous expression of NKG2C in g-NK cells from CMV+ healthy individuals, where cells expressing NKG2C were only found among g-NK cells. Therefore, I also looked for a correlation between the proportion of NKG2C expressing NK cells and CMV infection. The NKG2C+ NK cell population was associated with prior CMV infection among the healthy group, but no such correlation was found among the HCV patient group (**Figure 8A**). When I compared NKG2C expression between conventional NK cells and g-NK cells within each individual, I was not able to observe any difference (**Figure 8B**). Similar to the result with NKG2C, I found a correlation between the presence of an NK cell population lacking Syk and prior CMV infection among the healthy group, but no such correlation was found among the patient group (**Figure 9A**). However, unlike the result with NKG2C, there was a clear difference between conventional NK cells and g-NK cells in each individual when considering the proportion of NK cells lacking Syk (**Figure 9B**).

Taken together, I showed that among FcεRIγ, NKG2C and SYK markers that I tested, FcεRIγ shows the most association with prior CMV infection in the group of chronic HCV individuals. Furthermore, the novel g-NK cell subset, represented by an NK cell population that lacks FcεRIγ, observed in prior CMV exposed individuals was found at slightly reduced proportions in chronic HCV patients compared to the healthy group. No correlation between the age and the proportion of g-NK cells was found, suggesting that the g-NK cell proportion is marginally modulated during chronic HCV infection.

Figure 5. A strong association of g-NK cells with prior CMV infection is also found in chronic HCV patients. (A-C) Human PBMCs were analyzed using flow cytometry. **(A)** (Upper panels) Live cells that do not express CD3, CD14, and CD19 were further gated for cells expressing CD56 and CD16. (Lower panels) Among those gated, NK cells were further defined in accordance with the expression of FcεRIγ. Those that express FcεRIγ were classified as conventional NK cells, and those that do not express FcεRIγ as g-NK cells. Expression of NKG2C and SYK was then examined on conventional NK and g-NK cells. **(B)** Plots of NKG2C, FcεRIγ and SYK expression on $CD56^{dim}CD16^{+}$ NK cells from PBMCs of healthy and HCV individuals, each divided based on their CMV status. **(A and B)** Flow cytometry plots are representative of at least two independent experiments. **(C)** The percentage of g-NK cells was measured by flow cytometry. (healthy group, CMV- (n=19) and CMV+ (n=15); HCV group, CMV- (n=20) and CMV+ (n=27)). Data are shown as mean ± SEM and are representative of/pooled from at least two independent experiments. Data shown are representative of/pooled from at least two independent experiments. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. The p† value in **(C)** indicates the statistical result by excluding patient HCV37 (CMV- with g-NK cells). **(C)** Unpaired parametric Student's *t*-test with Welch's correction was used for the analysis of g-NK cell proportion between the defined groups.

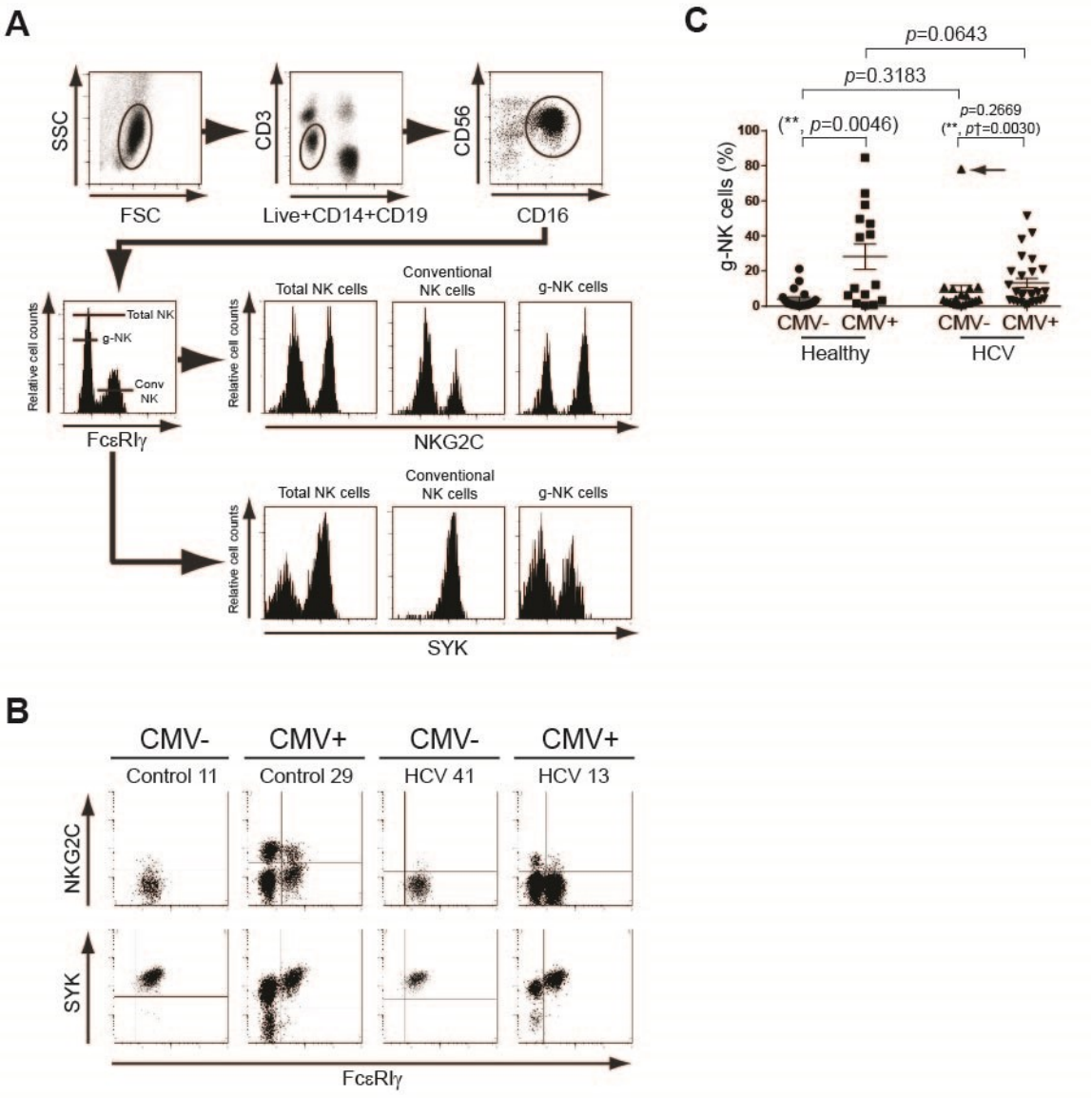

A
SSC
FSC
CD3
Live+CD14+CD19
CD56
CD16
Total NK
g-NK
Conv NK
Relative cell counts
FcεRIγ
Total NK cells
Conventional NK cells
g-NK cells
NKG2C
SYK
B
CMV-
CMV+
CMV-
CMV+
Control 11
Control 29
HCV 41
HCV 13
NKG2C
SYK
FcεRIγ
C
p=0.0643
p=0.3183
(**, p=0.0046)
p=0.2669
(**, p†=0.0030)
g-NK cells (%)
CMV-
CMV+
CMV-
CMV+
Healthy
HCV

Figure 6. Detection of HCMV pp65 specific T cell response using peptide stimulation. Along with the CMV specific IgG immunoassay on the serum of patient HCV37 who has developed a high percentage of g-NK cell population, the patient's PBMC was also incubated overnight and stimulated with HCMV specific pp65 peptide for 1 hour followed by an additional 6 hours stimulation in the presence of Brefeldin A. Positive controls contained control CEF peptides. Stimulation using CMV specific pp65 peptide on PBMC was performed in order to measure CMV specific memory T cell response. For comparison purposes, CMV-specific peptide stimulation was also performed on PMBCs of two controls who are CMV- and CMV+ and a HCV patient who is CMV+. The numerical values on each dot plot indicate the proportions of IFN-γ producing CD4 T cells (left) or CD8 T cells (right) from the total T cell population.

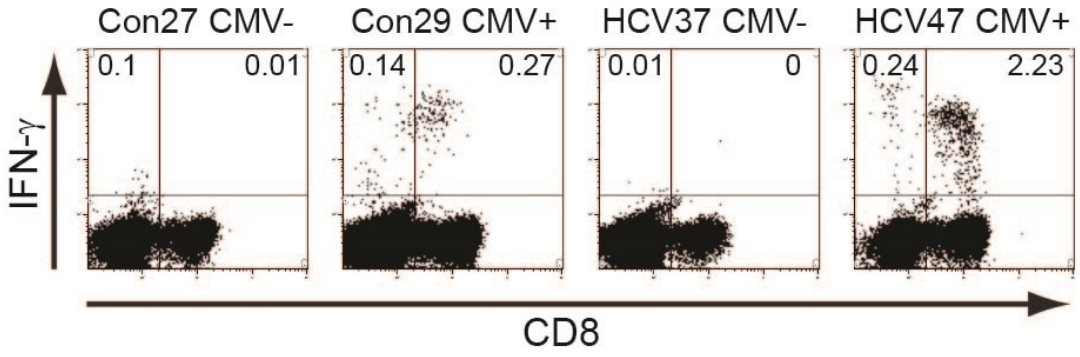

Con27 CMV-
Con29 CMV+
HCV37 CMV-
HCV47 CMV+
0.1
0.01
0.14
0.27
0.01
0
0.24
2.23
IFN-γ
CD8

Figure 7. No correlation between the proportions of g-NK cells and the age of subjects. The percentages of g-NK cells in healthy and HCV subjects' PBMC were compared with their ages. Pearson correlation was applied for statistical analysis. Controls on the top (n = 9). HCV patients on the bottom (n = 9).

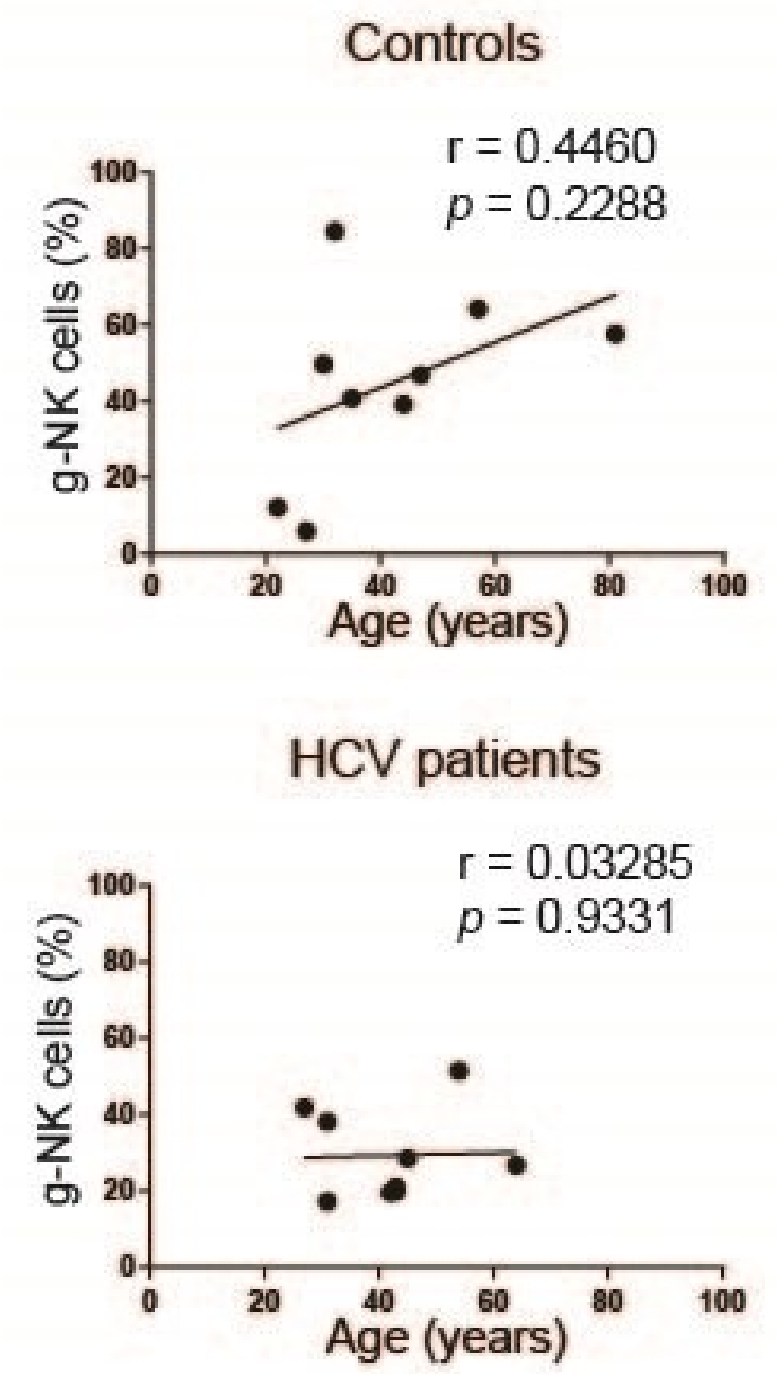
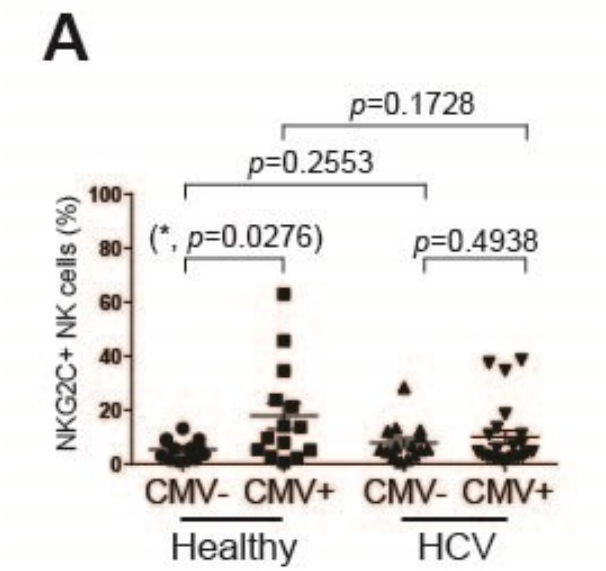

Controls
r = 0.4460
p = 0.2288
g-NK cells (%)
Age (years)
HCV patients
r = 0.03285
p = 0.9331
g-NK cells (%)
Age (years)

Figure 8. NKG2C expression on NK cells from healthy and chronic HCV patients. (A-B) Human PBMCs were analyzed using flow cytometry. **(A)** The percentage of NK cells expressing NKG2C (healthy group, CMV- (n=13) and CMV+ (n=14); HCV group, CMV- (n=15) and CMV+ (n=22)). Data are shown as mean ± SEM and are representative of/pooled from at least two independent experiments. **(B)** CMV+ healthy and HCV individuals were subdivided in respect to the presence of g-NK cells, and expression of NKG2C (healthy (n=8); HCV (n=6)) in each individual's NK cell population was compared. Data shown are representative of/pooled from at least two independent experiments. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. **(A)** Unpaired parametric Student's *t*-test with Welch's correction was used for NK cell phenotype analysis between the defined groups. **(B)** Ratio paired parametric *t*-test was used to compare conventional NK and g-NK cell phenotypes.


A
p=0.1728
p=0.2553
(*, p=0.0276)
p=0.4938
NKG2C+ NK cells (%)
100
80
60
40
20
0
CMV- CMV+ CMV- CMV+
Healthy
HCV

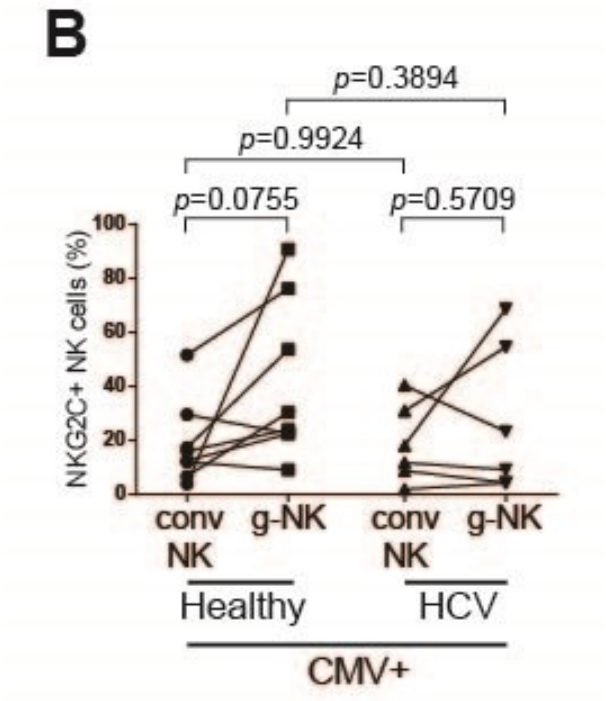
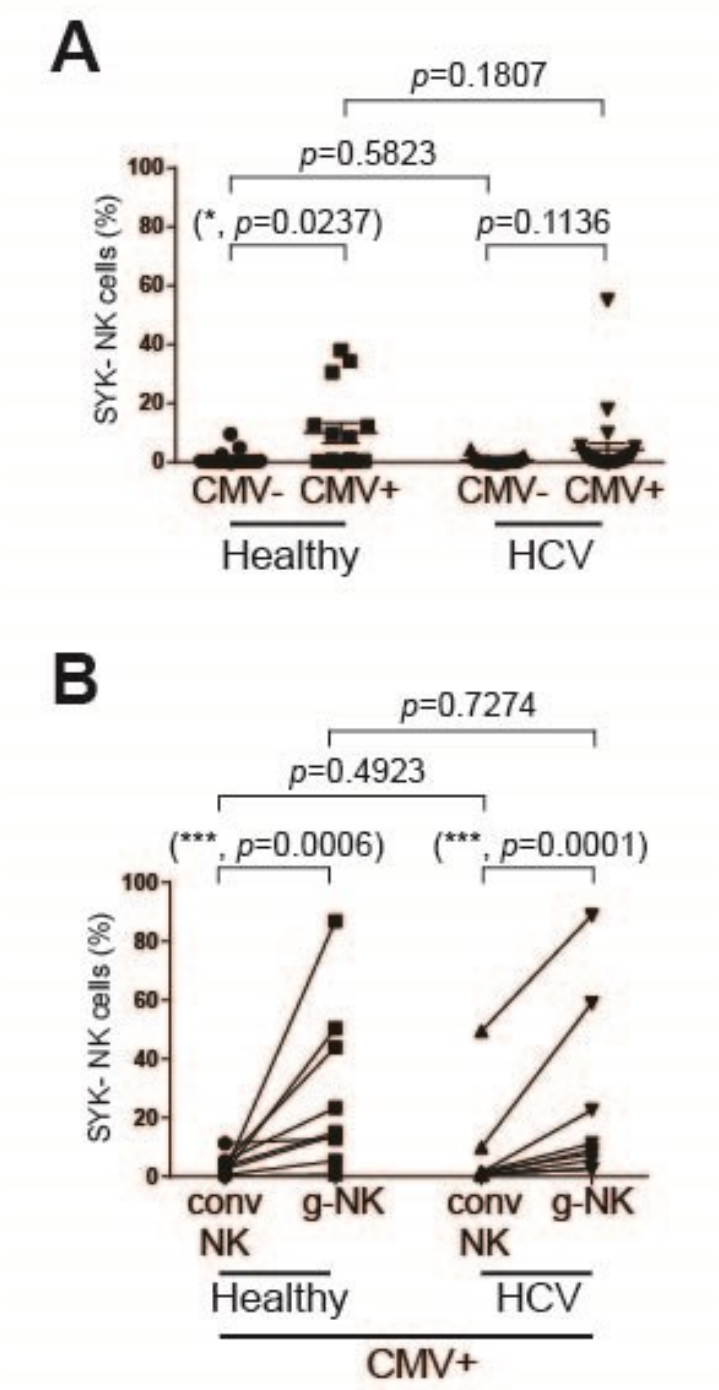

B
p=0.3894
p=0.9924
p=0.0755
p=0.5709
NKG2C+ NK cells (%)
100
80
60
40
20
0
conv NK
g-NK
conv NK
g-NK
Healthy
HCV
CMV+

Figure 9. SYK expression on NK cells from healthy and chronic HCV patients. (A-B) Human PBMCs were analyzed using flow cytometry. **(A)** NK cells lacking SYK were measured by flow cytometry (healthy group, CMV- (n=19) and CMV+ (n=15); HCV group, CMV- (n=19) and CMV+ (n=26)). Data are shown as mean ± SEM and are representative of/pooled from at least two independent experiments. **(B)** CMV+ healthy and HCV individuals were subdivided in respect to the presence of g-NK cells, and lack of SYK expression (n=9 for each of healthy and HCV groups) in each individual's NK cell population was compared. Data shown are representative of/pooled from at least two independent experiments. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. **(A)** Unpaired parametric Student's *t*-test with Welch's correction was used for NK cell phenotype analysis between the defined groups. **(B)** Ratio paired parametric *t*-test was used to compare conventional NK and g-NK cell phenotypes.

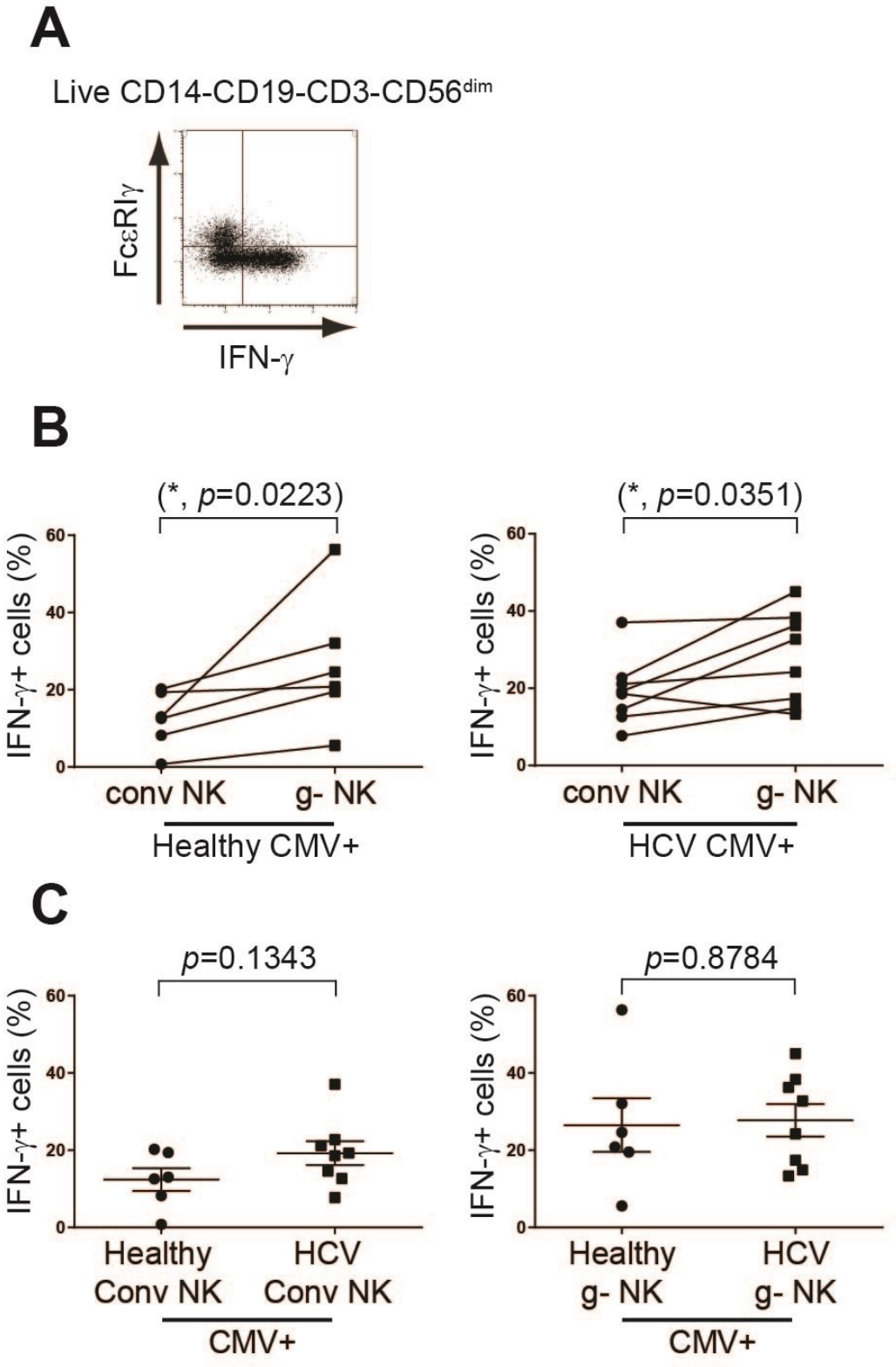

A
p=0.1807
p=0.5823
(*, p=0.0237)
p=0.1136
SYK- NK cells (%)
100
80
60
40
20
0
CMV- CMV+ CMV- CMV+
Healthy
HCV
B
p=0.7274
p=0.4923
(***, p=0.0006)
(***, p=0.0001)
SYK- NK cells (%)
100
80
60
40
20
0
conv NK
g-NK
conv NK
g-NK
Healthy
HCV
CMV+

3.3 Superior ADCC of g-NK cells is maintained in chronic HCV infection

Despite that the patients have a chronic HCV infection, the presence of g-NK cells in these individuals is still associated with prior CMV infection; although the proportion of g-NK cells was slightly reduced compared to the healthy group (**Figure 5C**). A key functional characteristic of the g-NK cell subset is its enhanced ADCC activity (Hwang et al., 2012; Lee et al., 2015; Zhang et al., 2013). While the results showed that chronic HCV patients maintain their g-NK cell population, it is also important for those g-NK cells to remain functionally capable. Hence, in order to determine whether g-NK cells found in HCV+ individuals retain this superior ADCC capability, IFN-γ production by conventional NK cells (expressing FcεRIγ) and g-NK cells upon stimulation with plate-bound α-CD16 was measured (**Figure 10A**). This assay has been used to evaluate the ADCC function in previous studies (Hwang et al., 2012; Lee et al., 2015). In both healthy and chronic HCV patients groups, g-NK cells showed superior ADCC than conventional NK cells (**Figure 10B**). The proportion of IFN-γ producing cells among g-NK cells was comparable in both control and HCV+ groups; while the proportion of IFN-γ producing cells among conventional NK cells was slightly higher in HCV+ individuals (**Figure 10C**).

Taken together, similar to that in the healthy control group, the g-NK cell subset in the HCV+ patients group retained its superior ADCC activity compared to conventional NK cells. Hence, it is possible that the maintenance of a functionally capable g-NK cell population in CMV+ chronic HCV patients may potentially have an effect on the patients' disease progression.

Figure 10. Superior ADCC of g-NK cell subset is maintained in individuals chronically infected with HCV. (A) The PBMCs of healthy and HCV individuals possessing g-NK cells were incubated in α-CD16 antibody coated plate in order to stimulate both the conventional and g-NK cells and compare their ADCC functions by measuring the IFN-γ producing cells via flow cytometry. Plot shows IFN-γ production in conventional and g-NK cells. Representative flow cytometry plot from at least two independent experiments is shown. **(B)** ADCC capabilities of conventional and g-NK cells in healthy and HCV individuals were compared. (healthy (n=6); HCV (n=8)). **(C)** The proportion of IFN-γ+ cells among conventional NK cells from healthy and HCV individuals were compared in regard to their ADCC capability. The same was done for the g-NK cell subset. (healthy (n=6); HCV (n=8)). Data are shown as mean ± SEM. **(B and C)** Data shown are representative of/pooled from at least two independent experiments. *$p \leq 0.05$. **(B)** Ratio paired parametric *t*-test was used to compare conventional NK and g-NK cell functions. **(C)** Unpaired parametric Student's *t*-test with Welch's correction was used to compare conventional NK and g-NK cell functions in healthy and HCV groups.


A
Live CD14-CD19-CD3-CD56dim
FcεRIγ
IFN-γ
B
(*, p=0.0223)
IFN-γ+ cells (%)
conv NK
g- NK
Healthy CMV+
(*, p=0.0351)
IFN-γ+ cells (%)
conv NK
g- NK
HCV CMV+
C
p=0.1343
IFN-γ+ cells (%)
Healthy Conv NK
HCV Conv NK
CMV+
p=0.8784
IFN-γ+ cells (%)
Healthy g- NK
HCV g- NK
CMV+

3.4 Reduced liver damage is associated with the presence of g-NK cells in CMV-infected HCV+ individuals

Although the patients have a chronic HCV infection, I showed that these patients still maintain the superior ADCC capability of g-NK cells compared to their conventional counterparts. It would be interesting to know whether these CMV-induced g-NK cells can lead to improved clinical outcomes in chronic HCV patients who maintain this unique subset of NK cells. Therefore, in order to first determine whether there is an association between prior CMV infection and the extent of hepatocellular injury in individuals chronically infected with HCV, the levels of ALT, AST, and GGT, measured at the Ottawa General Hospital, were compared between CMV+ and CMV- among chronic HCV patients. The levels of these liver enzymes are directly related to extent of hepatocellular injury. Based on my initial analysis, no correlations were observed between the liver enzyme levels and the status of CMV infection (**Figure 11A-C**). Nonetheless, it is noteworthy that not all CMV+ individuals developed the g-NK cell population (**Figure 5C**). Therefore, in order to determine whether the presence of g-NK cells influences the extent of liver damage in CMV+ individuals, the CMV+ HCV+ individuals were further subdivided into two groups based on either the presence or absence of g-NK cells. Upon comparison of the liver enzyme levels between these two subgroups, no statistically significant correlation was observed in regards to ALT, AST levels, although there is a trend of reduced levels of these enzymes in individuals possessing g-NK cells (**Figure 11A-B**). Notably, the GGT levels were found to be significantly lower in CMV+ HCV+ individuals possessing g-NK cells (**Figure 11C**).

Liver fibrosis was evaluated by liver biopsy or transient elastography (i.e. Fibroscan) which was done at the Ottawa General Hospital. The METAVIR staging system is commonly used for

evaluating liver fibrosis in patients with HCV (Goodman, 2007). Since the presence of g-NK cells is associated with a reduction of the GGT levels, it would be interesting to know whether a similar trend can be found in regards to the liver fibrosis. In fact, I found that the presence of g-NK cells was associated with lower METAVIR stages and all individuals showing severe liver fibrosis (stage 3 or 4) were only found in the group lacking g-NK cells (**Figure 11D**).

Next, I investigated whether the extent of g-NK cell proportion has an impact on the protection from liver damage. Even though there was slightly less GGT levels in individuals possessing high proportions of g-NK cells, no significant correlation was found between g-NK cell proportions and the levels of liver enzymes and/or liver fibrosis (**Figure 12A-D**). Since the presence of g-NK cells had an effect on the GGT level and the METAVIR stage (**Figure 11C-D**), I sought to find a relationship between the GGT level and the METAVIR stage. When we compared the GGT level with the METAVIR stage among all HCV patients, I did not find correlation between those two factors (**Figure 13A**). In addition, further subdividing the HCV patients into two groups based on CMV failed to show a correlation between the GGT level and the METAVIR stage in those groups (**Figure 13B-C**). Moreover, I did not observe a correlation between the METAVIR stages and the GGT levels with respect to either the presence or absence of g-NK cell population, indicating that the beneficial roles of g-NK cells is exclusively associated with either the fibrosis stages or the GGT levels (**Figure 13D-E**). It is also important to note that the difference was rather difficult to determine due to low values of GGT levels (**Figure 13E**).

Since I observed a reduction of the GGT level and the METAVIR stage among chronic HCV patients possessing g-NK cells, I questioned whether this protective effect is a result of g-NK cells' antiviral capability to control HCV replication. Upon analyzing the viral loads between

HCMV- g-NK-, HCMV+ g-NK-, and HCMV+ g-NK+ patient groups, I did not find any statistical difference in the viral loads between these groups; indicating that the presence of g-NK cells has no effect on controlling the viral loads in chronic HCV patients (**Figure 14A**). Because I was interested to know whether the proportion of g-NK cells has an effect on the viral load, I analyzed the viral loads among chronic HCV patients who possess g-NK cells. I was unable to identify a correlation between the proportion of g-NK cells and the viral load (**Figure 14B**), indicating that having increased numbers of g-NK cells does not impact the viral load.

In summary, I observed that chronic HCV patients, who possess g-NK cells, have a reduced GGT concentration in their blood and a lower METAVIR stage. This indicates that the patients with g-NK cells have reduced liver damage, potentially leading to a positive clinical outcome, although I was not able to determine the mechanism. I also found that neither the presence nor quantity of g-NK cells necessarily control the HCV viral load in the patients.

Figure 11. Reduced liver damage is associated with the presence of g-NK cells in CMV+ HCV+ individuals. Liver enzyme levels such as ALT (n = 15 and 24 for CMV- and CMV+ patients respectively. Among CMV+ group, 16 and 8 patients were g-NK- and g-NK+ respectively) **(A)**, AST (n = 15 and 24 for CMV- and CMV+ patients respectively. Among CMV+ group, 16 and 8 patients were g-NK- and g-NK+ respectively) **(B)**, GGT (n = 15 and 22 for CMV- and CMV+ patients respectively. Among CMV+ group, 14 and 8 patients were g-NK- and g-NK+ respectively) **(C)** as well as the METAVIR stage (n = 12 and 22 for CMV- and CMV+ patients respectively. Among CMV+ group, 16 and 6 patients were g-NK- and g-NK+ respectively) **(D)** were examined in HCV patients who are CMV- and CMV+. HCV patients who are CMV+ were further divided based on the presence of g-NK cells. **(A-D)** Data are shown as mean ± SEM and are representative of/pooled from at least two independent experiments. *$p \leq 0.05$. Unpaired parametric Student's *t*-test with Welch's correction was used for liver enzymes and METAVIR analysis between the defined groups.

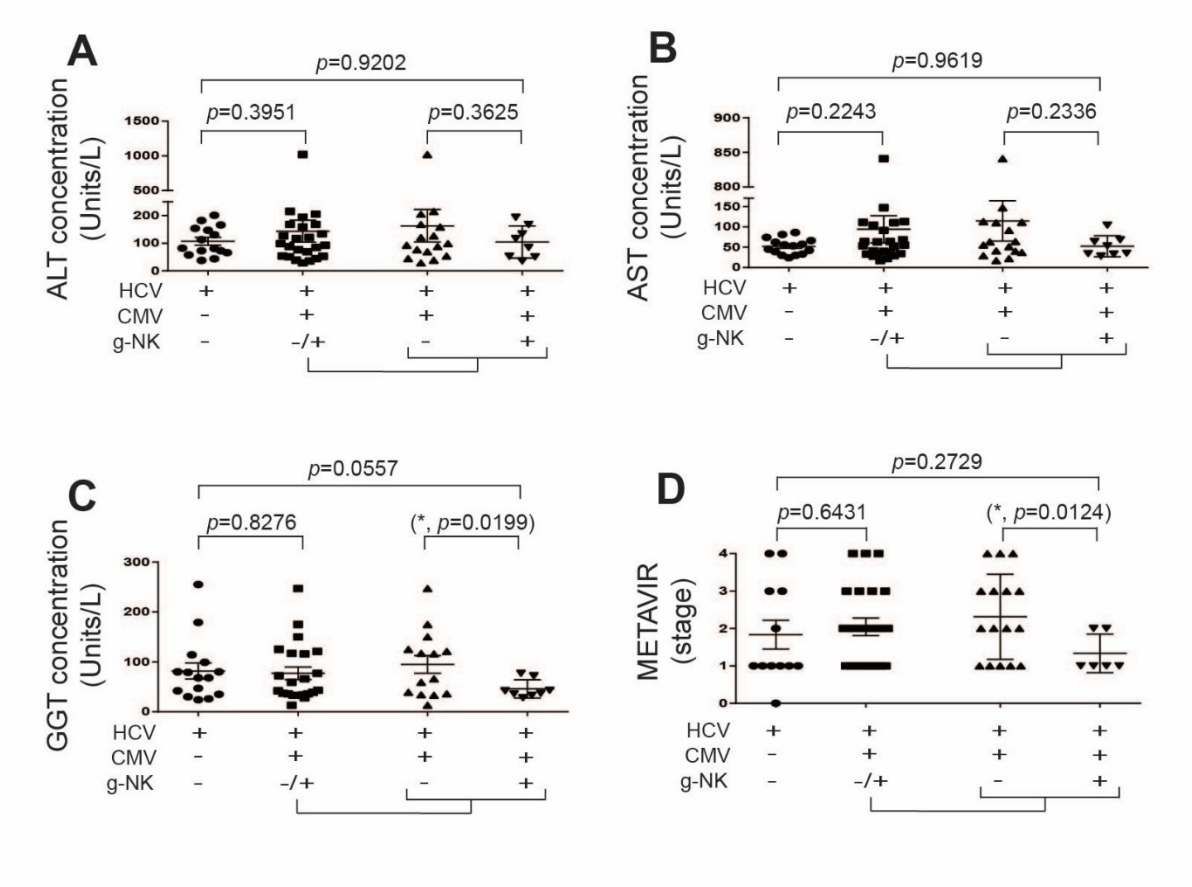

A
ALT concentration (Units/L)
p=0.9202
p=0.3951
p=0.3625
1500
1000
500
200
100
0
HCV + + + +
CMV - + + +
g-NK - -/+ - +
B
AST concentration (Units/L)
p=0.9619
p=0.2243
p=0.2336
900
850
800
150
100
50
0
HCV + + + +
CMV - + + +
g-NK - -/+ - +
C
GGT concentration (Units/L)
p=0.0557
p=0.8276
(*, p=0.0199)
300
200
100
0
HCV + + + +
CMV - + + +
g-NK - -/+ - +
D
METAVIR (stage)
p=0.2729
p=0.6431
(*, p=0.0124)
4
3
2
1
0
HCV + + + +
CMV - + + +
g-NK - -/+ - +

Figure 12. No correlation between the proportions of g-NK cells and the levels of liver enzymes and/or liver fibrosis. The percentages of g-NK cells in HCV patients' PBMC were compared with ALT (n = 8) **(A)**, AST (n = 8) **(B)**, GGT (n = 8) **(C)**, and METAVIR stage (n = 6) **(D)**. Pearson correlation was applied for statistical analysis.

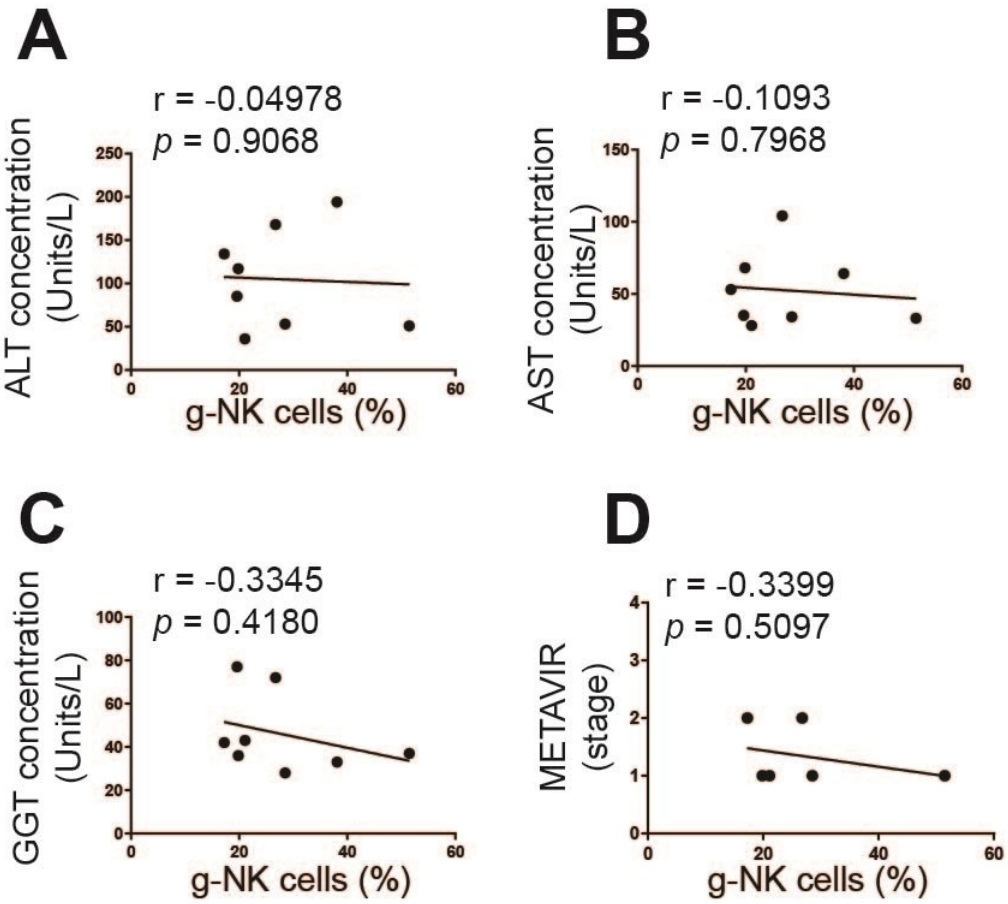

A
r = -0.04978
p = 0.9068
ALT concentration (Units/L)
g-NK cells (%)
B
r = -0.1093
p = 0.7968
AST concentration (Units/L)
g-NK cells (%)
C
r = -0.3345
p = 0.4180
GGT concentration (Units/L)
g-NK cells (%)
D
r = -0.3399
p = 0.5097
METAVIR (stage)
g-NK cells (%)

Figure 13. GGT enzyme level does not correlate with the stage of fibrosis. (A) GGT concentrations in HCV subjects were compared with the METAVIR stage (n = 31). **(B, C)** HCV subjects were subdivided based on their CMV status for comparison. (n = 12 and 19 for B and C respectively). **(D, E)** CMV+ HCV subjects were further subdivided based on the presence of g-NK cells. (n = 13 and 6 for D and E respectively). Pearson correlation was applied for statistical analysis.

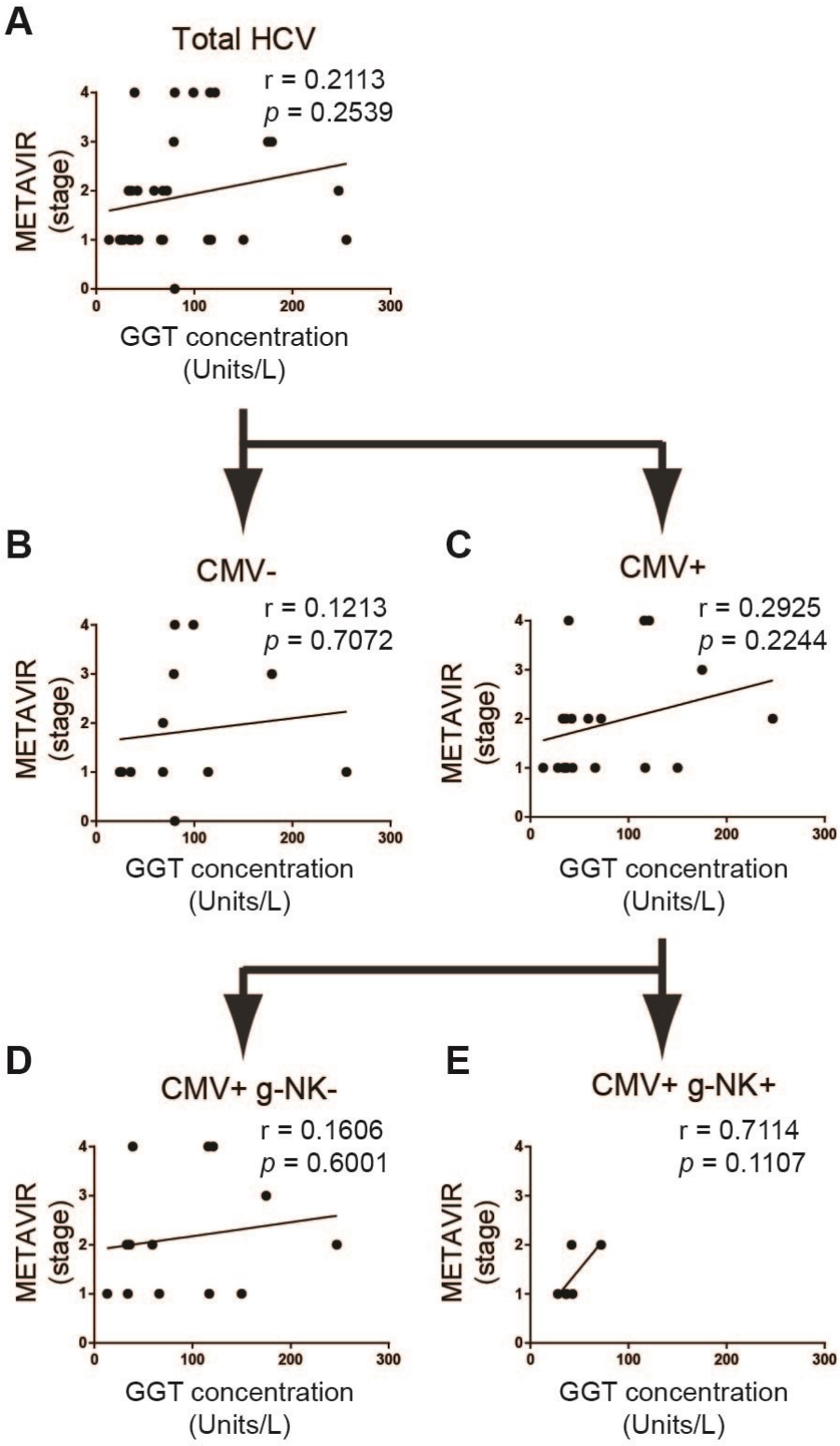
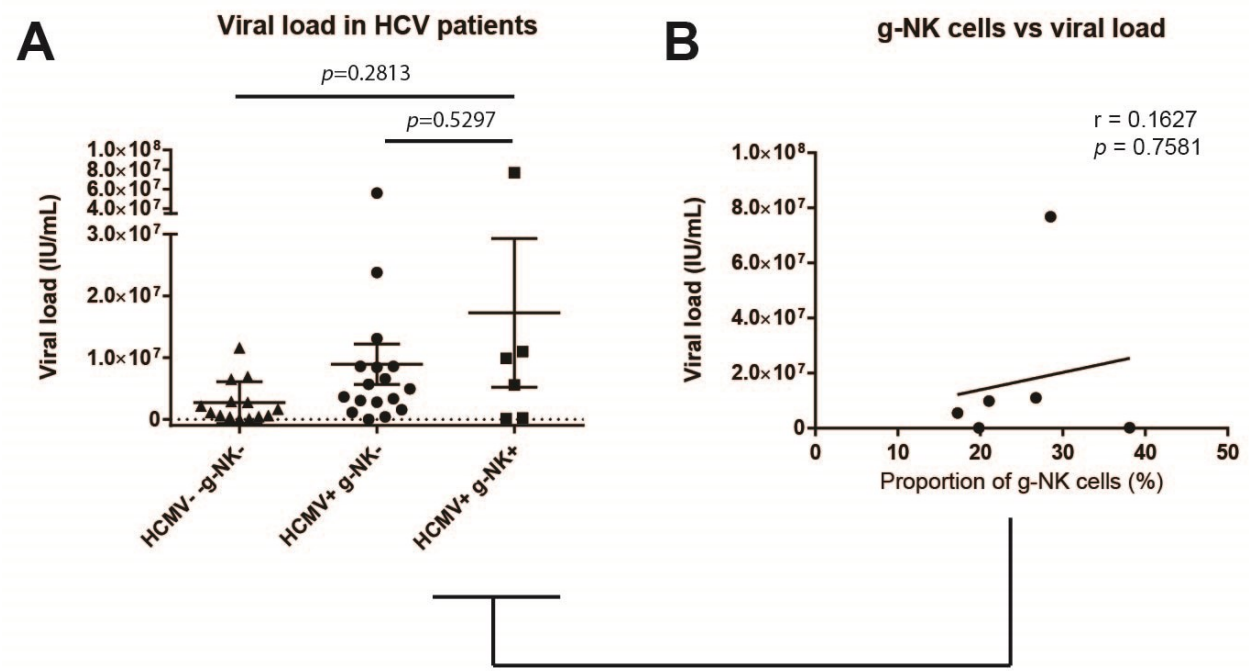

A
Total HCV
r = 0.2113
p = 0.2539
METAVIR (stage)
GGT concentration (Units/L)
B
CMV-
r = 0.1213
p = 0.7072
C
CMV+
r = 0.2925
p = 0.2244
D
CMV+ g-NK-
r = 0.1606
p = 0.6001
E
CMV+ g-NK+
r = 0.7114
p = 0.1107

Figure 14. Proportion of g-NK cells does not correlate with the viral load. (A) The proportion of g-NK cells in HCV subjects, divided based on HCMV serology and further subdivided based on absence or presence of g-NK cells, were compared with the viral load (n = 14, 17, and 6 for HCMV- g-NK-, HCMV+ g-NK- and HCMV+ g-NK+ respectively). **(B)** The proportion of g-NK cells in HCV patients' PBMC were compared with the viral load of HCV (n = 6). **(A)** Unpaired parametric Student's *t*-test with Welch's correction was used for statistical analysis. **(B)** Pearson correlation was applied for statistical analysis.


A
Viral load in HCV patients
p=0.2813
p=0.5297
Viral load (IU/mL)
1.0×10⁸
8.0×10⁷
6.0×10⁷
4.0×10⁷
3.0×10⁷
2.0×10⁷
1.0×10⁷
0
HCMV- g-NK-
HCMV+ g-NK-
HCMV+ g-NK+
B
g-NK cells vs viral load
r = 0.1627
p = 0.7581
Viral load (IU/mL)
1.0×10⁸
8.0×10⁷
6.0×10⁷
4.0×10⁷
2.0×10⁷
0
0
10
20
30
40
50
Proportion of g-NK cells (%)

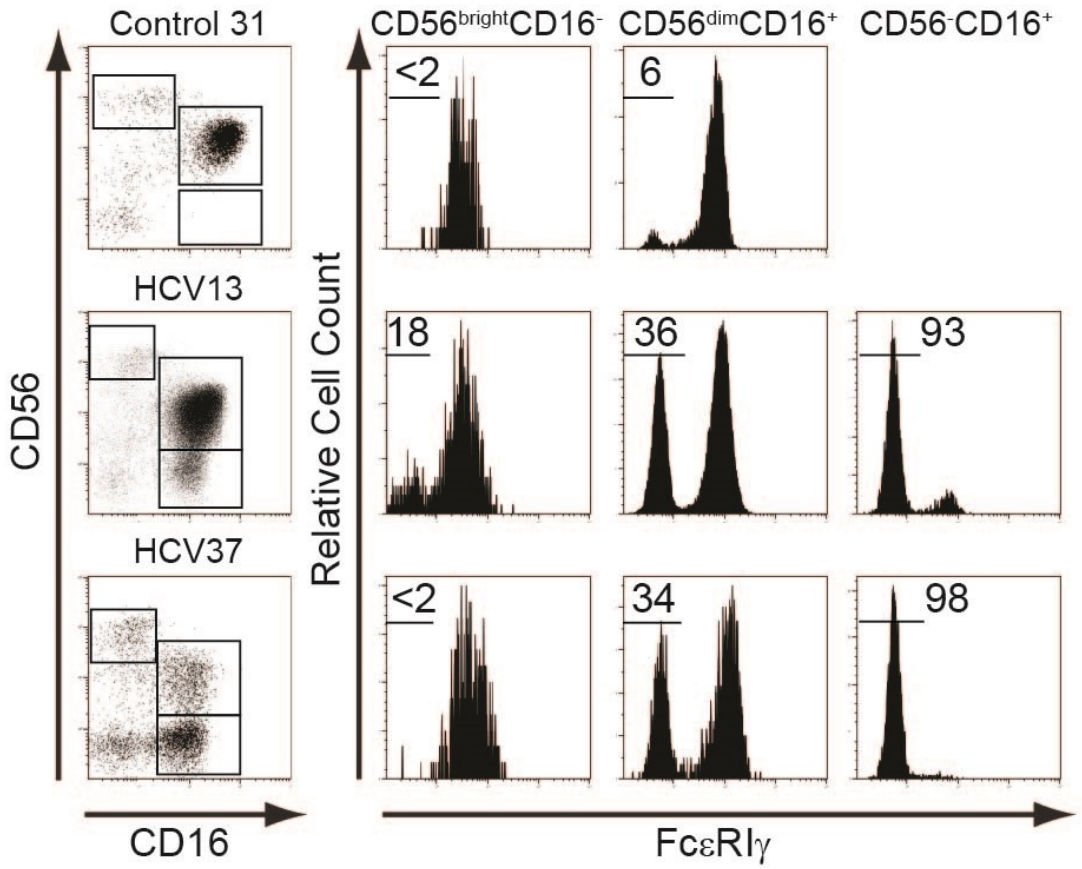
3.5 $CD56^{neg}CD16^{+}$ NK cells predominantly contain g-NK cells

Human NK cells in PBMC can generally be divided into two subsets based on their expression of CD56 and CD16, a major $CD56^{dim}CD16^{+}$ NK cell population and a minor $CD56^{bright}CD16^{-}$ NK cells. As previously reported (Hwang et al., 2012; Lee et al., 2015; Schlums et al., 2015; Zhang et al., 2013), $CD56^{bright}CD16^{-}$ NK cell population contains few g-NK cells (**Figure 15, second column**); while the majority of them are found among $CD56^{dim}CD16^{+}$ NK cells (**Figure 15, third column**). Interestingly, another $CD16^{+}$ NK cell population characterized by the absence of CD56 expression ($CD56^{neg}CD16^{+}$) was often observed in HIV-1 or HCV infected individuals (Bjorkstrom et al., 2010), and was suggested to be an exhausted NK cell population due to its persistent stimulation during the chronic state of infection (Gonzalez et al., 2009; Gonzalez et al., 2008). Since the association of g-NK cells with liver fibrosis and the GGT liver enzyme levels suggested beneficial roles of the g-NK cells in the immune response against chronic HCV infection, this led me to examine whether more g-NK cells are found among the $CD56^{neg}CD16^{+}$ NK cell population.

Among all HCV+ individuals, the $CD56^{neg}CD16^{+}$ NK cell population was present in two HCV+ individuals, HCV13, and HCV37, whereas none among the healthy control group possessed the population. Importantly, compared to $CD56^{dim}CD16^{+}$ NK population, the $CD56^{neg}CD16^{+}$ NK population is primarily composed of g-NK cells (**Figure 15, fourth column**). The NK cell phenotype of the HCV37 individual was particularly interesting due to the high proportion of $CD56^{neg}CD16^{+}$ NK cells. It is also noteworthy that the HCV37 individual is the outlier who possesses a high proportion of the g-NK cell subset without a sign of prior CMV exposure (**Figure 5C**). Consistently, the $CD56^{neg}CD16^{+}$ NK population did not exhibit CMV-associated NKG2C expression (Lopez-Verges et al., 2011). Instead, in agreement with the previously

demonstrated characteristics of the $CD56^{neg}CD16^{+}$ NK population (Bjorkstrom et al., 2010; Eller et al., 2009; Gonzalez et al., 2009; Mavilio et al., 2005), those NK cells in the HCV37 individual expressed NKG2D and showed IFN-γ production as well as a sign of degranulation via CD107a upon CD16 stimulation (**data not shown**), supporting the identity of this subset of cells as bona fide NK cells. Hence, these evidences identify the majority of $CD56^{neg}CD16^{+}$ population in two HCV+ individuals as g-NK cells and further support the active involvement of g-NK cells in chronic HCV infection.

Figure 15. $CD56^{neg}$ $CD16^{+}$ NK cells predominantly contain g-NK cells.
Flow cytometric analysis was performed on the PBMCs of one healthy and two HCV individuals in order to determine the proportion of g-NK cells in $CD56^{neg}CD16+$, $CD56^{dim}CD16+$, and $CD56^{bright}CD16-$ populations. Indicated numbers represent g-NK cell proportion. HCV13 patient is a CMV+ individual with g-NK cells; and HCV37 patient is a unique CMV- individual with g-NK cells. Representative flow cytometry plots from at least two independent experiments are shown.


Control 31
HCV13
HCV37
CD56
CD16
CD56brightCD16-
CD56dimCD16+
CD56-CD16+
Relative Cell Count
<2
6
18
36
93
<2
34
98
FcεRIγ

3.6 g-NK cells show enhanced ADCC against rituximab coated target cells

I was able to observe reduced GGT enzyme and fibrosis level in chronic HCV patients who possess g-NK cells (**Figure 11**). This indicates that the presence of g-NK cells brings a positive clinical outcome in chronic viral infection. I observed a superior ADCC response by g-NK cells upon cross-linking their surface CD16 with α-CD16 antibody (**Figure 10**). Furthermore, although g-NK cells are known to be highly associated with prior CMV infection, others have shown that g-NK cells can also respond to herpes simplex virus (HSV) infected or influenza virus infected cells in the presence of antibody specific to the respective viruses (Lee et al., 2015; Zhang et al., 2013). This suggests that the ADCC response by g-NK cells is not exclusive to CMV infection alone, and could be utilized to enhance NK cell based therapy in other diseases. Rituximab monoclonal antibody therapy is being used to treat B lymphoproliferative malignancies, such as NHL (Alderson and Sondel, 2011; Iannello and Ahmad, 2005); and ADCC has been suggested to be the principal mechanism of rituximab in its anti-tumor response (Clynes et al., 1998; Clynes et al., 2000). Therefore, I wanted to test whether g-NK cells can exhibit a superior anti-tumor response via ADCC compared to their conventional counterparts. For my preliminary study, the ADCC response of g-NK cells from healthy individuals was tested in vitro by stimulating NK cells with rituximab coated lymphoma cell lines and B cells from a CLL patient.

When enriched NK cells from healthy individuals were co-incubated with target lymphoma cell lines originated from patients with Burkitt lymphoma (type of NHL), such as Daudi, Raji, and RAMOS cells, no NK cells response, in the form of IFN-γ production, was observed in the absence of antibody (**Figure 16, first column**). In the presence of IgG antibody, no NK cell response was observed as well (**Figure 16, second column**). However, in the presence of

rituximab, the response by both conventional NK cells and g-NK cells was observed (**Figure 16, third column**). In addition, I observed that unlike conventional NK cells, g-NK cells responded more vigorously to all rituximab-coated CD20+ B lymphoma cell lines tested and predominantly produce IFN-γ (**Figure 16, third column**). It is also interesting to note that out of all three lymphoma cell lines tested, Daudi and RAMOS cells elicited the strongest g-NK cells response (**Figure 16**).

NK cells are one of the major producers of IFN-γ which can modulate the immune response of other cells. In addition, NK cells are also known for their cytotoxic function via the release of cytotoxic molecules such as granzyme and perforin. Hence, CD107a, a marker used to measure the exocytosis of granules by NK cells, was also measured upon stimulation by RAMOS cells, a lymphoma cell line that elicited the strongest IFN-γ response in g-NK cells. The degranulation by NK cells from two different healthy subjects was weak compared to the IFN-γ response (**Figure 17**). Interestingly, the NK cells from each individual showed a different response. While g-NK cells from eC041 responded better than conventional NK cells (**Figure 17A**), both NK cells from eC054 responded similarly (**Figure 17B**).

So far, I have tested the NK cells response against lymphoma cell lines. But the measurement of NK cell response against cancer cells from an actual CLL patient would provide better insights toward potential clinical outcome as it would be more clinically relevant. When NK cells were stimulated with the patient sample in the absence of antibody, no response by NK cells was observed (**Figure 18, first column**). Similarly, no NK cell response was observed when IgG antibody was added (**Figure 18, second column**). However, when rituximab was added, only g-NK cells showed a meaningful response while conventional NK cells showed a negligible response (**Figure 18, third column**). It is also interesting to note that the NK cell response,

based on IFN-γ production, elicited against B cells from the CLL patient was dramatically lower than the one elicited by lymphoma cell lines (**Figure 16 and Figure 18**).

In summary, I observed an ADCC response by NK cells only when they were stimulated by rituximab coated lymphoma cell lines and CD20+ B cells from CLL patient. The ADCC response was characterized by strong IFN-γ production but weak granule exocytosis. Among lymphoma cell lines, RAMOS and Daudi cells elicited a NK cell response stronger than that by Raji. Furthermore, the ADCC response by g-NK cells was substantially better than the conventional NK cells. A similar trend was observed when NK cells were stimulated with a CLL patient sample, but the response was dramatically lower than the one elicited by lymphoma cell lines. These results suggest a new therapeutic strategy where we could take advantage of the stronger CD16 mediated ADCC response by g-NK cells and enhance the rituximab treatment in CLL patients.

Figure 16. Proportion of NK cells producing IFN-γ upon co-incubation with rituximab treated lymphoma cell lines: Daudi, Raji and RAMOS cells. Enriched NK cells from healthy individual, who possesses g-NK cells, were co-cultured for 7 hours with rituximab treated B cell lymphoma cell lines Daudi, Raji and RAMOS at a 1 to 1 cell number ratio. Upon completion, cells were stained and the proportion of NK cells expressing IFN-γ was measured using flow cytometry. The numerical values on the dot plots indicate proportions of IFN-γ producing NK cells from the total NK cell population. The numerical values on the histograms indicate the proportion of IFN-γ producing NK cells from either conventional NK cells or g-NK cells.

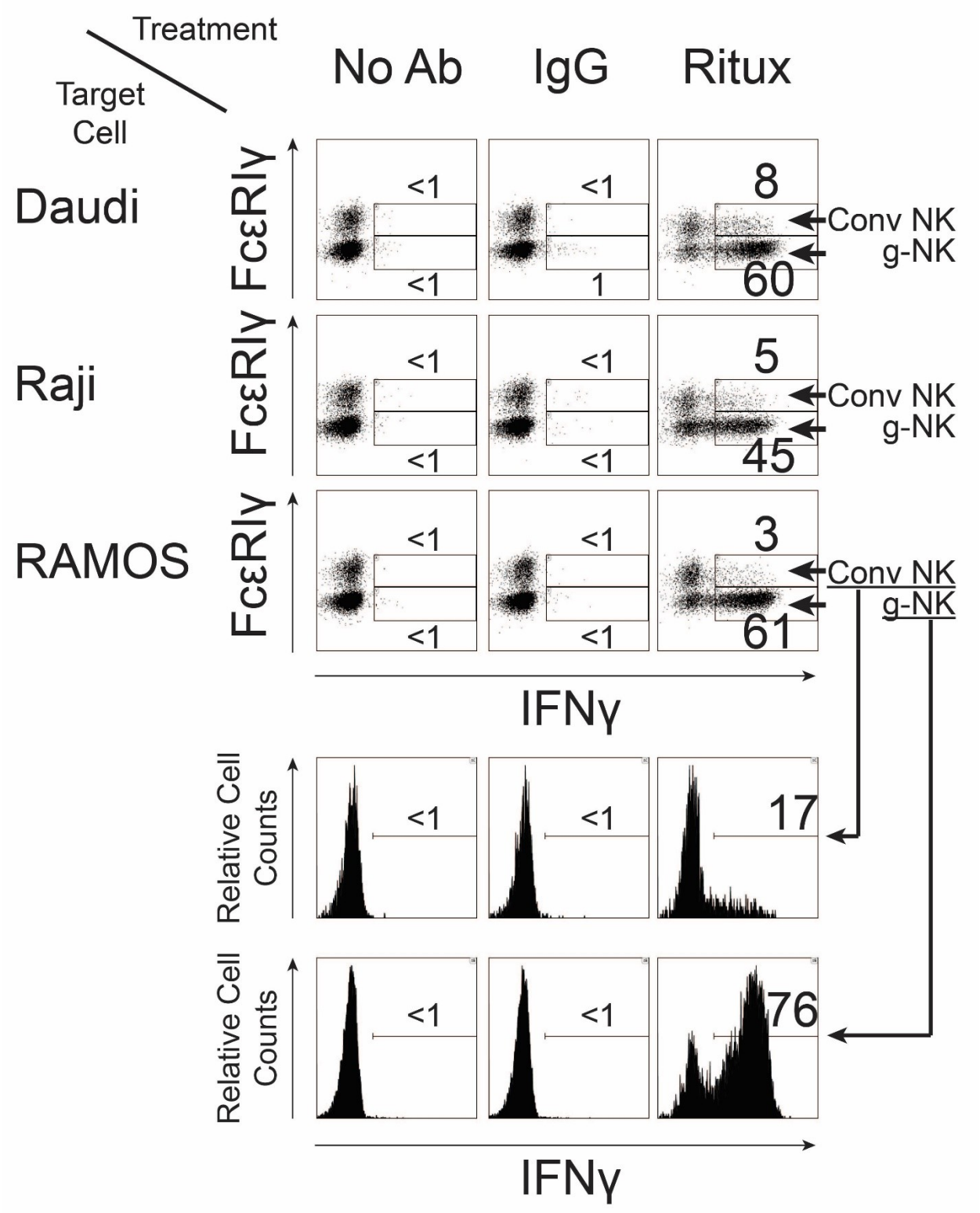

Treatment
Target Cell
No Ab
IgG
Ritux
Daudi
Raji
RAMOS
FcεRIγ
<1
8
60
1
5
45
3
61
Conv NK
g-NK
IFNγ
Relative Cell Counts
17
76

Figure 17. Proportion of NK cells producing CD107a (degranulation marker) upon co-incubation with rituximab treated RAMOS lymphoma cell line. Enriched NK cells from healthy individuals, who possess g-NK cells, were co-cultured for 7 hours with rituximab treated B cell lymphoma cell line RAMOS at a 1 to 1 cell number ratio. Upon completion, cells were stained and the proportion of NK cells expressing CD107a was measured using flow cytometry. The numerical values on the histograms indicate the proportions of CD107a expressing NK cells from either conventional NK cells or g-NK cells. The numerical values on the dot plots indicate the proportion of IFN-γ producing NK cells from total NK cells population.

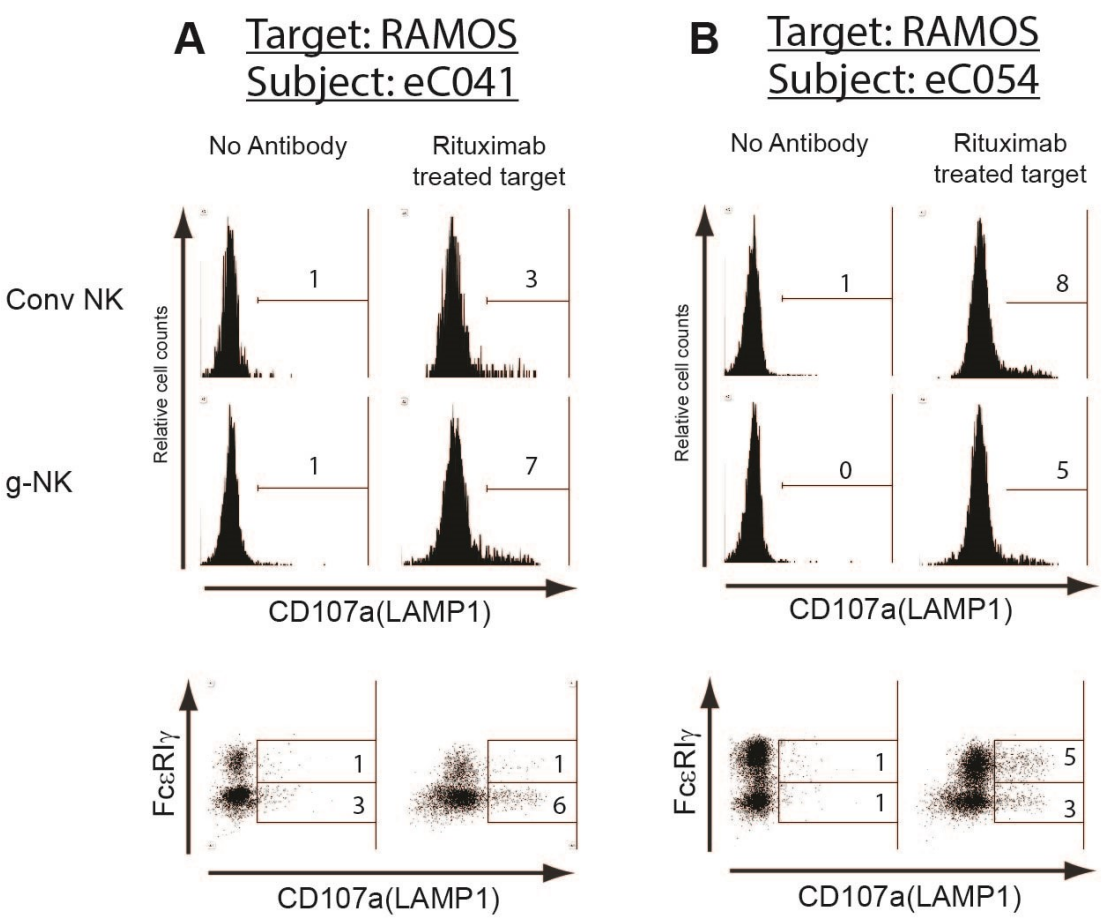
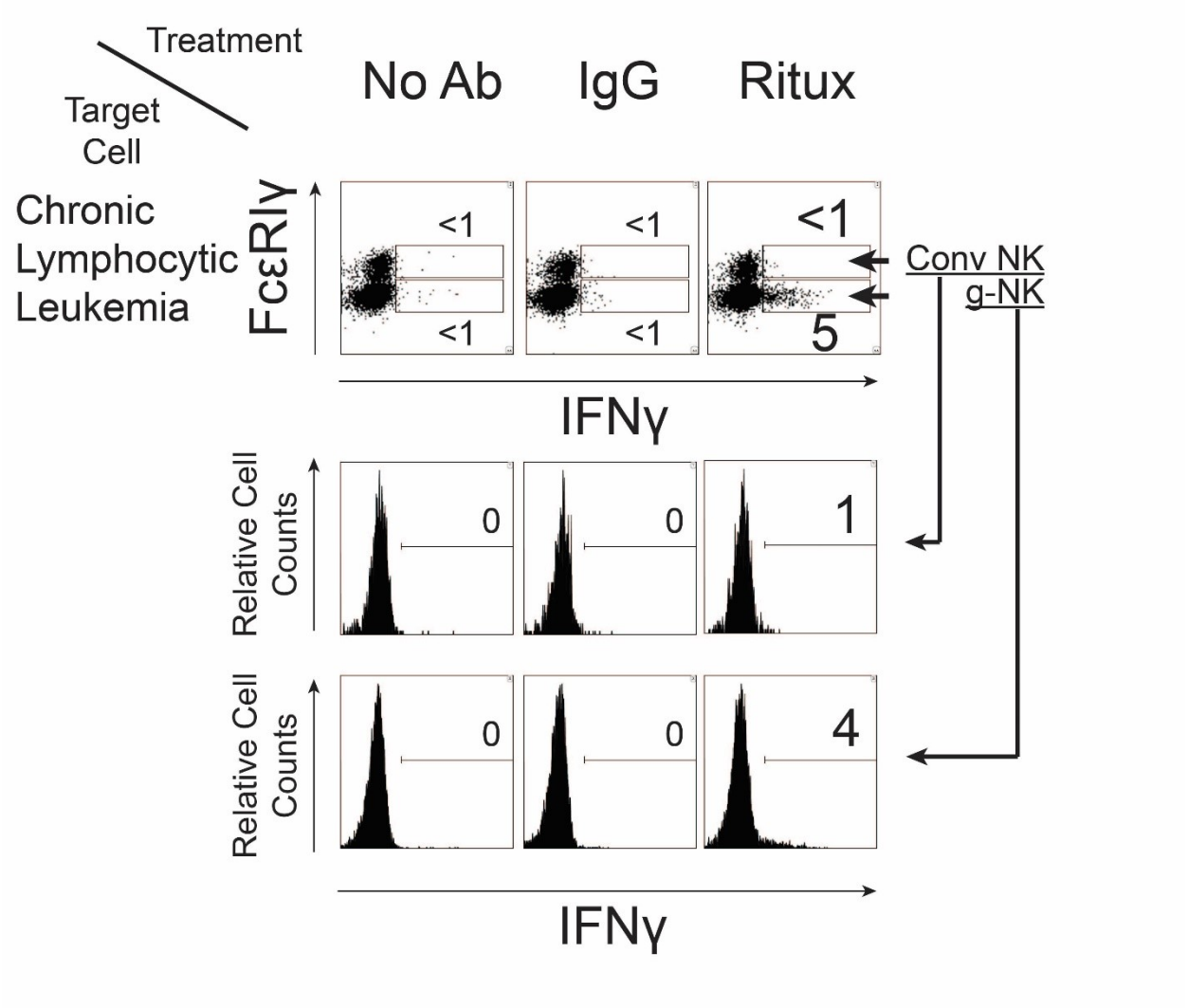

A
Target: RAMOS
Subject: eC041
No Antibody
Rituximab treated target
Conv NK
g-NK
Relative cell counts
1
3
1
7
CD107a(LAMP1)
FcεRIγ
1
3
1
6
CD107a(LAMP1)
B
Target: RAMOS
Subject: eC054
No Antibody
Rituximab treated target
Relative cell counts
1
8
0
5
CD107a(LAMP1)
FcεRIγ
1
1
5
3
CD107a(LAMP1)

Figure 18. Proportion of NK cells producing IFN-γ upon co-incubation with rituximab treated PBMC of CLL patient. Enriched NK cells from a healthy individual, who possesses g-NK cells, were co-cultured for 7 hours with rituximab treated PBMC of a CLL patient at a 1 to 1 cell number ratio. Upon completion, cells were stained and the proportion of NK cells expressing IFN-γ was measured using flow cytometry. The numerical values on the dot plots indicate the proportions of IFN-γ producing NK cells from total NK cells population. The numerical values on the histograms indicate the proportion of IFN-γ producing NK cells from either conventional NK cells or g-NK cells.

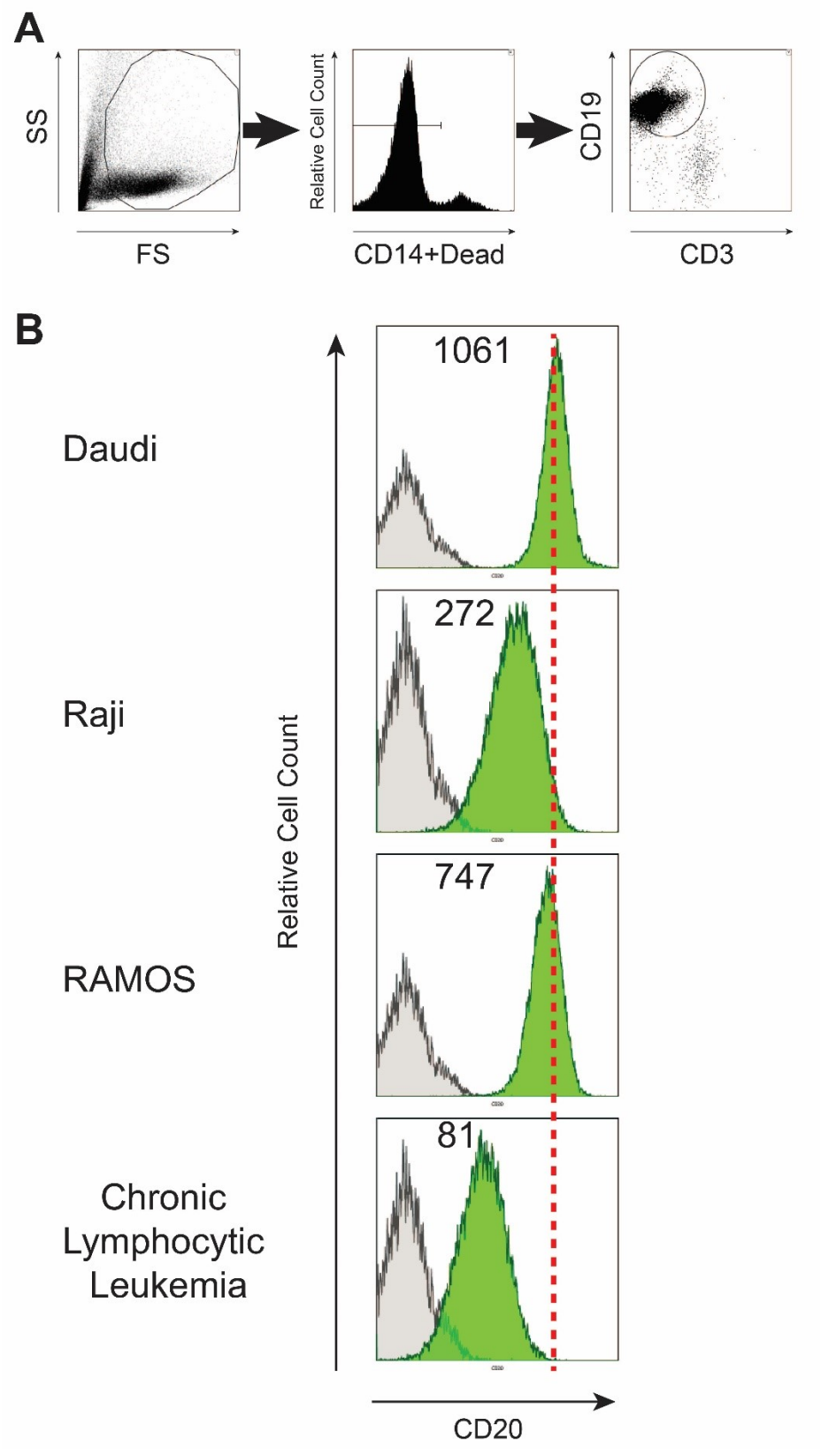

Treatment
Target Cell
No Ab
IgG
Ritux
Chronic Lymphocytic Leukemia
FcεRIγ
<1
<1
<1
<1
<1
5
IFNγ
Conv NK
g-NK
Relative Cell Counts
0
0
1
Relative Cell Counts
0
0
4
IFNγ

3.7 Evaluation of CD20 expression on target B cells

Previously, I observed the ADCC response by NK cells upon stimulation with rituximab coated lymphoma cell lines (Daudi, Raji and RAMOS) and CD20+ B cells from a CLL patient. It was interesting to note that the proportion of g-NK cells expressing IFN-γ differed between each of lymphoma cell lines and CLL despite using the same concentration of rituximab, a monoclonal antibody against CD20, in each test. RAMOS and Daudi cell lines were able to elicit the strongest ADCC response by g-NK cells followed by Raji. B cells from the CLL patient elicited a dramatically lower response (**Figure 16 and Figure 18**). Since rituximab is an anti-CD20 antibody, I suspected that the varied response of g-NK cells against rituximab coated target cells might be due to the differences in the expression of CD20 on each target cell. Therefore, in order to test whether this difference is a result of the differential expression of CD20 on each target cell, CD20 expression level was analyzed on all lymphoma cell lines, and B cells of CLL patient. Using multicolour flow cytometry, B cells were isolated as CD19-positive CD3-negative cells after gating on lymphocytes based on their characteristics of cell size and granularity, followed by the exclusion of $CD14^{+}$ monocytes, and dead cells (**Figure 19A**). Consistent with the NK cell ADCC response against antibody coated target cells, it was observed that Daudi and RAMOS cell lines showed the highest expression of CD20, followed by Raji then the B cells from CLL patient (**Figure 19B**).

This result strongly suggests that the NK cells' ADCC response against rituximab coated target cells is dependent on the level of CD20 surface expression on the target cells.

Figure 19. Expression of surface CD20 on Daudi, Raji, and RAMOS lymphoma cell lines, and B cells from CLL patient. The lymphoma cell lines Daudi, Raji and RAMOS and PBMC of CLL patient were stained using CD3, CD14, CD16, CD56, CD19 and CD20 antibodies. Following exclusion of T cells, monocytes, and NK cells, expression of CD20 was measured on CD19+ B cells. The numerical values on the histograms indicate mean intensity values of CD20 expression on B cells (from lymphoma cell lines or CLL patient).


A
SS
FS
Relative Cell Count
CD14+Dead
CD19
CD3
B
Daudi
Raji
RAMOS
Chronic
Lymphocytic
Leukemia
1061
272
747
81
Relative Cell Count
CD20

4. DISCUSSION

CMV has coevolved with humans for millions of years and expresses various encoded proteins, such as the pp65 tegument protein, which is directed to modulate both the innate and adaptive immune responses and allows the virus to establish a life-long latency (Engel and Angulo, 2012; Sun and Lanier, 2009). In particular, CMV infection is known to shape the phenotype and function of NK cells exemplified by the expansion and persistence of an NK cell subset displaying high surface levels of the NKG2C receptor (Muntasell et al., 2013). g-NK cells characterized by the lack in expression of the FcεRIγ adaptor protein are a newly identified NK cell subset, the expansion of which is also highly associated with prior CMV infection (Lee et al., 2015; Schlums et al., 2015; Zhang et al., 2013). Interestingly, the population is found to be stable for many years (Beziat et al., 2013; Lee et al., 2015; Schlums et al., 2015; Zhang et al., 2013), reminiscent of memory cell populations. Strong co-expression of NKG2C in the g-NK cell population was reported (Schlums et al., 2015; Zhang et al., 2013), suggesting that g-NK cells largely overlap with NKG2C+ NK cells. Considering the high and various prevalence of CMV infection in human populations (50-100%, depending on the geography) (Gaytant et al., 2002), it suggests that CMV can dramatically influence the immunological makeup of specific populations, and studying this modulation is important to understand the geographic or ethnic specific immune response.

NK cells exhibit a restricted recognition of target cells via their germline-encoded activating receptors (Lanier, 2008; Lee et al., 2007). This limitation could be exacerbated in cases of recognition of target cells infected with RNA viruses that show higher rates of mutations. ADCC function might represent an adaptation by which NK cells can recognize a broader range of target cells with the aid of antibodies. Interestingly, an in vitro study has revealed that g-NK cells

can respond to herpes simplex virus (HSV) infected or influenza virus infected cells in the presence of plasma (Lee et al., 2015; Zhang et al., 2013). The reactivity of g-NK cells against a broad range of targets is similar to the in vivo NKG2C+ NK cell expansion upon HIV-1, hantavirus, HBV, and HCV as previously reported (Beziat et al., 2012; Bjorkstrom et al., 2011; Brunetta et al., 2010; Guma et al., 2006; Petitdemange et al., 2011). Considering their long-lasting presence and the broad range of target cell recognition supported by antibodies, I hypothesized that g-NK cells are implicated in chronic infections such as HCV. In my analysis, slightly reduced proportions of the g-NK cell subset were found in chronic HCV subjects compared to healthy controls. Although the precise mechanism for the reduced g-NK cell proportions has yet to be identified, NK cells from chronically infected individuals showed diminished expression and/or function of NK cell activating receptors in several studies (Holder et al., 2014; Mela et al., 2005; Nattermann et al., 2005). To understand the interaction between g-NK cells and HCV infection, it would be very interesting to study the ADCC response of g-NK cells upon encountering antibody coated HCV infected hepatocytes. Unfortunately, the acquisition of HCV replicons in order to produce infectious HCV proved to be challenging; and in addition, my PBMC stocks of chronic HCV patients were depleted.

A mechanism by which g-NK cells are generated has recently been demonstrated, involving the epigenetic downregulation of FcεRIγ and SYK (Lee et al., 2015; Schlums et al., 2015). The altered phenotypes in NK cells are maintained for many years without a major fluctuation in healthy individuals. Thus far, CMV is the only identified driving force leading to the generation of the g-NK cell population, although only a few viruses such as HSV-1/2 have been assessed (Zhang et al., 2013). My results suggest that alternative pathways other than CMV infection might generate g-NK cells. One CMV seronegative patient who possesses a high g-NK cell

proportion was negative for both the CMV serology and the CMV pp65 specific T-cell response, suggesting that the patient had never been exposed to CMV. In addition, the g-NK cells of this patient did not express NKG2C (**data not shown**), supporting the possibility that the generation of g-NK cells is not due to CMV infection. Interestingly, the individual presented a high proportion of the $CD56^{neg}$ NK cells (**Figure 15**). The $CD56^{neg}$ NK cells were known to express lower levels of the natural cytotoxicity receptor NKp30 and NKp46 (Eller et al., 2009; Mavilio et al., 2005), consistent with the dramatically reduced expression of those receptors on g-NK cells. Therefore, in order to elucidate the mechanism of g-NK cell generation during chronic infections, investigating the relationship between g-NK cells and the $CD56^{neg}$ NK cells should be warranted. Possible driving forces for the generation of g-NK cells could be the virus infection itself or the inflammatory environment accompanied by the infection.

Although the roles of NK cell subsets expanded upon CMV infection are not fully resolved, several lines of evidence suggest a role in infection and cancer. In a T-cell deficient patient, a protective role for NKG2C+ NK cells has been proposed in the resolution of CMV infection (Kuijpers et al., 2008). Notably, a recent study reported a correlation between early CMV reactivation and a decrease in leukemia relapse after allogenic hematopoietic stem cell transplantation in adult patients (Elmaagacli et al., 2011; Green et al., 2013; Manjappa et al., 2014). Furthermore, Kim and colleagues demonstrated that g-NK cells exhibit enhanced ADCC capability through CD16 and can respond to not only CMV-infected cells but also HSV-infected and influenza virus infected cells in the presence of plasma from infected individuals, suggesting its potential to control a variety of virus-infected cells (Lee et al., 2015; Zhang et al., 2013). Thus I investigated whether the CMV-induced g-NK cell subset is associated with beneficial consequences in chronic HCV infection.

ALT, AST, and GGT are enzymes metabolically required by hepatocytes. Upon hepatocyte lysis, these enzymes are released into the serum and therefore the increased levels of the enzymes in the serum are considered as critical indicators of hepatocellular injury caused by hepatotrophic virus infections such as HBV and HCV (Guidotti and Chisari, 2006). Among chronic HCV+ individuals, no differences in the levels of liver enzymes or fibrosis between CMV+ and CMV- were identified in my study (**Figure 11**). These observations preclude us from drawing a simple conclusion regarding a beneficial effect of previous CMV exposure on the NK cell response to subsequent viral infections. Remarkably, my results showed that there was a trend of reduced liver enzyme levels in the g-NK+ group among CMV+ chronic HCV individuals. The most significant reduction was observed in the GGT levels of the g-NK+ group. Although these liver enzymes are routinely used to evaluate liver inflammation, less is known about the association between GGT levels and chronic HCV infection outcomes. Currently, the reason why a stronger correlation with g-NK cell population was found with GGT levels compared to ALT or AST levels remains unclear. One explanation could be that elevated GGT levels have been associated with staging 3 and 4 fibrosis, suggesting that the GGT level could be a marker of more advanced liver disease in chronic HCV (Silva et al., 2004). ALT or AST is considered to be more specific to HCV-induced hepatocellular liver disease (Afdhal and Nunes, 2004; Guidotti and Chisari, 2006). Furthermore, the associations between g-NK cell populations and liver fibrosis were primarily found in CMV+ chronic HCV subjects. In spite of the ameliorated liver damage observed in the presence of g-NK cells, I could not correlate the extent of g-NK cell proportions with the levels of liver enzymes (**Figure 12**), viral load (**Figure 14**) and/or HCV-RNA levels (**data not shown**). Previous studies demonstrated that CMV associated NK cells subset is expanded upon encounter with other viruses (Beziat et al., 2012; Bjorkstrom et al., 2011;

Brunetta et al., 2010; Guma et al., 2006; Petitdemange et al., 2011). In addition, the antibody dependent expansion of g-NK cells was also observed in response to CMV and influenza infected cells in the presence of plasma of infected individuals (Lee et al., 2015). Thus, the lack of expansion of g-NK cells in CMV+ chronic HCV subjects seems counterintuitive. One possible explanation for this discrepancy could be that PBMCs used in my study have been obtained from chronically infected HCV+ individuals (i.e. >6 months HCV RNA-positive). Even though stable maintenance of g-NK cells has been demonstrated in healthy controls (Lee et al., 2015; Schlums et al., 2015; Zhou et al., 2015), it is plausible that the proportion of the g-NK cell population might fluctuate in pathological conditions of chronic infections that cause liver damage during decades. In my cross-sectional study, it might be difficult to detect the peak responses of g-NK cells. Therefore, the definitive parameters that I mainly considered in this study were those of the presence of g-NK cells and liver damage. So far, the peak responses of NK cell expansion have only been observed in acute infections such as hantavirus and chikungunya infections (Bjorkstrom et al., 2011; Petitdemange et al., 2011). Another possible scenario could be that g-NK cells are required to protect the host from CMV reactivation during chronic HCV infection. Higher levels of CMV reactivation have been observed in chronic HCV subjects than self-resolving subjects (Tabll et al., 2011) and higher fibrosis was found in patients with reactivated CMV (Bader el-Din et al., 2011). In addition, the impact of heavy alcohol consumption on the differential levels of GGT or liver fibrosis between CMV+ g-NK- and CMV+ g-NK+ groups is expected to be minimal since both groups have similar ratios of individuals who daily consume alcohol excessively (62.5-64.3% and 50.0%, respectively). Even though the definitive role of g-NK cells during chronic HCV infection remains elusive, my results demonstrated that the presence of g-NK cells is associated with less hepatocellular injury

and liver fibrosis in chronic HCV patients. I believe that these data add novel information to the function of the newly discovered g-NK cells during pathological settings as shown here for HCV infection.

Several limitations of this study need to be considered. First, the direct killing of HCV-infected hepatoma cells by g-NK cell mediated ADCC in the presence of plasma from HCV+ individuals is plausible. However, due to limited resource, my current study did not directly test it. For an alternative study, I used lymphoma cell lines and B cells from CLL patient in order to test the ADCC capability of g-NK cells. As the results showed enhanced ADCC response by g-NK cells to rituximab coated target cells, including actual clinical patient sample (**Figure 16 and Figure 18**), it is plausible to hypothesize that ADCC by g-NK cells against infected hepatocytes could have also occurred in CMV+ g-NK+ chronic HCV patients. Interestingly, a report showed an association between the proportions of peripheral blood $CD56^{dim}CD16^{neg}$ NK cells and SVR (Nattermann et al., 2005; Oliviero et al., 2013). Since CD16 is downregulated on cells that have recently degranulated (Bowles and Weiner, 2005), it might suggest an active role of NK cell mediated ADCC in the killing of HCV-infected hepatocytes. In addition, ADCC against HCV-infected cells via antibody binding to a surface-expressed HCV envelope protein has been suggested (Nattermann et al., 2005). Second, I could not study the intrahepatic NK cells during chronic HCV infection because a noninvasive method for assessing liver fibrosis (i.e. Fibroscan) was utilized in most patients evaluated in this study. It is noteworthy that NK cells are enriched in the liver and therefore the investigation of the g-NK cell population in liver tissues is likely important to determine whether the g-NK cell population is found only in peripheral blood or also in tissues. Third, the presence of the g-NK cell population within the $CD56^{neg}$ NK cells questions the maturation and exhaustion status of g-NK cells. Further studies of the phenotype

and function of this subset of cells are required to understand the maturation process of g-NK cells during HCV pathogenesis.

The ADCC response by g-NK cells is not exclusive to CMV infection. Other reports have shown that g-NK cells can also respond to HSV infected or influenza virus infected cells in the presence of plasma (Lee et al., 2015; Zhang et al., 2013). Furthermore, I have shown that the presence of g-NK cells increases the resistance against chronic HCV induced liver damage. As ADCC has been suggested to be the principal mechanism of rituximab in its anti-tumor response (Clynes et al., 1998; Clynes et al., 2000), this raises an interesting question as to whether g-NK cells could be utilized to enhance the efficacy of rituximab in cancer therapy. My preliminary results showed a superior ADCC response by g-NK cells upon encountering rituximab coated lymphoma cell lines and B cells from a CLL patient. More tests with a larger sample pool are needed to confirm this observation, but the superior response was only limited to IFN-γ production and not degranulation (indicated by CD107a marker); which may affect NK cells' direct cytotoxicity. IFN-γ is known for its diverse immunomodulatory feature (Zaidi and Merlino, 2011). IFN-γ induces the differentiation and modulates the function of many types of immune cells. It regulates the differentiation, activation, and homeostasis of Th1-mediated immune responses and this regulation is mainly mediated by the activation of the dendritic cells' antigen presenting capability. It also activates macrophages and induces the production of chemokines, which recruit specific effector cells to the site of inflammation. In addition, IFN-γ is a potent antiviral cytokine capable of inducing nitric oxide (NO) production in infected cells. IFN-γ is the key cytokine for the induction of iNOS (inducible nitric oxide synthase) in activated macrophages and the generation of NO from the amino acid L-arginine, and thereby contributing to the control of replication or killing of intracellular microbial pathogens (Bogdan et al., 2000).

These activations are all associated with the anti-tumor mechanism during cell mediated adaptive immune response.

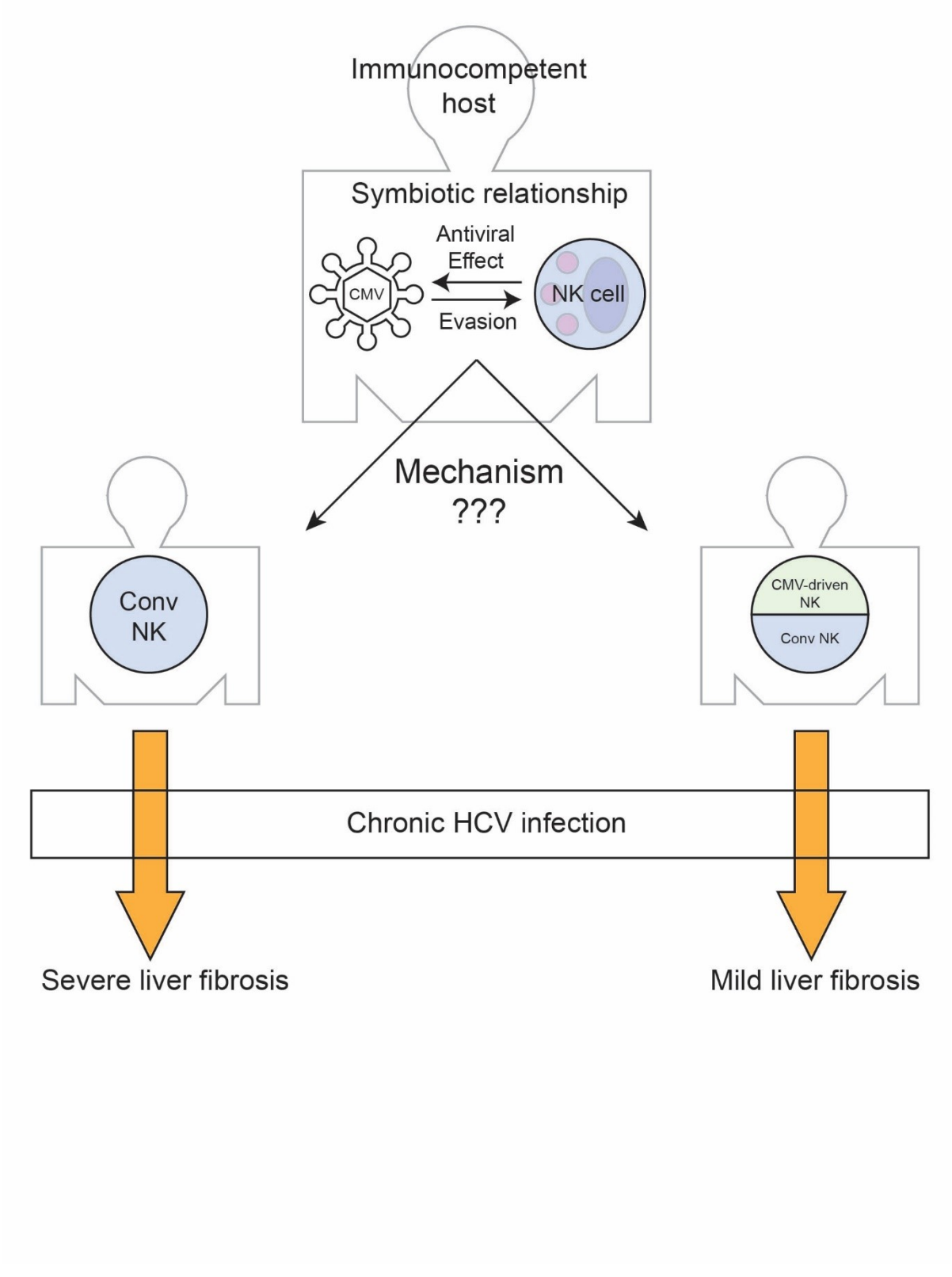
5. CONCLUDING REMARKS

In conclusion, I provided evidence that functionally capable g-NK cells are also present in chronic HCV patients, and their presence is associated with reduced liver damage. Based on this result, I propose a model where evolution plays an important role in the continual enhancement of the immune-evasive ability of CMV, and the antiviral ability of NK cells. In a way, this rivalry can be viewed as a symbiotic relationship, because in the end, they bring mutual benefit to each other. From the perspective of NK cells, an enhancement of NK cells is the generation of g-NK cells, a unique subset of NK cells with an extraordinary ADCC capability; and the presence of this unique subset of NK cells leads to mild liver fibrosis in chronic HCV patients, bringing a positive clinical outcome (**Figure 20**).

Thanks to the modular design of the mechanism of ADCC and the limitless antigen specificity of antibodies, the response of g-NK cells against infection is not strictly limited to CMV, but to a broad range of infections in the presence of effective antibodies. This was clearly seen in my experiment involving lymphoma cell lines, such Daudi, Raji and RAMOS cells, and CD20+ B cells from CLL patient, where a superior ADCC response by g-NK cells against rituximab coated lymphoma and leukemia target cells was observed.

For the first time, my data suggest that CMV influences the immune response by NK cells against chronic HCV infection and CLL. Hence, these results will provide insight in our understanding of the role of the long-lasting NK cell population in the context of a variety of pathological conditions such as chronic viral infections and cancers.

Figure 20. Summary diagram showing the beneficial role of g-NK cells in chronic HCV patients. The study has shown that similar to healthy individuals, prior encounter with CMV leads to development of g-NK cell population, and this population is maintained in chronic HCV patients. I observed that chronic HCV patients who possess this unique subset of NK cells have reduced liver fibrosis compared to those who do not; indicating that g-NK cells increase the host resistance against HCV induced liver damage.


Immunocompetent host
Symbiotic relationship
Antiviral Effect
CMV
NK cell
Evasion
Mechanism ???
Conv NK
CMV-driven NK
Conv NK
Chronic HCV infection
Severe liver fibrosis
Mild liver fibrosis

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7. APPENDIX

Ottawa Hospital Research Institute
Institut de recherche de l'Hôpital d'Ottawa

uOttawa

University of Ottawa Heart Institute
Institut de cardiologie de l'Université d'Ottawa

Ottawa Health Science Network Research Ethics Board/ Conseil d'éthique de la recherche du Réseau de science de la santé d'Ottawa
Civic Box 411 725 Parkdale Avenue, Ottawa, Ontario K1Y 4E9 613-798-5555 ext. 14902 Fax : 613-761-4311
http://www.ohri.ca/ohsn-reb

March 11, 2016

Dr. Curtis Cooper
Ottawa Hospital - General Campus
Division of Infectious Diseases
501 Smyth Road, Module G
Ottawa, ON
K1H 8L6

Dear Dr. Cooper:

RE: Protocol# - 20120009-01H **Immune and Metabolic Function During Viral Hepatitis Infection**

Renewal Expiry Date - April 04, 2017

Thank you for the letter from Mr. D. Mackie dated March 1, 2016. I am pleased to inform you that your Annual Renewal Request was reviewed by the Ottawa Health Science Network Research Ethics Board (OHSN-REB) and is approved. No changes, amendments or addenda may be made in the protocol or the consent form without the OHSN-REB's review and approval.

Your request to extend the projected date of study completion to May 2018 is approved.

Renewal is valid for a period of one year. Approximately one month prior to that time, a single renewal form should be sent to the REB office.

The REB no longer requires a 'valid until' date at the bottom of all approved informed consent forms. The consent forms currently approved for use by the REB are:
-English and French Information Sheet and Consent Form for Blood Collection, Version 2, dated June 5, 2012

OHSN-REB complies with the membership requirements and operates in compliance with the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans; the International Conference on Harmonization - Good Clinical Practice: Consolidated Guideline; and the provisions of the Personal Health Information Protection Act 2004.

Yours sincerely,

Raphael Saginur, M.D.
Chairperson
Ottawa Health Science Network Research Ethics Board

/cb

EDUCATION

University of Ottawa, Ottawa, ON **2014-Present**
MSc in Biochemistry, Microbiology and Immunology

Trent University, Peterborough, ON **2009-2014**
Honours BSc in Biochemistry and Molecular Biology
Dean's honour rolls
Thesis: "Investigation of ATF2 as a potential target of Cadmium induced pJNK in mouse forelimb bud cells"

RESEARCH EXPERIENCE AND REPERTOIRE

Master's Research Project **Present**
University of Ottawa, Ottawa, ON
Supervisor: Dr. Seung-Hwan Lee

- Peripheral blood mononuclear cell (PBMC) isolation of human blood samples
- Process PBMCs for cryopreservation
- Human and mice Natural Killer (NK) cell enrichment
- Ability to handle HCV, HIV, HCMV and CLL infected human blood samples
- Culture and maintain human MRC-5 cells, NK cells, and B-cell lymphoma
- Ability to propagate HCMV and influenza virus
- HCMV viral DNA isolation and purification
- Perform enzyme-linked immunosorbent assay (ELISA)
- Perform bead-based assay: cytometric bead array (CBA)
- Perform agarose gel electrophoresis
- Stimulate human PBMCs with various cytokines, antibodies, and peptides
- Extraction of mice DNA
- Mice dissection to extract spleen, lung, liver and brain
- Perform polymerase chain reaction (PCR), including nested PCR
- Perform surface and intracellular antibody staining for multicolor flow cytometry analysis (Fortessa, CyAn, FACSDiva, Summit, Kaluza)
- Familiarity with softwares such as Adobe Illustrator and GraphPad Prism

Student Research Project **2014**
Trent University, Peterborough, ON
Supervisor: Dr. Carolyn Kapron

- Perform mice husbandry, sacrifice, and dissection accordance to the Canadian Council on Animal Care guidelines
- Culture and maintain mice limb bud cells
- Perform western blotting

- Used ImageJ software to analyze raw intensity data

PRESENTATIONS

5th Annual Symposium on Cytokines in Inflammation, ageing, cancer and obesity **2016**
Immunology conference, Eastman, QC

- Performed poster presentation on current NK cells and cancer project
Title: Enhancing antibody-dependent cellular cytotoxicity of Natural Killer cells against cancer cells in monoclonal antibody-mediated immunotherapy

29th Annual Spring Meeting of the Canadian Society for Immunology **2016**
Immunology conference, Ottawa, ON

- Performed poster presentation on current NK cells and HCV project
Title: Investigating the role of NK cell subset lacking FcεRIγ in chronic hepatitis C virus infection

The Biochemistry, Microbiology and Immunology seminar day **2016**
University of Ottawa, Ottawa, ON

- Won 3rd place in oral presentation on current NK cells and HCV project
Title: Investigating the role of NK cell subset lacking FcεRIγ in chronic hepatitis C virus infection

5th Canadian Symposium on HCV (CanHepC) **2016**
Hepatitis C conference, Montreal, QC

- Performed poster presentation on current NK cells and HCV project
Title: NK cells lacking FcεRIγ are associated with reduced liver damage in chronic HCV infection

NK2015-15th Meeting of the Society for Natural Immunity **2015**
NK conference, Montreal, QC

- Performed poster presentation on current NK cells and cancer project
Title: Physical and functional characteristics, and maintenance of novel HCMV-associated ADCC-potent NK subset during chronic HCV infection

The Biochemistry, Microbiology and Immunology seminar day **2015**
University of Ottawa, Ottawa, ON

- Performed poster presentation on current NK cells and cancer project

Title: Physical and functional characteristics, and maintenance of novel HCMV-associated ADCC-potent NK subset during chronic HCV infection

LIST OF PUBLICATIONS

- **Jun S. Oh,** Alaa K. Ali, Sungjin Kim, Daniel J. Corsi, Curtis L. Cooper, Seung-Hwan Lee. NK cells lacking FcεRIγ are associated with reduced liver damage in chronic HCV infection. Eur J Immunol. 2016; 46(4):1020-1029.
  -European Journal of Immunology

- Alaa Kassim Ali, Jun Seok Oh, Eric Vivier, Meinrad Busslinger and Seung-Hwan Lee. NK Cell-Specific Gata3 Ablation Identifies the Maturation Program Required for Bone Marrow Exit and Control of Proliferation. J Immunol. 2016; 196(4):1753-1767.
  -The Journal of Immunology

CERTIFICATION AND TRAINING

- **Bio-containment level 3 facility training** **2014**
- **Lab safety** **2014**
- **WHIMIS certification** **2014**
- **Autoclave safety** **2014**
- **Animal care** **2013, 2014**
- **Radiation safety level I** **2013**
- **Biosafety level I** **2013**

EXTRA-CURRICULAR ACTIVITIES

Media technician **2014-2016**
Ottawa Korean Community Church, Ottawa, ON

Laboratory volunteer **2014**
Trent University, Peterborough, ON
Dr. Carolyn Kapron lab

Management and cashier **2004 – 2012**
Mr. Convenience, Peterborough, ON

Choir and audio/media technician **2009 - 2011**
Korean Paul's Mission Church, Peterborough, ON

Support worker **2003 - 2009**
Christian Horizons, Peterborough, ON

Hospital volunteer **2000 – 2001**
Peterborough Health Regional Centre, Peterborough, ON
Emergency department and surgical outpatient

Team leader **1999 – 2000**
Hope Valley Day Camp, Peterborough, ON