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Effect of NKG2C Absence on Natural Killer Cell Phenotype and Function in Human Immunodeficiency Virus/Cytomegalovirus Co-infection

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Abstract

Natural killer (NK) cells expressing NKG2C and CD57 expand following human cytomegalovirus (CMV) infection. This NK cell subset downregulates FcεR1γ and acquires enhanced capacity to mediate antibody-dependent cellular cytotoxicity (ADCC). Expansion of these differentiated NK cells is exaggerated in human immunodeficiency virus (HIV) infection. Individuals lacking the gene encoding NKG2C have diminished resistance to HIV, but it remains unclear whether differentiation into NK cells with superior ADCC is impaired in $NKG2C^{null}$ individuals. Therefore, our objective was to investigate if CMV-driven NK cell differentiation into enhanced killers is impaired in $NKG2C^{null}$ HIV-infected individuals. Phenotypic ($CD57^{+}$, $FcεR1γ^{-}$) and functional (IFN-γ, TNF-α induction and cytotoxicity) NK cell responses were compared between $NKG2C^{null}$ and matched NKG2C-expressing individuals by flow cytometry following stimulation through natural cytotoxicity receptors (using K562 cells) or CD16 (using monoclonal antibody, 3G8). Cytotoxicity was measured in ^{51}Chromium release assays against anti-CD16-coated P815 cells (redirected lysis) and anti-human leukocyte antigen (HLA) class I antibody-coated C1R cells (classical ADCC). Antibodies were titrated to determine concentrations producing half maximal responses (EC_{50}) to compare sensitivity. Our data indicate highly similar CMV-driven NK cell differentiation in terms of both general phenotype and function, regardless of NKG2C genotype. The observed equivalency between groups suggests alternate routes of CMV-driven NK cell differentiation, which are independent of NKG2C.

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List of Abbreviations and Symbols

%	percentage
vIL-10	viral interleukin
~	approximately
‘	prime
°C	degrees Celsius
µ	micro
ADCC	antibody-dependent cellular cytotoxicity
AIDS	acquired immunodeficiency syndrome
APC	antigen-presenting cell
APC	allophycocyanin
ART	antiretroviral therapy
ATCC	American Type Culture Collection
CD	cluster of differentiation
CI	confidence interval
Ci	curie
CMV	cytomegalovirus
CO_2	carbon dioxide
CRA	^{51}Chromium release assay
Da	Dalton
DAP10	DNAX-activating protein of 10kDa
DAP12	DNAX-activating protein of 12kDa
DC	dendritic cell
dH_2O	deionized molecular biology grade water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
Eat-2	Ewing’s sarcoma-associated transcript 2
EBV	Epstein-Barr virus
EC_{50}	half-maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fab	fragment antigen-binding
Fc	fragment cystallizable
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gram(s)
g	gravitational force
HA	hemagglutinin
HAART	highly active antiretroviral therapy
HCV	hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IE	immediate early

IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IQR	interquartile range
ITAM	immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
k	kilo
kb	kilobase
KIR	killer cell immunoglobulin-like receptor
L	litre(s)
LIR-1	leukocyte immunoglobulin-like receptor-1
M	molar
m	mili
mAb	monoclonal antibody
MAP	mitogen-activated protein
mCMV	murine cytomegalovirus
MHC I/II	major histocompatibility complex I/II
MICA	major histocompatibility complex class I chain-related molecule A
MICB	major histocompatibility complex class I chain-related molecule B
mTORC	mammalian target of rapamycin complex
N	nano
Na_2Cro_4	sodium chromate
NaAc	sodium acetate
NaCl	sodium chloride
NCR	natural cytotoxicity receptor
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
OD	optical density
P/S	penicillin/streptomycin
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
pH	potential of hydrogen
SHP1	Src homology region 2 domain-containing phosphatase-1
SHP2	Src homology region 2 domain-containing phosphatase-1
SIV	simian immunodeficiency virus
Syk	spleen tyrosine kinase
TE	tris EDTA
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
™	trade mark
UL	unique long
ULBP1	UL16 binding protein 1

WHO	World Health Organization
α	alpha
γ	gamma

Chapter 1: Introduction

1.1 Natural Killer Cells

Natural killer (NK) cells comprise 10-15% of the human lymphocyte population and act as a primary line of defense against pathogens[1]. These large granular lymphocytes represent a branch of the innate immune system and are able to kill infected, transformed, or otherwise stressed cells by targeted release of perforin and granzymes[2]. NK cells also release pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon gamma (IFN-γ), in response to aberrant cells. These cytokines help coordinate adaptive branches of host immunity to begin mounting a response[3].

NK cells are classically defined by expression of CD56 and absence of CD3[4]. Within this population, NK cells can be further subdivided into $CD56^{bright}$ and $CD56^{dim}$ groups. The former NK cell population displays enhanced production of cytokines and diminished cytotoxic capacity in comparison to the more highly differentiated $CD56^{dim}$ NK cells, which are more likely to express CD57 (a marker of maturation)[5]. $CD56^{dim}$ $CD57^{+}$ NK cells have high levels of surface CD16 expression and degranulate exceptionally well following stimulation through this receptor[5].

NK cells have common features with cells of the adaptive immune system, such as similar ontogeny[6]. For example, like B and T lymphocytes, NK cells arise from common lymphoid progenitor cells[7]. However, whereas B and T cells undergo somatic recombination to create immense receptor diversity, NK cells have a relatively limited repertoire of germline encoded receptors[8]. These receptors transduce either activating or inhibitory signals upon phosphorylation of immunoreceptor tyrosine-based activating

(ITAM) or inhibitory (ITIM) motifs on signaling proteins[9]. This results in NK cell-mediated killing, or activation-related cytokine release, only when the balance of activating receptor stimulation outweighs inhibitory signaling (see Section 1.1.4)[6].

1.1.2 Inhibitory NK Cell Receptors

If an NK cell receives equal stimulation through activating and inhibitory receptors, inhibitory signaling dominates. A major form of NK cell inhibitory signaling arises from killer cell immunoglobulin-like receptors (KIRs), which are characterized by either two (KIR2D) or three (KIR3D) extracellular Ig-like domains[10]. The inhibitory subset of KIRs recognize various human leukocyte antigen (HLA) class I molecules, have long (L) cytoplasmic tails, and associate with ITIMs[11]. Other important inhibitory NK cell receptors include leukocyte immunoglobulin-like receptor-1 (LIR-1), which recognizes a broad range of HLA class I molecules, and NKG2A/KLRD-1 (CD159a/CD94), which belongs to the C-type lectin-like family, forms a heterodimer with CD94, and transfers an inhibitory signal upon binding of HLA-E[12,13]. All of the inhibitory signals resulting from ligation of these receptors are transduced to the NK cell via the phosphorylation of tyrosine residues on ITIMs, which in turn recruit tyrosine phosphatases, Src homology region 2 domain-containing phosphatase (SHP) 1 and 2. See Table 1.1 for a list of inhibitory receptors and their ligands.

1.1.3 Activating NK Cell Receptors

NK cells are triggered to kill through ligation of activating receptors which transduce activating signals through the phosphorylation of ITAMs[14]. Upon phosphorylation, ITAMs recruit kinases that propagate activating signals. KIRs can be

Table 1.1 Inhibitory NK Cell Receptors

Receptor	Associated motifs	Ligand(s)
Inhibitory KIRs (except KIR2DL5 and 3DL3)	ITIMs	HLA class I molecules
LIR-1	ITIMs	HLA class I molecules
NKG2A	ITIMs	HLA-E

activating in nature if they associate with ITAMs instead of ITIMS. Numerous activating KIRs have undefined ligands and are thought to have evolved from inhibitory KIRs[15]. These activating KIRs have short (S) cytoplasmic tails, with the exception of KIR2DL4, which contains a long cytoplasmic tail despite transducing activating signals[16]. Activating and inhibitory KIRs are often highly homologous in their extracellular domains which makes differentiation using monoclonal antibodies (mAbs) challenging[17].

Natural cytotoxicity receptors (NCRs) are most often responsible for NK cell activation and these receptors include NKp46, 44, 30, and 80. NKp46 recognizes influenza hemagglutinin (HA) and parainfluenza HA-neuraminidase and is considered the main activating NK cell receptor[18]. Other NCRs and their ligands are summarized in Table 1.2. NKG2C/KLRD-1 (CD159c/CD94) is another important activating receptor which belongs to the C-type lectin-like family, forms a heterodimer with CD94, and associates with DNAX-activating protein of 12 kDa (DAP12), a transmembrane signaling protein which contains an ITAM[13]. Similar to NKG2A, NKG2C binds to cell surface HLA-E, but with lower affinity[19]. Expression of the activating receptor NKG2C and the inhibitory receptor NKG2A is thought to be mutually exclusive[18]. NKG2D is a homodimer which also belongs to the C-type lectin-like receptor family, however, it associates with the transmembrane signaling protein DNAX-activating protein of 10 kDa (DAP10)[20]. Ligation of NKG2D by major histocompatibility complex class I chain-related (MIC) molecules MICA, MICB, or UL16-binding protein 1 (ULBP1) results in NK cell activation[21].

The activating NK cell receptor which is most pertinent to our upcoming discussion of NK cell function is FcγRIIIa (CD16a), the low affinity IgG receptor present

on virtually all CD56^{dim} NK cells[22]. This receptor comes in two forms, CD16a and CD16b, however, only the former receptor is expressed on NK cells and will herein be referred to as CD16[23]. Two signaling adaptor molecules associate as a heterodimer with CD16 in the transmembrane region, CD3ζ, which contains three ITAMs, and FcεR1γ, which contains one ITAM[9,24]. CD16, also known as the immunoglobulin fragment crystallizable (Fc) receptor, is essential for NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC), a critical defense against viral invasion[25]. See Table 1.2 for a list of activating receptors and their ligands.

1.1.4 NK Cell-Mediated Killing

As mediators of innate immunity, NK cells are capable of lysing aberrant cells without prior sensitization[26]. The exocytosis of secretory granules containing perforin and granzymes is responsible for NK cell-mediated killing[2]. These lysosomes are readily available for targeted release toward aberrant cells – hence the appropriately named "natural killer" cell. Target cell lysis is mediated by perforin, which forms pores in the target cell membrane, leading to osmotic shock[27]. These perforin-mediated pores also allow granzymes into the cytosol, wherein granzymes A and B facilitate the cleavage of caspases, resulting in programmed cell death[28]. Perforin and granzyme-mediated killing only occurs when activating signaling outweighs the inhibitory signaling from NK cell interactions with a target cell.

NK cell-mediated lysis through direct interaction between an NK cell and a target cell is referred to as natural cytotoxicity[29]. NK cells are also triggered to kill through the crosslinking of an antigen-specific antibody to the target cell through the ligation of

Table 1.2 Activating NK Cell Receptors

Receptor	Adaptor molecules	Ligand(s)
KIR2DS2/2DS5	DAP12	Unknown
KIR2DS1	DAP12	HLA-C
KIR2DL4	FcεR1γ	HLA-G
KIR3DS1	DAP12	HLA-F
NKp46	CD3ζ and FcεR1γ	Viral HA
NKp30	CD3ζ and FcεR1γ	B7-H6, Cytomegalovirus pp65, HLA-B associated transcript 3 (BAT3)
NKp44	DAP12	Viral HA
NKG2C	DAP12	HLA-E
NKG2D	DAP10	MICA/MICB/ULBP1
CD16	CD3ζ and FcεR1γ	IgG

CD16, referred to as ADCC[29]. This takes non-pathogen-specific NK cell-mediated killing and renders it specific by harnessing the pathogen-specificity of antibodies to tag aberrant cells for lysis. Both natural cytotoxicity and ADCC are more potent if an NK cell expresses at least one self-specific inhibitory KIR. This effect is a result of classical NK cell licensing[30].

1.1.5 Classical NK Cell Licensing

Licensing refers to a maturation process wherein cells become fully competent in their functional role. During classical NK cell licensing, inhibitory KIRs are involved in assuring the NK cell can differentiate a healthy cell from an aberrant cell by engaging with self-major histocompatibility complex (MHC) class I molecules. The MHC complex refers to a set of proteins which bind and present peptides derived from self or pathogens and are recognized by T lymphocytes (T cells) and NK cells[31]. In humans, the MHC complex is referred to as the HLA complex. Engagement of inhibitory KIRs with either HLA A, B, or C (class I) molecules results in the transduction of inhibitory signals to the NK cell. HLA class I molecules serve as excellent "self" markers to avoid NK cell killing because they are expressed on almost all human cells[32].

Lack of engagement between inhibitory KIRs and HLA class I molecules during development results in hyporesponsive NK cells[33]. If a host cell is altered in a way that downregulates HLA class I expression, such as through viral invasion, NK cells lack the inhibitory stimulus necessary to suppress killing. Evidence for the presence of activating receptors on host cells came about in an experiment wherein NK cells were able to kill HLA class I-deficient target cells. This display of immunosurveillance is explained by the

"missing-self hypothesis", wherein infected or tumor cells, which evade T cell defenses by downregulating MHC class I, are recognized by NK cells[34].

1.1.6 NK Cell Stimulation Assays

A common experimental system to measure NK cell natural cytotoxicity is through incubation of peripheral blood mononuclear cells (PBMC) with natural NK cell targets. K562 cells are derived from immortalized human myelogenous leukemia cells and serve as natural NK cell targets due to their deficiency in HLA class I molecules. Stimulation through natural cytotoxicity receptors is mostly responsible for NK cell activation in this assay, resulting in K562 cell lysis, as well as pro-inflammatory cytokine expression. Intracellular expression of pro-inflammatory cytokines or surface expression of the degranulation marker, CD107a, is frequently measured by flow cytometry in these assays. Another way to measure NK cell activation is by ^{51}Chromium (Cr)-labeling target K562 cells before incubation with PBMC, and measuring ^{51}Cr release in supernatants post-incubation. The latter method was considered the gold standard for measuring NK cell killing, however, it comes with limitations[35]. ^{51}Chromium release assays (CRAs) involve hazardous radioactive materials and do not allow for the distinction of NK cell subsets involved, whereas flow cytometry allows the measurement of variegated responses among specific cell subsets by sorting based on expression profiles.

Another way to model NK cell activation is through the addition anti-CD16 monoclonal antibodies, such as 3G8, which directly stimulate NK cells. This type of activation is referred to as antibody-dependent NK cell activation. Intracellular expression

of pro-inflammatory cytokines or degranulation markers post stimulation using flow cytometric analysis is a common way to measure NK cell activation in this assay.

1.1.7 Pro-inflammatory cytokines

NK cells respond to activation signals by producing cytokines, which enable intercellular communication between cells of the immune system. The resulting "cytokine milieu" is important for the development of adaptive immune defenses and is comprised mostly of TNF-α and IFN-γ[36]. Although $CD56^{bright}$ NK cells have enhanced production of pro-inflammatory cytokines, our discussion focuses mainly on $CD56^{dim}$ NK cells, which are also able to produce cytokines after stimulation through activating or cytokine receptors[37].

IFN-γ is secreted primarily by T-$helper_1$ $CD4^+$ T cells, $CD8^+$ T cells, and NK cells[38]. There are many ways in which IFN-γ is able to interfere with viral replication, such as upregulating MHC I and MHC II expression, and subsequently allowing heightened presentation of viral proteins. IFN-γ also activates macrophages, causes vasodilation, and further induces production of pro-inflammatory cytokines from other immune cells[38]. TNF-α is known for causing necrosis of tumor cells, but contributes to host defense by generating inflammation[39]. The production of IFN-γ and TNF-α by NK cells is important for overall host defense and is used to mark activated NK cells.

Individuals with complete NK cell deficiencies lack NK cell-mediated cytolysis as well as the accompanying pro-inflammatory cytokine responses. These individuals, although extremely rare, are highly susceptible to certain infectious agents, such as

herpesviruses[4]. For the next section we will focus on cytomegalovirus (CMV), a beta-herpesvirus which elicits a profound immune response, even in healthy individuals.

1.2 Overview of Human Cytomegalovirus

Human cytomegalovirus (CMV) is a double stranded DNA beta-herpesvirus that establishes a persistent, lifelong, and primarily latent infection[40]. CMV is the largest human herpes virus and has coevolved with its host for millions of years[41]. Primary infection with CMV occurs through contact with infected body fluids such as semen, vaginal secretions, breast milk, urine, and saliva[42]. Although mucosal epithelial cells serve as primary targets, endothelial cells, smooth muscle cells, dendritic cells (DC), fibroblasts and leukocytes are also permissive to CMV infection[43].

Primary infection, reactivation or de novo reinfection with CMV results in extensive transcription of the viral genome, compared to a more restricted transcriptional profile during CMV latency [44]. Reactivations are generally controlled by a robust immune response in healthy hosts and, therefore, remain asymptomatic[45]. Conversely, in immunocompromised individuals, active infection can cause serious health complications.[46]. The most common populations at risk for CMV-related complications are transplant recipients, those co-infected with human immunodeficiency virus (HIV), and developing fetuses following congenital transmission[47]. The deleterious consequences of CMV infection among immunocompromised adults include serious end-organ diseases, whereas newborns display symptoms such as hearing loss and neurodevelopmental delays[48,49]. Even healthy hosts are not spared from the effects of CMV. In a process termed “memory inflation”, the immune system becomes highly

preoccupied CMV and slowly dedicates an inordinate fraction of immune cells to its control. This can be seen in the overwhelming responses from both adaptive and innate branches of the human immune system[50].

1.2.1 CMV & Adaptive Immunity

1.2.1.1 Humoral Immunity

The adaptive immune system mounts a dramatic CMV-specific response within both its cellular and humoral arms. CMV-infected hosts attempt to control viral dissemination by generating a diverse set of antibodies toward various CMV proteins, such as pp65, pp150, gB, gH, and immediate early (IE)-1[51]. The presence of CMV-specific antibodies are used to diagnose CMV infection and researchers often apply anti-CMV IgG levels as surrogate markers of CMV infection control[52].

Evidence for antibody-mediated viral control can be seen in the context of congenital transmission. There is approximately a 40% chance of a fetus becoming infected with CMV if the mother develops primary infection during pregnancy, however, if the mother transfers CMV-specific IgG antibodies across the placenta, the fetus is less likely to become infected[47,53]. Many studies have shown that treatment with CMV-specific hyperimmune globulin reduces congenital transmission compared to those treated with a placebo[54,55].

1.2.1.2 Cellular Immunity

In addition to humoral immunity, a strong CMV-specific T cell response emerges and is maintained, even in the absence of detectable viremia[36]. CMV-specific $CD4^+$ and $CD8^+$ T cell populations commonly occupy ~10% of the entire T cell repertoire, which is

an incredibly high percentage considering the overall diversity of the repertoire and number of pathogens constantly challenging the human immune system[56]. In more extreme cases of memory inflation, the $CD8^+$ effector memory T cell compartment alone can reach up to 30% of total $CD8^+$ T cells in circulation[52]. These T cell populations mostly target the immunodominant proteins pp65 and IE-1[56]. Researchers are currently debating whether viral reactivations are the underlying cause of this highly expanded T cell population, which is both cytotoxic and produces high levels of IFN-γ and TNF-α upon activation[57]. Memory inflation is characterized by the emergence of CMV-specific $CD8^+$ effector memory T cells which lack CD28, a costimulatory molecule and express CD57. These two features in combination denote terminal differentiation and define a more phenotypically mature and pro-inflammatory effector memory T cell population[52]. $CD4^+$ T cells also dedicate a large portion of their resources to CMV control, however, in comparison to the fraction of CMV-specific $CD8^+$ T cells, their response is generally much less extravagant[52].

The innate immune response to CMV infection is also dramatic. A specific population of differentiated NK cells is uniquely associated with CMV infection (see section 1.2.3). However, unlike the expanded T cell population that emerges in CMV infection, the lack of CMV-specific receptors on NK cells obscures the driving forces behind the expansion and maintenance of this population.

1.2.3 CMV & Innate Immunity

Pattern recognition receptors, such as toll-like receptor 2 (TLR-2) on fibroblasts, are among the first to signal CMV invasion. TLR-2 recognizes CMV surface

glycoproteins gB and gH, which activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway and recruits antigen-presenting cells (APCs), phagocytes, and NK cells[36,58]. CMV has evolved many immune evasion strategies, one of which is an interleukin (IL)-10 viral homologue (vIL-10), which mimics the anti-inflammatory properties of IL-10[36,59]. This immune evasion strategy operates by reducing the effector NK cell pool in rhesus macaques, providing evidence for the crucial role of NK cells in CMV defense[60]. The interaction between CMV and host NK cells is highly complex and is still quite poorly understood. Although NK cells have the capacity to control CMV, the reverse appears to hold true, as CMV can leave a lasting mark on host immune cell populations.

A striking example of how CMV infection can dramatically shape the host immune system is seen in the high proportion of peripheral blood NK cells expressing the activating receptor NKG2C, compared to CMV-negative individuals[61]. Previous reports describe a "dose effect" with regards to the copy number of NKG2C, wherein the highest numbers of circulating $NKG2C^{+}$ NK cells are present in $NKG2C^{+/+}$ individuals, followed by $NKG2C^{+/-}$ individuals[62,63,64]. This dose effect is more obvious in CMV-infected hosts because they have larger populations of NK cells expressing NKG2C. Despite the associations made between CMV infection and an expanded population of $NKG2C^{+}$ NK cells, the interactions driving this expansion, such as a CMV-encoded ligand for NKG2C, have yet to be elucidated[65].

In contrast with the current uncertainty regarding human responses to CMV, the interactions driving expansion of murine CMV (mCMV)-specific NK cells are clearly defined. The observation that certain strains of mice displayed resistance to mCMV

infection led to a breakthrough in the understanding of murine NK cell responses to mCMV. BALB/c mice are susceptible to mCMV infection whereas C57BL/6 mice are resistant. By genetically analyzing these mice, Brown *et al.* were able to pinpoint *Ly49H,* a gene encoding an activating receptor found on the surface of murine NK cells, as a driver of mCMV resistance[66]. This receptor directly interacts with the mCMV-encoded m157 glycoprotein. Ly49H$^+$ NK cells expand and persist following primary infection and are able to control future encounters with mCMV[67].

Researchers have described the CMV-driven NKG2C$^+$ NK cell population as reminiscent of the murine Ly49H$^+$ NK cell population, however, these populations are fundamentally quite different[64]. The expression of NKG2C does not provide resistance to hCMV infection, as evidenced by the millions of NKG2C-expressing individuals who are CMV-positive. Additionally, there is no evidence for a CMV-derived ligand for NKG2C[68]. Despite these differences, NKG2C-expressing NK cells are still thought to play a pivotal role in CMV control, and for this reason have been studied extensively.

1.2.4 Characterization of NKG2C$^+$ NK Cells

NKG2C$^+$ NK cells emerge during CMV infection and often express CD57, a maturation marker. There are many functional and phenotypical features which describe this expanded population.

1.2.4.1 Functional Characteristics of NKG2C+ NK Cells

NKG2C$^+$ NK cells are characterized by enhanced ADCC through CD16[64,69]. This population tends to downregulate main activating receptors such as NKp46 and NKp30, and as a result, exhibits less effective natural cytotoxicity[64,69]. NKG2C$^+$ NK cells also

demonstrate upregulated IFN-γ expression through epigenetic remodeling similar to what occurs in CMV-specific $CD8^+$ T cells[70]. The IFN-γ promoter region is hypomethylated, which leads to quicker and more accessible stimulation of IFN-γ production. This enables NK cells to adopt adaptive features despite their innate nature and explains the increased production of IFN-γ observed in $NKG2C^+$ NK cells from CMV-seropositive donors[70].

1.2.4.2 KIR Expression on Adaptive NK Cells

Adaptive NK cells are further characterized by the selective expression of certain KIRs[64]. Beziat *et al.* showed that both activating and inhibitory KIR expression patterns were skewed in expanded $NKG2C^+$ NK cell populations[71]. They found a selective expansion of $NKG2C^+$ NK cells expressing the activating receptors KIR2DS2, 2DS4, and 3DS1. They also found an association between the expansion of inhibitory KIRs if their cognate HLA class I ligands were present within the host. Beziat *et al*. proposed KIR-mediated NK cell education as a mechanism behind the expansion of licensed self-specific KIR^+ NK cells co-expressing NKG2C in CMV-infected hosts[71].

1.2.4.3 LIR-1 Expression on Adaptive NK Cells

LIR-1 is an inhibitory receptor found on subsets of monocytes, B cells, T cells, and NK cells which binds MHC class I molecules (HLA A, B, C and G) as well as the CMV-encoded MHC class I homolog, unique long (UL) region protein 18 (UL18)[72,73]. UL18 has been shown to bind LIR-1 with one thousand times stronger affinity than the MHC class I molecules[12,73].

In a cohort of CMV-infected children, researchers observed an increase in the percentage of $LIR\text{-}1^+$ NK cells within the NKG2C-expressing subset. This enrichment of

LIR-1^{+} NK cells was not observed among NKG2C^{-} NK cells, and was significantly higher than within the subset of NKG2A-expressing NK cells[74]. There are mixed reports regarding the action of UL18 on NK cell function, wherein certain publications report LIR-1-mediated inhibition of NK cells, while others reveal the opposite[75,76].

1.2.4.4 CD2 Expression on Adaptive NK Cells

CD2 serves as a co-stimulatory receptor on NK cells and is upregulated on adaptive NK cells after *in vitro* expansion. The interaction between CD2 on NK cells and CD58, which is expressed on infected cells, has been proposed as a contributor to adaptive NK cell activation. In a co-culture system, the production of IFN-γ and TNF-α by adaptive NK cells diminished after blocking with anti-CD2 or anti-CD58 monoclonal antibodies[77].

1.2.4.5 Expression of NKG2A & Other Intracellular Proteins on Adaptive NK Cells

Another stable feature of expanded NKG2C^{+} cells is the absence of NKG2A, and downregulation of certain intracellular signaling proteins such as FcεR1γ, spleen tyrosine kinase (Syk), promyelocytic leukemia zinc finger (PLZF) and Ewing's sarcoma-associated transcript 2 (Eat-2)[64,78,79]. Schlums *et al.* observed a substantial downregulation of these proteins among CMV-seropositive hosts, primarily within the CD56^{dim} NK cell subset[80]. Among these proteins, downregulation of FcεR1γ was the most pronounced[80].

These features (NKG2C^{+} NKG2A^{-} KIR^{+} LIR-1^{+} FcεR1γ^{-} Syk^{-} Eat-2^{-} IFN-γ^{+++}), paired with enhanced ADCC, define the expansion of an adaptive NK cell population wherein CMV is thought to be the main driving force. This expansion, as well as the expansion of CMV-specific CD8^{+} T cells, is notably more prominent in HIV-infected

individuals[81]. Contrary to initial assumptions, these adaptive NK cell features do not appear to be restricted to NKG2C-expressing NK cells. This implies that interactions involving NKG2C are not necessary in driving this expansion, and that NKG2C-deficient NK cell populations also participate in CMV control.

1.2.5 Characterization of NKG2C^{null} NK Cells

Miyashita *et al*. identified the relatively common homozygous deletion of a 16kb region of the genome that encompasses the NKG2C gene[82]. Follow up studies, using their uniquely designed PCR primers, revealed that up to 1 in 20 individuals have a homozygous deletion of the NKG2C gene. Again, many NKG2C^{null} individuals are still able to maintain a subclinical CMV infection, even in the case of those co-infected with HIV. The maintenance of subclinical infection affirms existence of CMV control mechanisms entirely independent of NKG2C expression.

1.2.5.1 Functional Characteristics of NKG2C^{null} NK Cells

In a cohort of NKG2C-deficient Gambian children, researchers found significant elevation in the level of anti-CMV IgG from serum samples[83]. This display of heightened humoral responses could be a compensatory measure for slight impairment in innate control, or more specifically, in the functional capacity of NK cells from NKG2C-deficient hosts. The same study also demonstrated an attenuation of CD57^{+} NK cell generation among NKG2C-deficient children, implying a potential delay in overall NK cell maturation[83]. CMV-driven maturation may not be optimal in NKG2C-deficient individuals if somehow NKG2C expression drives NK cell maturation as a whole. A consensus has yet to be reached on whether there is a phenotypical or functional

impairment in the maturation of NK cells from NKG2C-deficient individuals, and even less is known within the context of an immunocompromised host.

1.2.5.2 KIR Expression on NKG2C^{null} NK Cells

Specific constellations of NK cell receptors have been proposed as alternative methods of CMV control[17,84, 85]. KIRs are grouped into haplotypes A or B. In the context of solid organ transplantation, the presence of a group B (predominantly activating) haplotype has been associated with a reduced risk of CMV reactivation[17]. Furthermore, a protective effect has been demonstrated wherein individuals with more than five activating KIRs, or the simultaneous expression of both KIR2DS2 and 2DS4, have a reduced risk of CMV infection[84]. In a cleverly designed study by Chiesa *et al*., the transplantation of cord blood from an NKG2C^{null} donor resulted in a population of CD56^{dim} NKG2A^{-} activating KIR^{+} NK cells that were able to degranulate, kill targets, and release IFN-γ via stimulation through activating KIRs[85]. This phenotype is similar to the mature NKG2C-expressing NK cell population which is associated with CMV infection, and implies the potential role of activating KIRs as a substitute for NKG2C in driving the expansion of adaptive NK cells.

1.2.5.3 CD2, LIR-1 & FcεR1γ Expression on NKG2C^{null} NK Cells

A research group based out of the Karolinska Institutet investigated NKG2C deletion in the context of CMV infection within the largest reported cohort of NKG2C-deficient individuals (n=60). Their results suggested a role for CD2 as "signal 2" in the activation of ADCC among differentiated NK cells by boosting the CD16 signaling cascade[86]. CD2 is present on both T and NK cells and serves as a co-stimulatory molecule

on NK cells specifically. Liu *et al.* found that the observed upregulation of CD2 on NKG2C^{null} NK cells resulted in the synergistic induction, alongside CD16, of the mitogen-activated protein (MAP) kinase and the mammalian target of rapamycin complex (mTORC) pathways[86]. Another group brought forth similar findings by showing that blocking CD2 or CD58 resulted in diminished antibody-mediated NK cell activation[77].

There is little knowledge with regard to NK cell expression of LIR-1 or CD2 within HIV/CMV co-infected individuals lacking the NKG2C gene. The phenotype of NK cells from NKG2C^{null} hosts is an important clue to understanding how adaptive NK cells control CMV infection. In a study by Makwana *et al*., researchers found that the most functionally active cells, in terms of CD107a and TNF-α induction, were FcεR1γ^{-} LIR-1^{+} NKG2C^{-} NK cells[87]. These results contrast with the popular assumption that NKG2C^{+} NK cells selectively display enhanced ADCC functionality. Ultimately, our research aims to understand whether NK cells from NKG2C^{null} hosts are as efficient in their functional role.

As the literature suggests, NKG2C appears to be dispensable in the case of overall CMV control, however, alternate routes of CMV-driven NK cell maturation are yet to be elucidated. Healthy individuals do not form the ideal cohort to decipher if and how NKG2C^{null} individuals control CMV infection, as they likely maintain CMV infection under tight control. In order to investigate phenotype and potential functional impairment of NK cells from NKG2C-deficient hosts we will use a cohort of immunologically affected adult individuals as they are the most likely cohort to display altered CMV control, highlighting any impairment or compensatory measures.

1.2.6 Epidemiology of CMV/HIV Co-infection

Up to 90 percent of the worldwide population is infected with CMV, depending on geographic location and socioeconomic status[88]. The World Health Organization (WHO) estimates 36.7 million people are infected with HIV, and certain groups suggest nearly all HIV-positive individuals are co-infected with CMV[89,88]. Among our cohort of HIV-infected individuals, 84% are CMV-positive, which is slightly above the average infection rate among non-HIV-infected individuals, but is significantly lower than a typical cohort of HIV-infected individuals wherein reports of up to 100% infection rates are common[90].

1.3 Overview of Human Immunodeficiency Virus Infection

Human Immunodeficiency Virus-1 (HIV-1) is a sexually transmitted virus that is the result of a cross-species transmission from simian immunodeficiency virus (SIV)-infected chimpanzees in Southern Africa. Although less common and less pathogenic, HIV-2 is endemic in Western Africa and also results in disease[91]. HIV-1 (herein referred to as HIV) is the causative agent for acquired immunodeficiency syndrome (AIDS) and has resulted in over 35 million deaths, according to the WHO. With the advent of antiretroviral therapy (ART), and the resulting decline in progression towards AIDS, mortality and morbidity rates are lower in populations with access to ART. This has shifted HIV research from focusing on AIDS-related morbidities towards non-AIDS defining illnesses, such as cardiovascular and neurological diseases. These non-AIDS defining illnesses are typically age-related in the general population, yet appear much earlier in HIV-infected individuals on ART. The early appearance of age-related

morbidities seen in HIV-infected individuals is likely accelerated by co-infection with CMV[92,93]. Understanding the host response to CMV is important for the general population, but is especially important for HIV-infected individuals.

1.3.1 CMV/HIV Co-Infection

CMV infection can increase susceptibility to HIV infection, enhance untreated HIV disease progression towards AIDS, and exacerbate the effects of non-HIV/AIDS associated morbidities, even among populations receiving ART[90]. Before researchers established CMV as the causative agent behind the emergence of an adaptive NK cell population, these adaptive cells were considered an artifact of HIV infection.

Various studies performed in early 2000 described phenotypic and functional features of NK cells within the context of HIV infection. There was an observed downregulation of NKp46, NKp30, and NKp44 expression within HIV-infected viremic patients, compared to highly active ART (HAART)-treated aviremic patients[94,95]. Another group published their findings regarding a switch from NK cells expressing NKG2A to NKG2C in HIV-infected patients[96]. NK cell cytotoxicity against K562 cells, which serve as natural NK cell targets, was shown to be decreased in HIV-infected viremic patients, versus either HIV-infected aviremic individuals or healthy controls[94].

The notion that HIV infection was responsible for these specific changes in the NK cell repertoire partially changed in 2006 when Guma *et al.* published a paper encouraging researchers to consider CMV serostatus when analyzing phenotypical and functional immune characteristics in cases of immunosuppression, such as HIV infection[97]. Guma *et al.* pointed out that many of these associations, such as their findings

regarding a switch from NKG2A to NKG2C-expressing NK cells in HIV-infected individuals, or others involving NK cell characterization in HIV-infected patients, often dissipated when accounting for CMV serostatus. Four years later, a study revealed that the switch from NKG2A to NKG2C-expressing NK cells in HIV-infected individuals only held true with a concurrent CMV infection[98]. The observed downregulation of NKp46 and NKp30 in HIV infection was also subsequently reported as an imprint of CMV infection[99]. Finally, less effective natural cytotoxicity is now reported as a hallmark of expanded NK cells in CMV infection rather than HIV infection[69]. It is not surprising that the expansion of this subset of NK cells was initially identified and labeled as a hallmark of HIV infection, as these infections typically coexist in the human host and evidence remains that the expansion of $NKG2C^{+}$ NK cells in CMV infection is amplified in HIV-infected individuals, compared to HIV-uninfected controls[81].

Although many associations have been established between expansion of $NKG2C^{+}$ NK cells and CMV/HIV co-infection, little research has been done with a focus on the expansion and differentiation of $NKG2C^{null}$ NK cells. To date, no research has been published with a focus on $NKG2C^{null}$ NK cells from CMV-infected individuals who are also HIV-positive. Certain studies have investigated $NKG2C^{null}$ individuals in CMV infection, or $NKG2C^{null}$ individuals in HIV infection, but never a combination of both. For these reasons, we selected a cohort of HIV-infected patients for our study, as the effects of CMV are amplified in this setting. We hypothesize that any differences in CMV control, or in NK cell differentiation, will be amplified in this setting. Additionally, HIV-infected individuals have larger populations of differentiated NK cells for study purposes.

Our study will investigate the effect of NKG2C deletion on phenotypic and functional NK cell maturation in the context of HIV-infection by comparing NK cells from NKG2C^{null} and NKG2C-expressing hosts, matched for age, CD57^{+} NK cell population, and CMV/HIV infection history. We will investigate phenotype using flow cytometric analysis of specific NK cell receptors and intracellular proteins (aim 1). Functional maturation will be compared after stimulation through CD16 in anti-CD16 redirected lysis and through natural cytotoxicity assays (aim 2). If those individuals who are NKG2C-deficient have lesser differentiated NK cells, or a diminished ability to perform effector functions, this could explain the fact that NKG2C-deficient individuals have a more difficult time controlling HIV. Conversely, if these individuals have similar phenotypic and functional NK cells, this would point to the undoubted existence of alternate pathways of CMV-driven NK cell differentiation, aside from NKG2C.

In summary, our hypothesis is that the lack of NKG2C could impair CMV-driven NK cell differentiation into mature effectors with superior ADCC. Therefore, our overall objective is to investigate if CMV-driven NK cell differentiation is impaired in HIV-infected NKG2C^{null} individuals through phenotypical (aim 1) and functional (aim 2) analysis.

2. Materials and methods

2.1 Sample Collection and Peripheral Blood Mononuclear Cell Isolation

This study received ethical approval from the Health Research Ethics Authority of Newfoundland and Labrador. All donors provided informed consent before blood collection at the Newfoundland and Labrador Provincial HIV Clinic (St. John's, NL, Canada), or by a trained phlebotomist at Memorial University's Faculty of Medicine (St. John's, NL, Canada). Blood was collected by forearm venipuncture into acid-citrate dextrose-containing vacutainers.

Peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn whole blood by density gradient centrifugation at 400 *g* using Ficoll-Paque PLUS lymphocyte isolation solution (GE Healthcare, Chicago, IL, USA). Briefly, separation of PBMC from whole blood involved layering Ficoll-Paque beneath the blood sample. This occurred after an initial centrifugation at 400 *g* and removal of the uppermost plasma-containing layer using a 5 mL pipette. The remaining blood sample was reconstituted to 2X the original blood volume with phosphate buffered saline (PBS) (see Table 2.1 for preparation of PBS). Next, centrifugation at 400 *g* for 30 minutes with no brake yielded a PBMC-containing layer at the interface between the two resulting phases. The interface was removed and washed with PBS containing 1% fetal calf/bovine serum (FCS) (Gibco, Gaithersburg, MD, USA). Freshly collected PBMC intended for immediate use were resuspended in lymphocyte medium made using Roswell Park Memorial Institute (RPMI)-1640 (Gibco) supplemented with 10% FCS, 200 IU/mL penicillin/streptomycin (P/S), 1% L-glutamine (all from Gibco), 1% 1 M 4-(2-hydroxyethyl)- 1-

Table 2.1 Solution Preparations

Solution	Ingredients
PBS	8 g of NaCl (Sigma-Aldrich)
	0.2 g of KCl (Sigma-Aldrich)
	1.44 g of Na_2HPO_4 (Sigma-Aldrich)
	0.24 g of KH_2PO_4 (Sigma-Aldrich)
	1 L dH_20 (made to pH 7.4)
Lysis buffer	10 mM Tris (J. T. Baker, Phillipsburg, NJ, USA)
	10 mM Sodium Chloride (ThermoFisher Scientific)
	1 mM EDTA (Sigma-Aldrich)
	0.5% Sodium dodecyl sulfate (Sigma-Aldrich)
	dH_20 (made to pH 8.0)
TE buffer	10 mM Tris
	0.1 mM EDTA
	dH_20 (made to pH 8.0)
Flow buffer	1.04 g EDTA
	0.5% FCS (Gibco)
	1 g Sodium azide (Sigma-Aldrich)
	500 mL PBS (made to pH 7.0-7.2)

piperazineethanesulfonic acid (HEPES), and 5.5 x 10^{-5} M 2-mercaptoethanol (both from Sigma-Aldrich, St. Louis, MO, USA). Additionally, in order to develop a continuously growing cell line which could serve as a primary source of deoxyribonucleic acid (DNA) for genome analysis, study participants had their peripheral blood B cells immortalized using Epstein-Barr Virus (EBV) as described[101]. Once established, EBV-transformed B lymphoblastoid cells were stored in liquid nitrogen until needed.

2.2 Cryopreservation of PBMC

If PBMC were not used within 1 day post-isolation, cells were resuspended in freezing medium, made using lymphocyte medium supplemented to 20% FCS with 10% dimethyl sulfoxide (Sigma-Aldrich), in aliquots of 1.0-2.5 x 10^7 PBMC/mL. These samples were placed in 1.5 mL Nalgene cryotubes (Sigma-Aldrich) for overnight storage at -70°C in a Mr. Frosty™ (ThermoFisher Scientific, Waltham, MA, USA) and were transferred to liquid nitrogen the following day. Samples remained in liquid nitrogen until needed.

2.3 Thawing PBMC

Cryogenically frozen PBMC were removed from liquid nitrogen and transported on ice to a 37˚C water bath where samples were quickly thawed. After immediate transfer to a 15 mL tube containing 10 mL lymphocyte medium (see Section 2.1), samples were centrifuged for 5 minutes, decanted, and resuspended in 4 mL lymphocyte medium for overnight recovery at 37°C with 5% CO_2.

2.4 Identification of NKG2C^{null} Individuals by Flow Cytometry

PBMC from HIV-infected individuals were separated from whole blood (as described in Section 2.1) for analysis by flow cytometry (see Section 2.7 for detailed flow cytometry procedures). PBMC were labeled with anti-CD3, -CD56, -CD57, and -NKG2C fluorescence-conjugated antibodies (see Table 2.2 for antibody conjugates and suppliers) which allowed identification of various cell subsets. Individuals with <1% of NK cells ($CD3^-$ $CD56^+$) expressing NKG2C were identified by analysis with Kaluza Flow Cytometry Software 1.2 (Beckman Coulter, Brea, CA, USA).

2.5 Confirmation of NKG2C^{null} Genotype by Polymerase Chain Reaction

Individuals with <1% of NK cells expressing NKG2C were considered "suspect" NKG2C^{null} and were genotyped using polymerase chain reaction (PCR) to confirm homozygous NKG2C gene deletion. For primers and PCR amplification conditions see Section 2.5.2.

2.5.1 Deoxyribonucleic Acid Isolation

Isolation of genomic DNA from cryogenically stored samples was performed by thawing EBV-transformed B lymphoblastoid cells and immediately adding them to 10 mL lymphocyte medium. Following a centrifugation at 290 *g*, the supernatant was decanted and B cells were resuspended in lymphocyte medium (see Section 2.1) for culture. Once viability was over 50%, ~4 million B lymphoblastoid cells were taken for DNA extraction, centrifuged for 5 minutes, decanted, and 200 µL lysis buffer (see Table 2.1 for preparation of lysis buffer) was added directly to the B cell pellet. Next, the suspension was pipetted up and down to ensure adequate mixing before transfer to a 1.5 mL tube.

Table 2.2 PCR Cocktail Components

PCR Cocktail[a] (per 1 reaction)	
PCR buffer	5 µL
dNTPs (10 µM)	1 µL
Magnesium Chloride	1.5 µL
NKG2C **or** Break Primers	2 µL
Taq DNA polymerase	0.5 µL
dH_20	38.5 µL

[a]All products were purchased from Invitrogen

After adding 5 µL proteinase K (Invitrogen, Waltham, MA, USA) the suspension was vortexed and placed in a 42˚C water bath overnight.

The following day, 300 µL phenol (Anachemia, Laval, QC, Canada), maintained at pH ≥7.8, was added and the suspension was manually shaken for 5 minutes. Next, the suspension was centrifuged for 4 minutes at 14,000*g*. The DNA-containing upper aqueous layer was removed and placed in a 1.5 mL tube into which 300 µL chloroform:isoamyl alcohol (Baxter Healthcare Corporation, Muskegon, MI, USA, and Sigma-Aldrich, respectively) at a ratio of 24:1, was added. This was shaken manually for 5 minutes and centrifuged for 2 minutes at 14,000 *g*. The top layer was removed and placed in a new 1.5 mL tube. Finally, 750 µL of 100% ethanol (Commercial Alcohols, Brampton, ON, Canada) was added, along with 30 µL 3M sodium acetate (NaAc, Sigma-Aldrich) and the sample was placed at -20˚C overnight.

On the third day, the sample was centrifuged for 15 minutes at 14,000 *g*, the upper layer was carefully removed using a pipette, and 1 mL 70% ethanol was added. After another 15 minute centrifugation at 14,000 *g*, the supernatant was removed with a pipette and the pellet of genomic DNA was air dried for 10 minutes. Finally, DNA was diluted to a concentration between 20-200 ng/µL in Tris ethylenediaminetetraacetic acid (EDTA) buffer (see Table 2.1 for preparation of tris EDTA [TE] buffer) and DNA content was measured using a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific).

2.5.2 NKG2C PCR

PCR amplification involved two sets of forward and reverse primers, "NKG2C" and "Break", designed by Miyashita *et al.* to detect NKG2C deletions[82]. Break primers

(designed to reveal NKG2C deletion) were forward (5'-ACTCGGATTTCTATTTGATGC-3') and reverse (5'- AGTGATGTATAAGAAAAAG-3'), and NKG2C primers were forward (5'-ATCAATTATTGAAATAGGATGC-3') and reverse (5'- CGCAAAGTTACAACCATCACCAT-3'). Stock primer solutions (Integrated DNA Technologies, Coralville, IA, USA) were made up in deionized (d)H_20 at a concentration of 6.0 µM and 7.5 µM for NKG2C and Break, respectively. Separate PCR cocktails were created for NKG2C and Break primer sets as per Table 2.2.

The PCR cocktails were combined with 2 µL DNA (or dH_20 for negative control) and placed in 0.2 mL thin-walled PCR tubes. DNA from a known heterozygous individual ($NKG2C^{-/+}$) was used as a positive control. The PCR conditions were similar to those described by Miyashita *et al*, beginning with a denaturation step at 96˚C for 10 minutes[82]. The following 35 cycles were carried out at 96˚C for 30 seconds (denaturation), 45˚C for 30 seconds (annealing), and at 72˚C for 40 seconds (extension). This protocol differed from that used by Miyashita *et al.* in the annealing temperature, and that both NKG2C and Break reactions were done under identical conditions. This protocol was adapted from Miyashita and adapted such that the conditions could be identical without affecting the accuracy of the results. These differences in our protocol did not affect the accuracy of our results, as demonstrated by previous members of our research group (results not shown).

Following PCR, samples were subjected to electrophoresis on a 2% agarose gel in TE buffer at 110V for 15-20 minutes. DNA bands in the gels were visualized with SYBR Safe™ DNA gel stain (Invitrogen) using the Kodak Gel Logic 440 Imager (Kodak, Rochester, NY, USA).

2.6 Matching NKG2C^{null} Individuals to NKG2C-Expressing Control Group

Eight individuals were identified as homozygous NKG2C^{null} and, therefore, we required a matched control group for comparison purposes. Individuals were matched based on age, %CD57^{+} NK cell population, CMV status (based on enzyme-linked immunosorbent assay [ELISA] results), CMV-specific CD8^{+} T cell response, and duration and severity of HIV disease. These characteristics were obtained from previous experiments in the lab (see Table 3.1).

2.7 Flow Cytometry

PBMC from healthy controls were used for the optimization of intra- and extracellular staining with fluorescence-conjugated antibodies. Extracellular staining of resuspended PBMC took place in ~200 µL flow buffer (see Table 2.1 for preparation of flow buffer) in the dark at 4˚C for 30 minutes using anti-CD3, -CD56, -CD57, and -NKG2C antibodies. Following staining, PBMC were washed with flow buffer. Intracellular staining was performed using MACS Inside Stain Kit (Miltenyi Biotec, Cologne, Germany). Briefly, 250 µL Inside Fix (Miltenyi Biotec) was added, along with 250 µL flow buffer to form a final concentration of 2 x 10^6 PBMC/mL. This suspension was left to incubate for 20 minutes at room temperature and then washed with 1 mL flow buffer. After decanting, 100 µL Inside Perm (Miltenyi Biotec) was added, followed by an appropriate volume of antibody (Table 2.3). Antibodies used for intracellular staining included anti-FcεR1γ, -IFN-γ, and -TNF-α. Next, the sample was placed in the dark at 4°C for 10 minutes. Finally, the sample was washed once with 1 mL Inside Perm (Miltenyi Biotec) and twice with flow buffer. In preparation for analysis by flow

Table 2.3 Antibody-Conjugated Fluorochromes

Antibody Specificity	Fluorochrome Conjugate	Volume Added (µL/2.0 x 10^6 PBMC)	Supplier and Clone
Anti-Human CD3	PE-Cyanine5.5	5	eBioscience[a] (Clone: SK7)
Anti-Human CD56	APC-Vio770	5	Miltenyi Biotec (Clone: REA196)
Anti-Human CD57	PE-Vio770	5	Miltenyi Biotec (Clone: TB03)
Anti-Human NKG2C	APC	5	R&D Systems[b] (Clone: 134591)
Anti-Human FcεR1γ	FITC	3	Milli-Mark® Millipore Sigma[c] (polyclonal rabbit anti-human)
Anti-Human IFN-γ	PE	7.5	eBioscience (Clone: 4S.B3)
Anti-Human TNF-α	Brilliant Violet	7.5	BioLegend (Clone: MAb11)

[a]Waltham, MA, USA
[b] Minneapolis, MN, USA
[c]Etobicoke, ON, Canada

cytometry, cells were fixed in 250 µL of 1% paraformaldehyde (1% PFA) (Sigma-Aldrich) and stored in the dark at 4°C.

Analysis was done using Kaluza Flow Cytometry Software 1.2 (Beckman Coulter, Brea, CA, USA) after data acquisition on the MoFlo Astrios EQ flow cytometer (Beckman Coulter).

2.8 NK Cell Stimulations

To measure general NK cell activation through natural cytotoxicity receptors, the MHC class I-deficient human erythromyeloblastoid leukemia K562 cell line (American Type Culture Collection (ATCC)® #CCL 243™) was incubated with PBMC at a 1:5 ratio (400,000 K562 cells: 2 million PBMC). Pro-inflammatory cytokines were detected post-stimulation by flow cytometry after intracellular cytokine staining. Expression of IFN-γ and TNF-α was measured, as their production is a marker of NK cell activation. In addition to NK cell activation, cellular cytotoxicity was confirmed with NK cell-mediated killing of radioactively-labeled K562 targets by ^{51}CRAs (results not shown). Alternatively, to measure antibody-dependent NK cell activation *in vitro*, the anti-CD16 monoclonal antibody, 3G8 (BioLegend, San Diego, CA, USA), was used at a concentration of 1 µg/1 x 10^6 PBMC. Again, the resulting pro-inflammatory cytokine response was measured by flow cytometry following intracellular staining of IFN-γ and TNF-α.

Stimulations with the K562 cell line and the anti-CD16 monoclonal antibody took place at a concentration of 2 million PBMC/mL lymphocyte medium. After a one hour incubation, Brefeldin A (Sigma-Aldrich) was added to a concentration of 10 µg/mL

followed by an additional 15-hour incubation at 37˚C with a 5% carbon dioxide (CO_2) atmosphere.

All cell lines used in this project were cultured at 37°C in 5% CO_2 in lymphocyte medium and maintained at a concentration between 0.2 and 1.0 x 10^6/mL.

2.9 Cytotoxicity Assays

Two models of CD16-mediated NK cell cytotoxicity were investigated in this project by 5-hour ^{51}CRAs. The first model of ADCC used the C1R-B27 B cell line (a gift from Kelly MacDonald, University of Toronto, ON, Canada) coated with anti-HLA class I antibodies, which served as NK cell targets. The pan-anti-HLA class I antibodies used in this assay were produced by the W6/32 murine B cell hybridoma (a gift from Dr. Sheila Drover, Memorial University, St. John's, NL, Canada).

The second assay measured killing of the murine mastocytoma cell line, P815 (ATCC® TIB-64™) by anti-CD16 redirected lysis using the 3G8 monoclonal antibody. P815 cells express fragment crystallizable (Fc) receptors on their cell surface, which recognizes the Fc portion of the 3G8 antibody, resulting in exposed CD16-specific fragment antigen-binding (Fab) regions able to stimulate NK cells via CD16 and mediate P815 cell lysis. See Figure 2.1 for an overview of these stimulations.

Approximately 1 million target cells were pelleted and labeled with approximately 100 µCi sodium chromate ($Na_2{}^{51}CrO_4$, Perkin-Elmer, Waltham, MA, USA) for 90 minutes at 37˚C in 5% CO_2. Post-labeling, target cells were washed three times with PBS supplemented with 1% FCS. The CRAs were performed in duplicate in

U-bottom 96-well microtiter plates (BD Biosciences, San Jose, CA, USA) with an effector to target ratio of 50:1 for both the C1R and P815 assays.

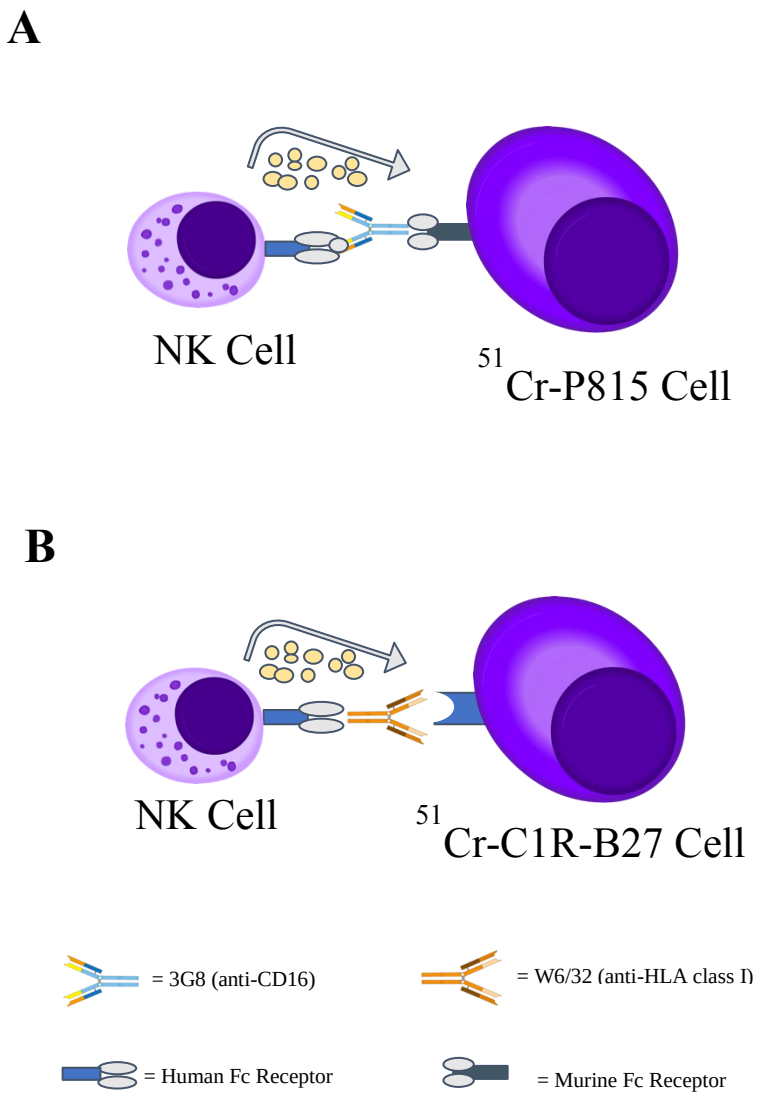

Figure 2.1 NK Cell Cytotoxicity Assays

NK cells kill ^{51}Chromium-labeled target P815 cells through crosslinking mediated by anti-CD16 antibodies in an anti-CD16 redirected lysis assay [A]. NK cell-mediated ADCC was modeled using anti-HLA class I-coated ^{51}Chromium-labeled C1R cells [B].

The cytotoxicity measured in these assays was mediated by the antibodies which cross link effectors (NK cells) to their ^{51}Chromium-labeled target cells. P815 cells were plated with 12 doubling dilutions of 3G8 antibody at an initial concentration of 100 ng/mL. C1R cells were plated with 6 doubling dilutions of the W6/32 supernatant, which was at an initial concentration of 1000 ng/mL. A "no-antibody control" was also used for each cell line, wherein a matched volume of lymphocyte medium was added. Dilutions of the anti-CD16 and anti-HLA class I antibodies allowed for the calculation of half-maximal effective concentrations (EC_{50}).

Each well contained 200 µL of [antibody-containing]-lymphocyte medium, 50 µL target cells (at a concentration of 100,000 target cells/mL), as well as 50 µL PBMC (at a concentration of 5,000,000/mL). The maximum release well contained 250 µL of 1 N hydrochloric acid along with 5,000 ^{51}Chromium-labeled target cells. Finally, the minimum release well contained 250 µL lymphocyte medium and 5,000 target cells. After 5-hour incubation, 100 µL of sample from each well was removed and placed in kimble tubes (ThermoFisher Scientific) containing 50 µL bleach. These samples were analysed using the Wallac 1480 Wizard Gamma Counter (Perkin Elmer) which provides percent lysis as an output. Percent lysis was calculated as follows:

$$\% \text{ lysis} = 100x \frac{(\text{specific lysis} - \text{minimum lysis})}{(\text{maximum lysis} - \text{minimum lysis})}$$

2.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Inter-group comparisons were tested using the Mann-Whitney *U* test when results were not normally distributed. If the results were normally

distributed, an unpaired student's *t*-test was performed. Normal distribution was determined using the D'Agostino-Pearson omnibus test, Shapiro-Wilk test, and the Kolmogorov-Smirnov test. Results were considered statistically significant when $P \leq 0.05$.

3. Results

3.1 Identification of NKG2C^{null} subjects

3.1.1 Rationale

NK cells expressing NKG2C selectively expand following CMV infection. This NK cell subset is associated with a differentiated phenotype including expression of CD57 and loss of FcεR1γ. Expansion of these differentiated NK cells is exaggerated in HIV infection compared to uninfected controls[81]. Additionally, absolute lack of NKG2C expression affects susceptibility to HIV infection and disease progression which may be a result of compromised NK cell function[97]. NKG2C expression could be partially responsible for driving the expansion of differentiated NK cells that display enhanced ADCC compared to undifferentiated NK cells which lack these phenotypic markers. For this reason, we chose to compare phenotypic NK cell differentiation between HIV-infected NKG2C^{null} donors and a group of NKG2C-expressing matched donors to investigate any variance in differentiation potential associated with the absence of NKG2C. An HIV/CMV co-infected cohort serves as an ideal setting for our investigation, as it is most likely to reveal any functional discrepancies between NK cells from NKG2C^{null} and NKG2C-expressing individuals.

3.1.2 Identifying NKG2C^{null} Individuals

Individuals with <1% of their NK cells (CD3- CD56+) expressing NKG2C by flow cytometry following extracellular staining for anti-NKG2C and anti-CD57 were categorized as "suspect" NKG2C^{null} individuals (see Figure 3.1.1). Suspect NKG2C^{null}

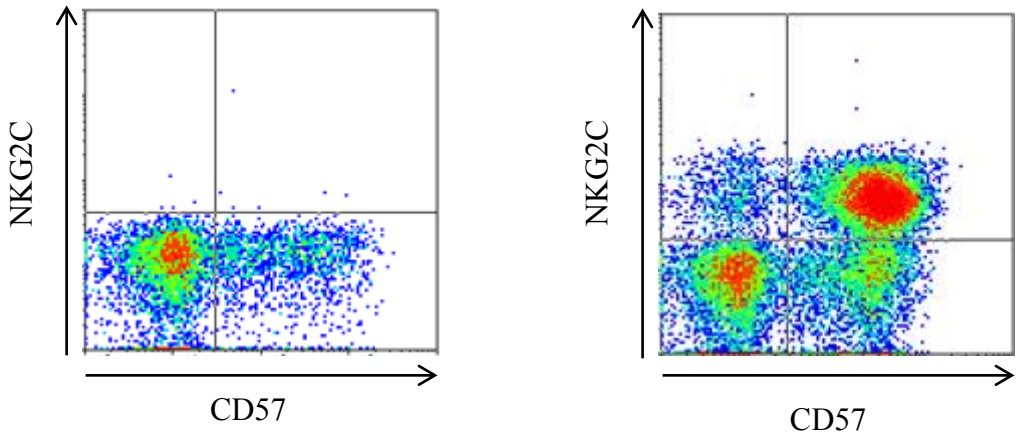

Figure 3.1.1 Identification of Suspect NKG2C^{null} Individuals by Flow Cytometry

HIV/CMV co-infected individuals typically have an expanded population of NKG2C^{+} NK cells (right), whereas NKG2C^{null} individuals lack this expansion (left). PBMC were isolated from whole blood donor samples and stained using anti-CD3, CD56, CD57, and NKG2C fluorescence-conjugated antibodies for analysis by flow cytometry. NK cells (CD3+ CD56-) were gated on for analysis of NKG2C and CD57 expression.

individuals were genotyped using NKG2C- and NKG2C deletion-specific primers designed by Miyashita *et al.*[82]. Genotypic analysis included two sets of PCR, one which identified the presence of the NKG2C gene (wild type NKG2C) and another which identified its absence by amplification of a segment brought into proximity by the deletion of a segment of DNA including the coding sequence for NKG2C (NKG2C deletion) (see Figure 3.1.2). A combination of these tests allowed determination of NKG2C genotype ($NKG2C^{-/-}$, $NKG2C^{-/+}$, or $NKG2C^{+/+}$). We identified 8 homozygous null ($NKG2C^{-/-}$) individuals in our study cohort of HIV-infected individuals. All were co-infected with CMV (see Figure 3.1.3).

3.1.3 Assigning an NKG2C-Expressing Matched Group

All donors were assigned identification numbers upon entry into the study. $NKG2C^{null}$ individuals were matched to HIV-infected NKG2C-expressing controls based primarily on age, $\%CD57^+$ NK cell population identified by flow cytometry, and anti-CMV IgG responses measured by ELISA. Other parameters considered were duration of HIV infection, nadir $CD4^+$ T cell count (cells/mm^3 peripheral blood) and anti-CMV $CD8^+$ T cell responses identified as IFN-γ-expressing $CD8^+$ T cells post-stimulation with overlapping CMV peptides from immunodominant pp65 and IE-1 proteins (see Table 3.1 for $NKG2C^{null}$ and NKG2C-expressing participant characteristics). This resulted in 8 $NKG2C^{null}$ male subjects being matched to 1 female and 7 male NKG2C-expressing controls based on these parameters. The one female included was the closest match available in our cohort (See Table 3.1). There was no significant difference between groups with regard to matching parameters (data not shown, Mann-Whitney *U* test, ns).

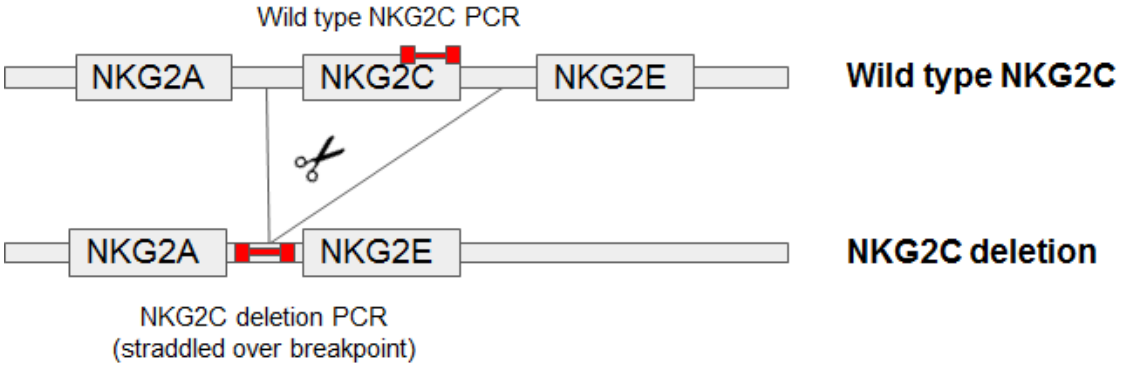

Figure 3.1.2 Description of PCR Amplifications

Wild type NKG2C PCR amplifications involved a common 362-base pair fragment within the NKG2C gene (primer sites shown in red). NKG2C deletions were identified using primer sets which only allow for amplification of a 411-base pair fragment if the NKG2C gene is absent from the genome. These primers straddled either side of NKG2C, therefore, if NKG2C was present the sequence would be too long to be amplified by standard PCR. Scissors indicate deletion sites of the NKG2C gene as occurs in NKG2C^{null} individuals.

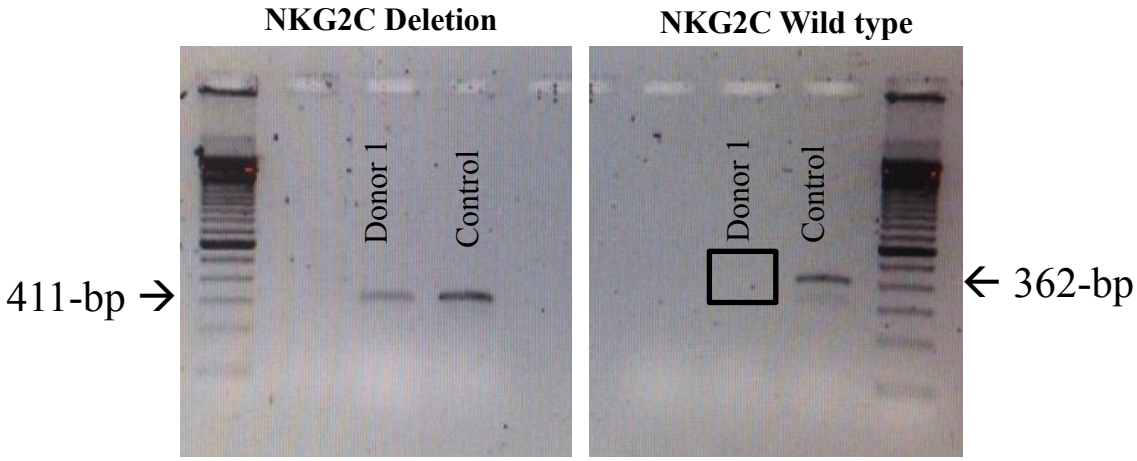

Figure 3.1.3 Confirmation of NKG2C^{null} Status by PCR

An example NKG2C^{null} individual (Donor 1) showed a band indicating amplification within the NKG2C deletion PCR while lacking a band in the wild type NKG2C amplification. The heterozygous positive control expressed a band in each region (NKG2C$^{-/+}$).

Table 3.1 NKG2C^{null} and Matched Group Characteristics

Nulls (ID #)[a]	Sex	Age	$CD57^+$ NK (%)	Anti-CMV IgG (OD)[b]	CMV-specific $CD8^+$ T cells[c] (%)	Nadir[d]	**Matches** (ID #)[a]	Sex	Age	$CD57^+$NK (%)	Anti-CMV IgG (OD)[b]	CMV-specific $CD8^+$ T cells[c] (%)	Nadir[d]
21	M	54	20	1.8	6.7	6	138	F	52	18.7	0.8	0.2	147
28	M	51	24	2.1	11.2	0	78	M	47	24	1.4	4.5	32
192	M	58	65	1.3	18.3	120	201	M	58	61.1	0.8	4.4	95
237	M	62	56	1.2	11.4	480	196	M	60	58.7	1.0	14.9	63
245	M	56	55	1.3	6.3	390	209	M	55	51	1.8	0.2	276
259	M	35	42	1.0	1.4	323	264	M	34	39	0.8	0.7	660
270	M	38	31	0.9	1.5	480	174	M	34	32	0.9	0.8	380
294	M	47	48	1.1	8.1	558	229	M	46	50	1.3	2.3	264

[a]Null and matched group patient identification number

[b]Anti-CMV IgG responses against CMV AD169-infected MRC-5 cell lysate were measured using an ELISA with patient plasma diluted 1:500 (OD=optical density)

[c]Detection of CMV-specific $CD8^+$ T cell responses was performed in a 5-hour stimulation with overlapping CMV peptides from the immunodominant pp65 and IE-1 proteins, followed by intracellular staining for IFN-γ^+ $CD8^+$ T cells

[d]Lowest recorded $CD4^+$ T cell count (cells/mm^3 peripheral blood)

3.2 Comparison of NK Cell Population Size and Phenotype between NKG2C^{null} and NKG2C-Expressing Matched Groups

3.2.1 Rationale

Exact mechanisms of NK cell-mediated herpesvirus control remain unclear, however, some studies directly implicate NK cells in control of CMV infection. For example, an individual devoid of NK cells suffered severe recurrent herpesvirus infections, including CMV infection[102]. Furthermore, an individual lacking T cells displayed a decrease in CMV viral load concurrent with the emergence of an expanded NK cell population homologous to the differentiated NK cell population with enhanced ADCC that emerges in chronic CMV infection[103]. This population is marked by the expression of CD57 [and NKG2C] and is often accompanied by the loss of FcεR1γ and NKG2A. CMV is the only virus that dramatically shapes the host NK cell population in such a profound fashion, and this shaping is further amplified in HIV infection[72,81]. For these reasons, NK cell constitution and phenotypic differentiation are of particular interest in the context of CMV and HIV infection. This is an optimal setting to investigate the role of NKG2C.

3.2.2 Comparison of Percent NK Cells within the Total Lymphocyte Population between Groups

The first way we compared NKG2C^{null} and NKG2C-expressing groups was by measuring the overall proportion of NK cells ($CD3^-$ $CD56^+$) within the lymphocyte population (%NK cells/total lymphocytes, see Figure 3.2.1 for gating strategy).

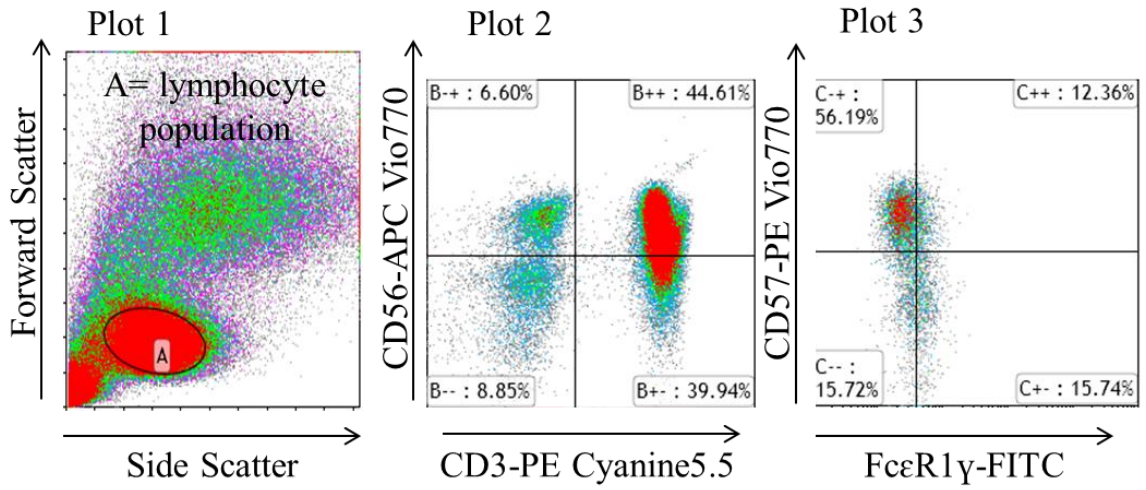

Figure 3.2.1 Natural Killer Cell Gating Strategy

PBMC were isolated from whole blood and stained with anti-CD3, CD56, and CD57 (extracellular) and anti-FcεR1γ (intracellular) fluorescence-conjugated antibodies for analysis by flow cytometry (see Table 2.2 for list of antibody-conjugated fluorochromes and their concentrations). This representative gating strategy begins with total PBMC (Plot 1) and the lymphocyte population is encircled in [A]. Plot 2 reveals lymphocyte expression of CD3 and CD56. Quadrant B-/+ encompasses NK cells (CD3- $CD56^{+}$). Plot 3 characterizes NK cell expression of CD57 and FcεR1γ. Quadrant C-/+ encompasses mature ($CD57^{+}$) NK cells which have lost expression of FcεR1γ. Plots were generated using Kaluza Flow Cytometry Software version 1.2.

Our results were normally distributed, therefore, we compared groups using a Student's *t*-test and reported mean values with a 95% CI. The mean NK cell population was 8.7%, 95% CI [4.8, 12.6] and 11.2%, 95% CI [6.7, 15.7] of total PBMC for the NKG2C^{null} and matched donor group, respectively (Figure 3.2.2). There was no significant difference in NK cell population sizes between groups (Student's *t*-test, ns). Therefore, in this setting, it appears that NKG2C expression or lack thereof has no obvious effect on NK cell numbers.

3.2.3 Comparison of FcεR1γ & CD57 Expression between Groups

Loss of FcεR1γ from NK cells is associated with more potent ADCC and is more common among mature (CD57^{+}) NK cells[79,104,105]. First, we confirmed that both groups had similar populations of mature NK cells (Figure 3.2.3A). Next, we measured the extent of FcεR1γ loss from the overall (Figure 3.2.3B) and mature (Figure 3.2.3C) NK cell populations.

PBMC were isolated from whole blood and labeled for analysis by flow cytometry. Our results in Figure 3.2.3A are normally distributed and reveal similar CD57^{+} NK cell populations between groups, wherein 62.3%, 95% CI [47.4, 77.1] of NKG2C^{null} NK cells expressed CD57, compared to 57.7%, 95% CI [49.9, 65.6] in the matched group. There was no significant difference between groups with regard to CD57 expression (Student's *t*-test, ns). Figure 3.2.3B depicts a broad range of FcεR1γ^{-} NK cells from participants in both groups, which are normally distributed and, therefore, reported

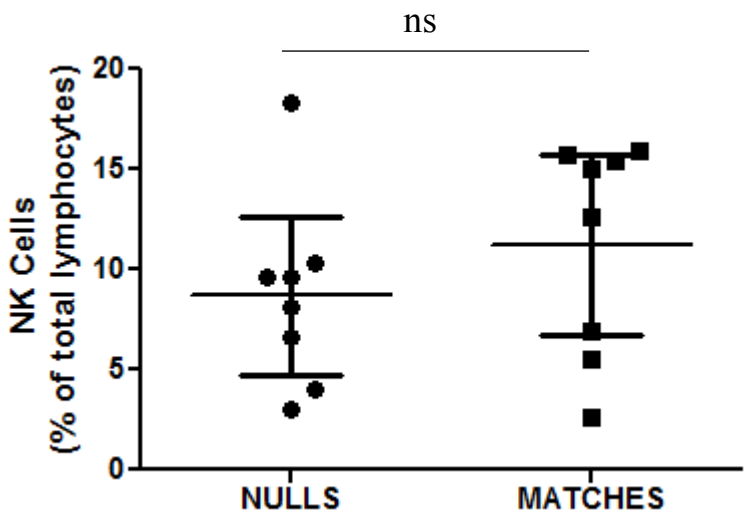

Figure 3.2.2 NK Cell Population in NKG2C^{null} and Matched Groups

NK cell ($CD3^-$ $CD56^+$) frequency represented as percent of total lymphocyte population. PBMC were stained with anti-CD3 and CD56 fluorescence-conjugated antibodies for analysis by flow cytometry. Data was generated by gating on quadrant $B^{-/+}$ as shown in Figure 3.2.1. Results are displayed as mean values with a 95% CI (Student's *t*-test, ns).

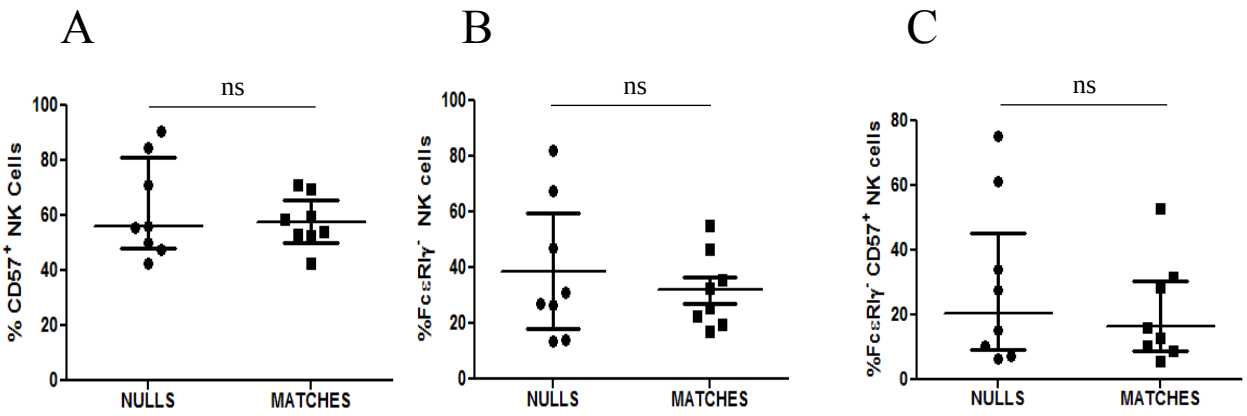

Figure 3.2.3 Loss of FcεR1γ Among NKG2C^{null} and Matched Groups

CD57^{+} NK cells were measured as a percent of total NK cells and data were generated by gating on quadrants C$^{-/+}$ and C$^{+/+}$ as shown in Figure 3.2.1. Results are displayed as mean values with a 95% CI (Student's *t*-test, ns) [A]. FcεR1γ^{-} NK cells were measured as a percent of total NK cells for both groups and data was generated by gating on quadrants C$^{-/+}$ and C$^{-/-}$ as shown in Figure 3.2.1. Results are displayed as mean values with a 95% CI (Student's *t*-test, ns) [B]. FcεR1γ^{-} NK cells were measured as a percent of mature (CD57^{+}) NK cells and data was generated by gating on quadrant C$^{-/+}$ as shown in Figure 3.2.1. Results are displayed as mean values with a 95% CI (Student's *t*-test, ns) [C]. PBMC were stained with anti-CD3, -CD56, -CD57 and FcεR1γ fluorescence-conjugated antibodies for analysis by flow cytometry after separation from whole blood samples [Panels A-C].

as mean [95% CI] values. 38.7%, 95% CI [17.8, 59.5] of NK cells from the NKG2C^{null} group lost FcεR1γ expression compared to 31.9%, 95% CI [20.6, 43.1] in the matched group. There was no significant difference in mean FcεR1γ^{-} NK cell values between groups (Student's *t*-test, ns). Data in Figure 3.2.3C also followed a normal distribution, and contrary to current literature, there was no enhancement of FcεR1γ^{-} NK cells in mature NK cell populations[79]. 29.7%, 95% CI [8.0, 51.4] of mature NK cells from the NKG2C^{null} group lost expression of FcεR1γ, compared to 21.0%, 95% CI [7.7, 34.3] in the matched group (Student's *t*-test, ns). Together these phenotypic data suggest equivalent phenotypic NK cell differentiation regardless of NKG2C genotype and lack of FcεR1γ^{-} NK cell enrichments among mature NK populations in both groups.

3.3 NK Cell Cytokine Responses to Stimulation (K562 and 3G8)

3.3.1 Rationale

Data presented in section 3.2 showed no significant difference in NK cell population size or degree of phenotypic maturation, marked by CD57 expression and loss of FcεR1γ, between NKG2C^{null} and NKG2C-expressing groups. In this section, we investigated whether the observed phenotypic equivalence translated into a similar capacity for NK cell activation between groups. To this end, we measured pro-inflammatory cytokine expression, specifically TNF-α and IFN-γ expression, as markers of NK cell activation in response to stimulation.

3.3.2 NK Cell Cytokine Production in Response to NCR Stimulation (K562)

The first stimulation we tested served as a baseline measurement of general NK cell cytotoxicity. NK cell activation was measured against erythromyeloblastoid leukemia K562 cells, which serve as classical NK cell targets by activation through NCRs in the absence of inhibition through HLA class I receptors. Both groups responded by expressing equivalent amounts of IFN-γ and TNF-α post-stimulation with K562 cells (Figure 3.3.1A and B, respectively). These data are not normally distributed, therefore, results are reported as median values followed by interquartile range (IQR). NKG2C^{null} median IFN-γ expression following exposure to K562 cells was 3.3%, (IQR=1.7-4.2) whereas the median expression of IFN-γ among the matched group was 0.9%, IQR=0.4-4.4. There was no significant difference between groups (Mann-Whitney test, ns).

TNF-α expression was normally distributed and therefore reported as mean values with a 95% CI. Mean TNF-α expression within the NKG2C^{null} group was 3.3%, 95% CI [0.9, 5.7], compared to 3.1%, 95% CI [1.7, 4.5] in the matched group. Again, there was no significant difference between groups (Student's *t*-test, ns). These data show that NK cells from both groups were equally competent in their general activation, as measured by cytokine production in response to stimulation through NCRs by K562 cells.

3.3.3 NK Cell Cytokine Production in Response to CD16 Stimulation (3G8)

Our next goal was to measure NK cell activation through CD16 since differentiated NK cells are reportedly better at ADCC. We investigated the relative strength of NK cell activation from either group to direct stimulation through CD16 using the 3G8 (anti-CD16) monoclonal antibody. The results obtained in these 16-hour assays

are displayed in Figure 3.3.2. Our results suggest equivalency between groups in pro-inflammatory cytokine response to 3G8 antibody-mediated stimulation, as measured by IFN-γ (Figure 3.3.2A) and TNF-α expression (Figure 3.3.2B).

The data in Figure 3.3.2A and B are normally distributed, therefore, statistical analysis was performed using Student's *t*-tests. Mean NKG2C^{null} donor group NK cell expression of IFN-γ following 3G8 stimulation is 11.0%, 95% CI [5.4, 16.7], compared to 9.4%, 95% CI [4.6, 14.3] in the matched group. Mean TNF-α^{+} NK cells, reported in Figure 3.3.2B, is 8.7%, 95% CI [3.2, 14.6] among NKG2C^{null} donors and 12.2%, 95% CI [4.2, 20] in the matched group. There was no significant difference between groups in IFN-γ or TNF-α expression in response to 3G8 stimulation (Student's *t*-test, ns). These data show that both groups are equivalent in their capacity for CD16-mediated activation by 3G8 stimulation and that mean NK responses to stimulation through CD16 are notably stronger than through NCRs by K562 cells (Figure 3.3.1 vs Figure 3.3.2).

3.4 Measuring NK Cell Cytotoxicity in Models of ADCC

3.4.1 Rationale

Thus far we have observed equivalency between groups in NK cell population size, phenotype and pro-inflammatory cytokine responses to stimulation through NCRs (K562) and CD16 (3G8). To extend these findings, we measured NK cell cytotoxicity against antibody-treated cell lines in 5-hour ^{51}Chromium release assays involving redirected and classical ADCC (refer to Figure 2.1A & B, respectively).

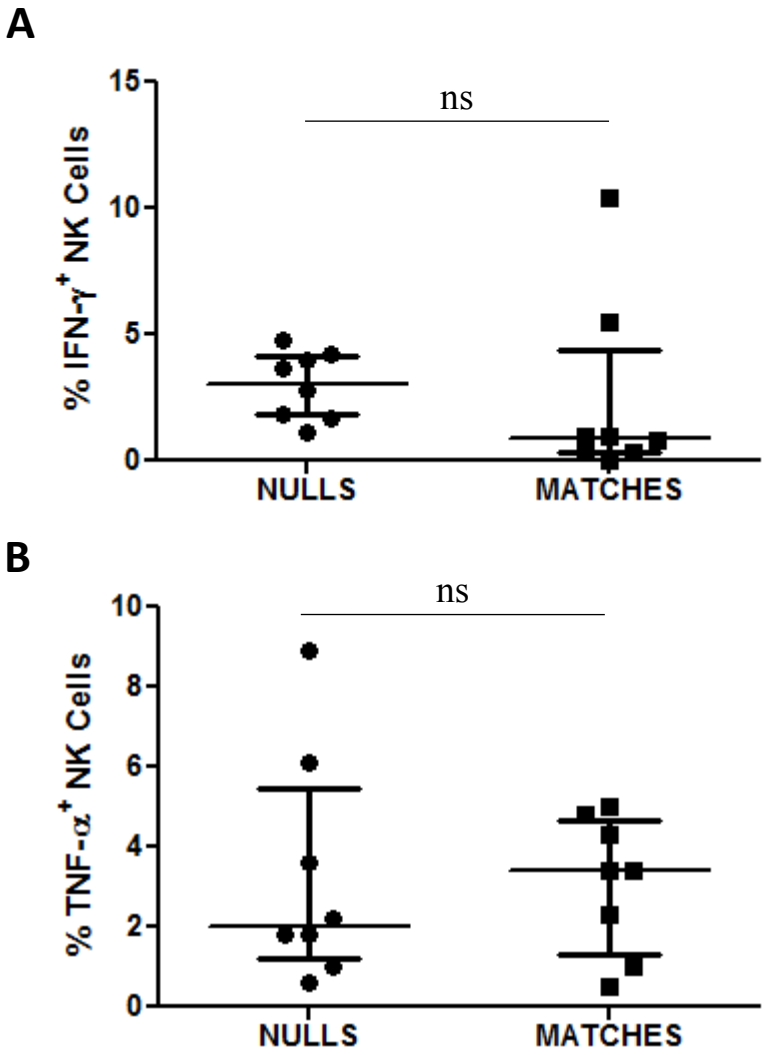

Figure 3.3.1 Pro-Inflammatory Cytokine Responses to K562 Stimulation

PBMC (2 x 10^6/mL) from NKG2C^{null} and NKG2C-expressing individuals were isolated and incubated with K562 cells at a ratio of 2 million PBMC: 400,000 K562 cells for 16 hours at 37˚C in a 5% CO_2 atmosphere (Brefeldin A was added after 1 hour). Post-stimulation, PBMC were labeled with fluorescence-conjugated antibodies to identify NK cells expressing IFN-γ [A] and TNF-α [B]. There was no significant difference between groups in median expression of IFN-γ (Mann-Whitney test, ns) or mean expression of TNF-α (Student's *t*-test, ns) among NK cells between groups.

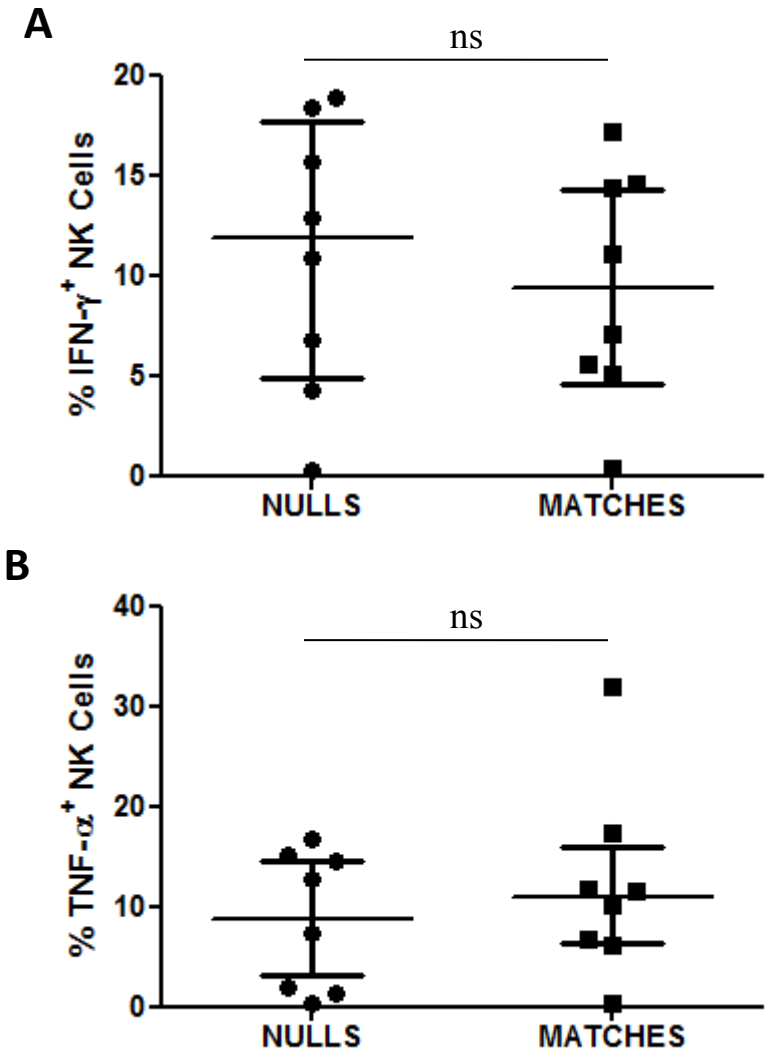

Figure 3.3.2 Pro-Inflammatory Cytokine Responses to 3G8 Stimulation

PBMC (2 x 10^6/mL) from NKG2C^{null} and NKG2C-expressing individuals were isolated and incubated with the 3G8 monoclonal antibody at a concentration of 1 µg/1 x 10^6 PBMC for 16 hours at 37˚C in a 5% CO_2 atmosphere (Brefeldin A was added after 1 hour). Post-stimulation, PBMC were labeled with fluorescence-conjugated antibodies to identify NK cells expressing IFN-γ [A] and TNF-α [B]. Results are shown as mean values with 95% CI (Student's *t*-test, ns).

3.4.2 NK Cell Cytotoxicity against Antibody-Coated P815 and C1R Cell Lines

NK cells from NKG2C^{null} and NKG2C-expressing groups were equivalent in their ability to lyse antibody-coated target cells in models of ADCC and anti-CD16 redirected lysis. Data are not normally distributed in either assay. There was no significant difference between the NKG2C^{null} and matched group in their capacity to mediate anti-CD16 redirected lysis of ^{51}Chromium-labeled P815 cells at the highest concentration of 3G8 antibody (Mann-Whitney *U* test, ns). NKG2C^{null} donor group median killing of P815 cells was 20.7%, 95% CI [5.2, 29.1] whereas the matched group was 10.4%, 95% CI [2.6, 32.0] (see Figure 3.4.1A).

We found no significant difference between groups in their capacity for lysis of ^{51}Chromium-labeled C1R cells coated with the highest concentration of W6-32 antibody (Mann-Whitney *U* test, ns). NKG2C^{null} donor median killing of C1Rs was 28.1%, 95% CI [15.0, 37.3] compared to matched group killing of 18.2%, 95% CI [19.5, 30.6] (see Figure 3.4.1B).

These data indicate equivalence between groups in their capacity for ADCC and anti-CD16 redirected lysis regardless of NKG2C expression, however, this applies only to stimulations wherein target cells are coated with a saturated concentration of antibody. Additionally, both groups appeared to display greater effector function against C1Rs (ADCC) relative to stimulation through CD16 by Fc receptor-expressing P815s (anti-CD16 redirected lysis), although these results are not statistically significant (see Figure 3.4.1 B vs A).

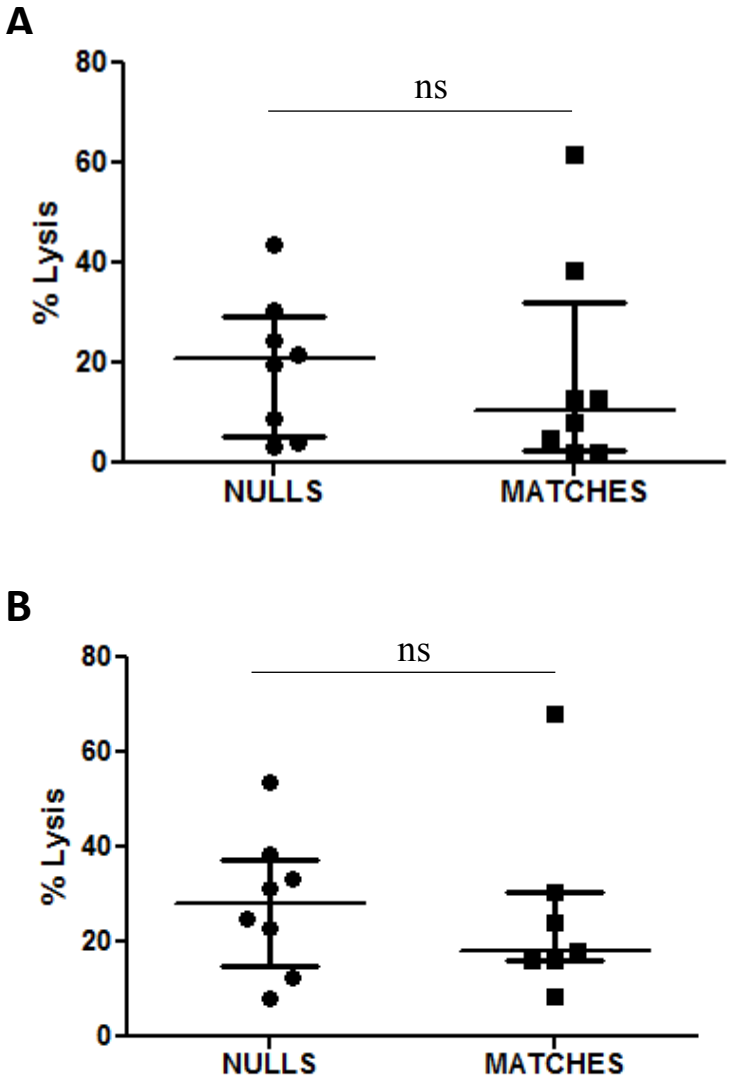

Figure 3.4.1 ^{51}Chromium Release Assays at Saturating Ab Concentrations

PBMC (2.5 x10^5) were incubated with ^{51}Chromium-labeled, anti-CD16-coated P815 cells [A] or ^{51}Chromium-labeled, anti-HLA class I-coated C1R-B57 cells [B] at an effector to target ratio of 50:1. Percent lysis was calculated as follows:

$$100x\frac{(\text{specific lysis} - \text{minimum lysis})}{(\text{maximum lysis} - \text{minimum lysis})}$$

3.4.3 NK Cell Sensitivity to Triggering Through CD16 (3G8) and W6/32 (C1R)

We reasoned that although both groups displayed equivalent capacity for ADCC and anti-CD16 redirected lysis at a saturated concentration of cross-linking antibody, there could be differences in sensitivity related to differentiation and maturation in the two groups. For example, heightened sensitivity to triggering through CD16 could increase target cell killing capacity at lower concentrations of antibody (3G8 or W6/32). To investigate differential sensitivity to triggering via CD16 between groups we performed doubling dilutions of 3G8 and W6/32 antibodies. Figure 3.4.2 displays a representative example of killing over various antibody titrations against P815 [A] and C1R [B] cell lines in the assays previously described in Section 2.9.

In order to convey the similarity we observed between groups in their sensitivity to triggering through CD16 in both assays, despite variability in absolute levels of killing, we normalized our results (see Figures 3.4.3 and 3.4.4). The EC_{50}, defined as the amount of antibody required to elicit a half maximal response, was calculated for both groups. NK cell-mediated ADCC followed a similar pattern in both groups.

W6/32 antibody began at a concentration of 1000 ng/mL and doubling dilutions were performed 6 times, resulting in a minimum concentration of 31.25 ng/mL. The 4^{th} dilution of W6/32 resulted in the EC_{50} for both the $NKG2C^{null}$ group and matched group (Figure 3.4.3). This dilution is equivalent to a concentration of 125 ng/mL W6/32 antibody.

3G8 antibody started at a maximum concentration of 100 ng/mL and doubling dilutions were performed 12 times to a final concentration of 0.05 ng/mL. We chose to perform additional dilutions with 3G8 because it elicited a stronger pro-inflammatory response than W6/32-coated C1Rs at lower concentrations (data not shown). This may be due to a higher affinity of 3G8 compared to W6/32 antibodies with donor CD16 receptors. The mean EC_{50} from the $NKG2C^{null}$ group and the NKG2C-expressing group was at the 7.4 and 7.5^{th} dilution of 3G8, respectively (Figure 3.4.4). These dilutions are equivalent to ~1.2 ng/mL W6/32.

These data suggest equivalent sensitivity to triggering through CD16 between $NKG2C^{null}$ and NKG2C-expressing groups as seen through highly similar EC_{50} values.

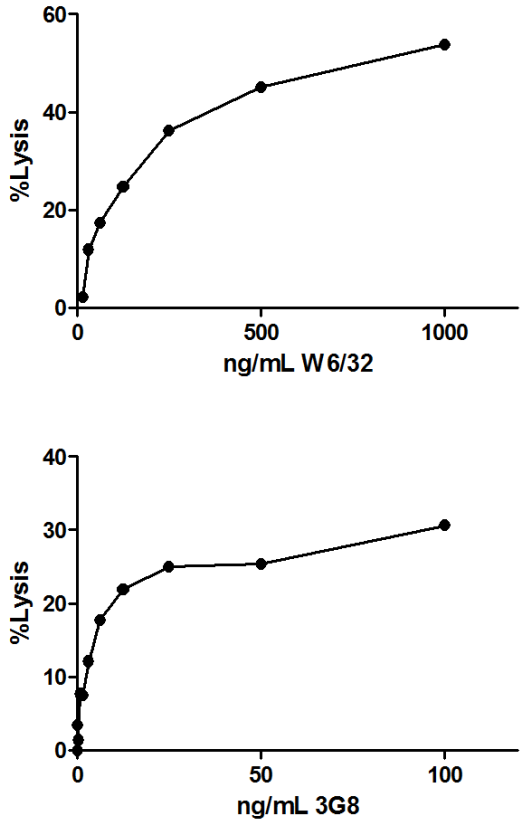

Figure 3.4.2 Representative Ab Dilution Assays

Representative examples of donor responses throughout titrations of 3G8 and W6/32 antibodies in 5-hour ^{51}CRAs. PBMC (2.5 x10^5) were incubated with ^{51}Chromium-labeled, anti-CD16 (3G8)-coated P815 cells at antibody concentrations ranging from 0.05-100 ng/mL [A]. PBMC were incubated with ^{51}Chromium-labeled, anti-HLA class I (W6/32)-coated C1R cells at antibody concentrations ranging from 17-1000 ng/mL [B].

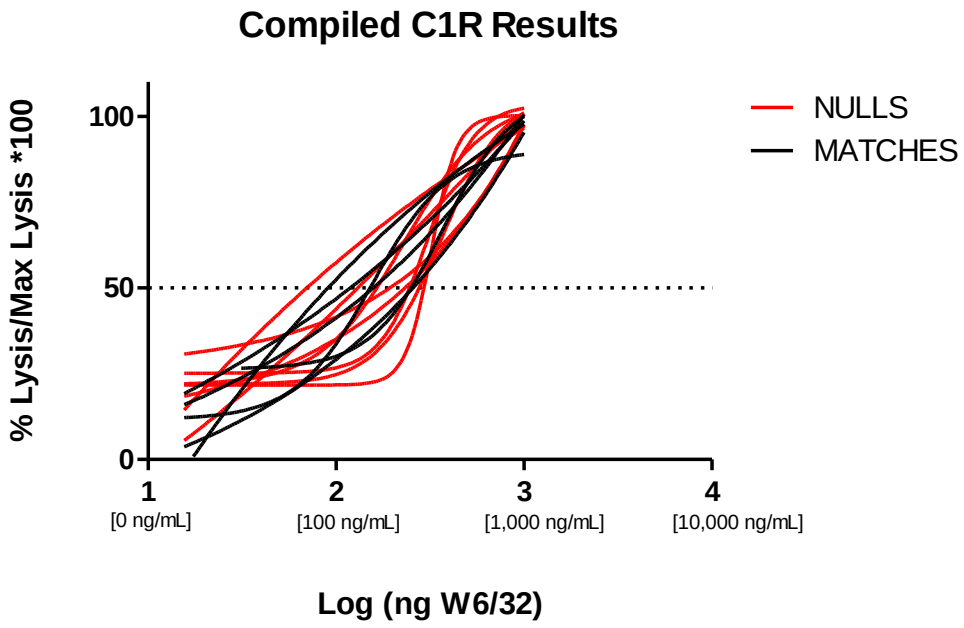

Figure 3.4.3 W6/31 ^{51}Chromium Release Assays at Diluted Ab Concentrations

PBMC (2.5 x10^5) were incubated with ^{51}Chromium-labeled, anti-HLA class I-coated C1R cells at antibody concentrations ranging from 17-1000 ng/mL in 5-hour CRAs. EC_{50} values were manually calculated for each donor using the concentration resulting in half-maximal target cell lysis.

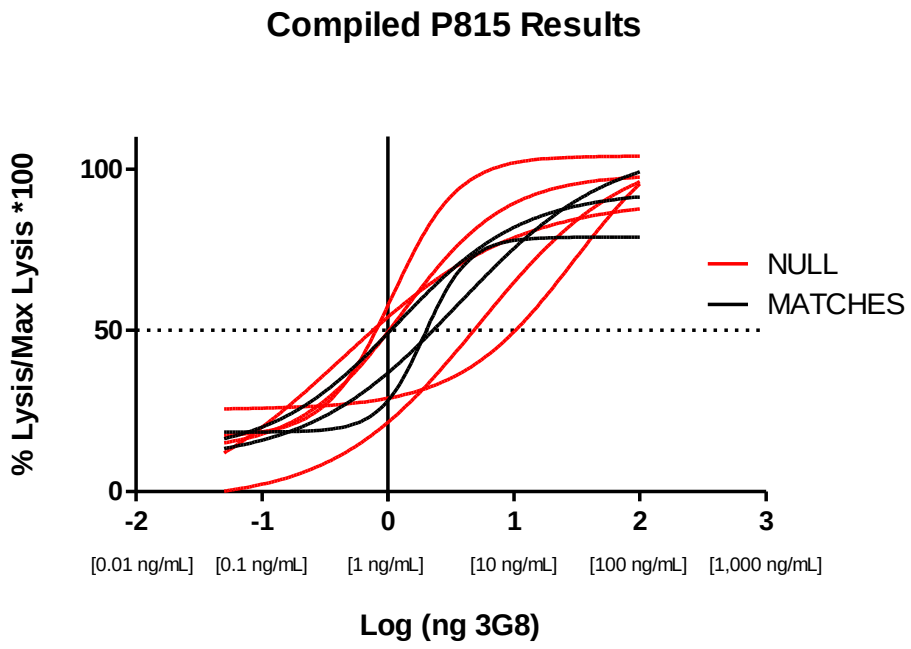

Figure 3.4.4 P815 ^{51}Chromium Release Assays at Diluted Ab Concentrations

PBMC (2.5 x10^5) were incubated with ^{51}Chromium-labeled, anti-CD16-coated P815 cells at antibody concentrations ranging from 0.05-100 ng/mL in 5-hour ^{51}CRAs. EC_{50} values were manually calculated for each donor from the concentration resulting in half-maximal target cell lysis.

4. Discussion

4.1 Summary of Results

NK cells expressing the activating receptor NKG2C expand following CMV infection[104]. This expanded subset of NK cells is further characterized by expression of the maturation marker CD57, loss of FcεR1γ and an enhanced capacity to mediate signaling through CD16. The results of this research study provide evidence against dependence on NKG2C expression for CMV-driven NK cell differentiation into mature effector cells. Our data indicate general equivalency in NK cell population size, phenotypic character, cytokine induction and cytotoxic function between NKG2C^{null} and NKG2C-expressing donors co-infected with CMV and HIV.

More specifically, phenotypic NK cell maturation, as measured by the expression of CD57 and loss of FcεR1γ, occurred to a similar extent in both groups. The phenotypic similarities observed between groups extended to functional equivalency in terms of cytokine induction, wherein NKG2C^{null} donors mounted equivalent IFN-γ and TNF-α responses to triggering through CD16 (3G8 antibody stimulation) or NCRs (K562 stimulation). Finally, NK cells from both groups were equally capable of, and equally sensitive to ADCC against ^{51}Chromium-labeled targets cells (anti-CD16 redirected lysis of P815 or ADCC against antibody-coated C1R cell lines) in the presence of saturating or limiting antibody concentrations (3G8 or W6/32, respectively). These data indicate general equivalency in CMV-driven NK cell differentiation, independent of NKG2C expression.

Considering the association between NKG2C expression and CMV-driven NK cell differentiation, a logical question to pose is whether NKG2C is required for CMV-driven NK cell differentiation[85,104]. To answer this question, our investigation details whether NK cells from NKG2C^{null} individuals can mediate comparable differentiation to that observed among NKG2C-expressing individuals. In the context of primary HIV infection, homozygosity for NKG2C$^{(+/+)}$ is protective in reducing susceptibility to HIV infection and is also protective against disease progression[106]. We reasoned that without NKG2C, which appears to mark potent effector cells and diminished risk of HIV infection and disease progression, NK cells may not mirror the same phenotypic differentiation in the context of CMV infection[106].

Our investigation began by comparing absolute NK cell population size between groups to exclude the possibility of differential absolute NK cell numbers affecting overall NK function and complicating comparison between study groups. We measured average NK cell populations among NKG2C^{null} and NKG2C-expressing groups as 8.7% and 11.2% of total lymphocytes, respectively, with no significant difference between groups. The NK cell population fractions among our cohort were slightly reduced compared to healthy controls, wherein NK cells typically comprise 10-15% of total lymphocytes. However, reduced NK cell (CD16^{+} CD56^{dim}) fractions are typical of HIV infection[107]. After controlling for NK cell population size between groups, we proceeded to investigate phenotypic characteristics of NK cell differentiation between groups.

There are estimated to be between 6,000 and 30,000 NK cell subsets per individual, each likely bearing a slightly different functional capacity[108]. Infection with CMV drives the expansion of a phenotypically distinct NK cell subset which can expand

to take up a large proportion of the overall NK cell population. As previously stated, CD57 is preferentially expressed on NKG2C^{+} NK cells expanded in CMV infection, and FcεR1γ is lost[81,78,109]. Although there are other markers of this expanded NK cell population, such as loss of NKG2A and signaling molecules including PLZF, SYK and Eat-2, we chose to characterize NKG2C^{null} NK cell differentiation based solely on expression of CD57 and loss of FcεR1γ[79,85]. CD57 is a well-known surface marker associated with NK cell maturation and FcεR1γ is initially associated with the CD16 receptor that appears to increase in activity on differentiated NK cells.

We observed no significant difference in NK cell expression of CD57 or in loss of FcεR1γ between groups. Our results complement those from a recent study, which also reported similar NK cell phenotypes between NKG2C^{null} and NKG2C-expressing groups[86]. These findings suggest that there is at least redundancy in NKG2C expression for driving NK cell differentiation in response to CMV infection.

Although studies often do not compare FcεR1γ expression among various NK cell subsets, multiple groups have reported accumulation of FcεR1γ^{-} NK cells among mature (CD57^{+}) NK cell subsets in CMV infection[78,81,109]. Our results are discordant with these reports in that we observed a slightly decreased fraction of FcεR1γ^{-} NK cells among mature populations (results not shown). We speculate that FcεR1γ can be lost in response to various stimuli such as stimulation with 3G8, even in the absence of CMV infection. This could explain why we did not observe an accumulation of FcεR1γ^{-} cells among mature (CMV-driven) NK cells.

In summary, speculation that NKG2C gene deletion may affect phenotypic NK cell differentiation was not supported by our results, which suggest highly similar CMV-

driven phenotypic NK cell differentiation between NKG2C^{null} and NKG2C expressing groups. Next, we compared NK cell function between groups.

We chose to measure IFN-γ expression in response to direct stimulation through CD16 using the monoclonal antibody, 3G8. Differentiated NK cells upregulate IFN-γ expression in response to stimulation through CD16 more readily than undifferentiated, or naïve NK cells[70]. This has been attributed to demethylation of CpG motifs in conserved non-coding sequences of the IFN-γ gene region, leading to elevated gene expression[70]. Any differences in CMV-driven NK cell differentiation towards enhanced ADCC or antibody-dependent cytokine release should be highlighted in this assay. We also included a baseline measure of NK cell function involving incubation with K562 cells, which cause NK cell activation through NCRs, modeling natural cytotoxicity. A second marker of NK cell activation, TNF-α, was added in both of these sets of experiments to broaden analysis of pro-inflammatory responses between groups.

We anticipated similar cytokine expression in response to NCR stimulation between groups, as there is no evidence that NKG2C expression affects NK cell capacity to mediate natural cytotoxicity. Conversely, we suspected NK cells from NKG2C^{null} individuals might display diminished cytokine expression in response to CD16 stimulation, as differentiated NK cells, which typically express NKG2C, respond more robustly to stimulation through CD16[70].

Our results again indicated equivalency between groups in terms of cytokine expression in response to stimulation through NCRs. This validates our selection of a matched group in terms of general NK cell function. Contrary to our second expectation, both groups were also equivalent in terms of activation through CD16. These results

support findings published in 2016 by Liu *et al*., which reported similar epigenetic remodeling of the IFNG promoter in NKG2C^{null} healthy donors, resulting in heightened IFN- γ responses following infection with CMV[86].

Although these assays do not allow interaction between NKG2C and its ligand, we conclude that general CMV-related differentiation into effectors with heightened pro-inflammatory cytokine production in response to stimulation through CD16 occurs in NKG2C^{null} individuals. In agreement with most current literature, NK cells from both groups responded more robustly to stimulation through CD16 compared to stimulation through NCRs[24]. Indeed, average IFN-γ and TNF-α expression from either group in response to K562 stimulation was only ~5% of NK cells compared to ~10% of NK cells post-stimulation with 3G8.

Of note, when we compared cytokine responses to stimulation via CD16 across various NK cell subsets, the CD57^{+} NK cell subset expressed the highest amount of IFN-γ (data not shown), irrespective of NKG2C expression. This finding was unexpected, as most groups report NKG2C^{+} NK cells as the most robust responders to stimulation through CD16[61]. However, our findings are in line with a study by Makwana *et al.* which excluded NKG2C as a marker of the most highly activated NK cells in response to stimulation (in terms of CD107a expression)[87]. Both Makwana's and our findings constitute further evidence of redundancy to NKG2C expression in driving expansion of NK cells with heightened cytokine responses.

Next, we chose to test whether NK cell-mediated cytotoxic function was similar between groups. Indeed, we found that ADCC and anti-CD16 redirected lysis was equivalent between groups at a saturating concentration of antibody. Despite the inability

of NKG2C to interact with its ligand in these CMV-independent assays using CMV-infected donor samples, we conclude that a lack of NKG2C expression does not affect general NK cell phenotypic differentiation or capacity to mediate target cell lysis through CD16. At this point we considered the effect of saturating antibody concentrations and questioned whether NKG2C absence during NK cell differentiation into mature effectors could more subtly affect the development of NK cell sensitivity to triggering through CD16. To this end, we carried out twelve (3G8 assays) and six (C1R assays) doubling dilutions of our sensitizing antibodies to observe cytotoxic responses over a broad range of concentrations.

These cytotoxicity assays were normalized to the maximum amount of killing observed for each individual. The maximum amount of killing did not always occur at the highest concentration of antibody, which was likely a manifestation of the prozone effect (a poorly understood phenomenon in which inaccurately low responses are observed at high antibody concentrations due to interference or other unknown issues). Indeed, numerous donors displayed the highest amount of target cell killing in the anti-CD16 redirected lysis assay at the 2^{nd} or 3^{rd} doubling dilution of antibody. We chose to normalize our results by assigning 100% lysis as the maximum amount of killing reported for each donor at any antibody concentration. Certain donor NK cells did not lyse target cells, regardless of the presence of antibodies and these results (3 NKG2C^{null} and 4 matched donors) were excluded from our analysis of EC_{50} values. Genetic background and/or chronic infection with HIV and CMV may contribute to weak or impaired NK cell responses. These results visually depict the extent of overlap in responses between groups, despite not being statistically significant due to the low number of donors in each

group. The antibody dilution producing a half maximal response (EC_{50}) was calculated from raw data for each individual and our results indicated no significant difference in EC_{50} values between groups. This finalized our assessment of NKG2C^{null} NK cells as equivalent in phenotype, cytokine induction, and sensitivity to triggering through CD16 in comparison to their NKG2C-expressing matched counterparts in a CMV-independent assay system setting.

As with most human research, there were limitations to our study. Our analysis focused solely on equivalency in phenotype and general function, however, our findings do not demonstrate equivalency in a CMV-specific assay setting. This could be a relevant issue as CMV-encoded UL40 stabilizes expression of HLA-E on CMV-infected cells which, in concert with NKG2C expression on NK cells, may synergize with CD16 triggering to evoke greater cytotoxic function (ADCC) than observed in our assays. To address this limitation, we are developing a CMV-specific ^{51}Chromium release assay (described in section 4.2), however, further optimization is required for sensitive comparison of CMV-specific NK cell ADCC function between our two study groups.

The participants in our study were recruited through the Newfoundland and Labrador Provincial HIV Clinic and despite a relatively small sample size (n=8 out of 160 donors), this reflects the global distribution of NKG2C^{null} individuals, wherein 5% of individuals carry a homozygous deletion of NKG2C[82]. We emphasize that our study was solely concerned with HIV-infected individuals. The setting of HIV infection made our study more feasible because it amplifies CMV-driven NK cell differentiation compared to HIV-uninfected controls[81]. In other words, HIV infection serves as a magnifying glass by way of exaggerating the expansion of differentiated NK cells for analysis.

In addition, lack of NKG2C has deleterious effects in HIV infection, therefore, an HIV-infected study cohort is logical to investigate the impact of NKG2C absence on NK cell differentiation, especially considering the nearly universal rates of CMV co-infection[90]. Within this specific setting, our study revealed no disadvantage among NKG2C^{null} individuals in terms of CMV-driven NK cell differentiation, suggesting that NK cell differentiation does not underlie the observed advantage of NKG2C-expressing individuals in HIV infection.

Both HIV infection itself and the ART regimens used to treat HIV infection affect general NK cell phenotype and function[95,97,107,110]. For example, in aviremic HIV infection the potency of NK cell-mediated ADCC decreases due to reduced expression of CD16[110]. Additionally, although ART regimens restore CD4^{+} T cell counts and decrease viral replication, NK cell function often does not fully recover[95]. For these reasons, our findings are not necessarily reflective of NK cell differentiation in the absence of HIV infection and a parallel study including HIV-negative donors either supporting or refuting the findings of Liu *et al*. would complement this study.

Despite these limitations, phenotypic and functional equivalency between the NK cells of NKG2C^{null} and NKG2C-expressing donors was demonstrated for multiple measures in CMV-independent assays. This equivalence implies existence of NKG2C-independent routes to CMV-driven NK cell differentiation.

4.2 Future Directions & Significance

The absolute necessity for NKG2C in driving NK cell maturation in response to CMV infection is clearly not supported by our results. Thus, it is unlikely that failure of

NK cells to mature in response to CMV infection is related to the increased sensitivity to HIV infection described for the NKG2C^{null} population, unless NKG2C is somehow directly involved in mediating an anti-HIV effect. Our findings add to the body of knowledge concerning NK cell maturation in CMV infection and may direct research towards uncovering NKG2C-independent routes of maturation. These routes could be harnessed to create NK cells with enhanced function against altered cells in settings of infection and cancer.

In fact, research investigating NKG2C-independent pathways to CMV-driven NK cell differentiation has already begun. Liu *et al*. recently published a study including 60 NKG2C^{null} healthy controls, which reported similar CMV-driven NK cell differentiation over various phenotypic and functional parameters[86]. These findings pressed the discovery of alternate routes to NK cell differentiation, wherein they identified CD2 as a critical receptor in boosting the CD16 signaling cascade among adaptive NK cells[86]. Shortly thereafter, Rolle *et al.* reported that blocking CD2 in co-culture assays severely diminished adaptive NK cell responses[77].

Another receptor that has recently been investigated in this setting is LIR-1, as its expression is reportedly higher on NK cells in CMV infection compared to CMV-negative controls[73]. A CMV-encoded MHC I homolog, UL18, binds LIR-1 with much higher affinity than its natural ligands[111]. Incubation of PBMC with CMV-AD169-infected fibroblasts resulted in heightened pro-inflammatory cytokine responses in comparison to when a virus with UL18 deleted from the viral genome was used[73]. Thus, it appears that interaction between LIR-1 and UL18 results in immune activation in this setting. Makwana *et al.* found that LIR-1^{+} NK cells were the most active subset in terms

of CMV-specific ADCC responses in renal transplant recipients[87]. These results are especially interesting as LIR-1 is an inhibitory receptor. In line with its inhibitory function, but in contrast with the previously mentioned reports, Prod'homme *et al.* claim that UL18 inhibits LIR-1^+ NK cells but activates LIR^- NK cells[112]. A clear consensus has not yet been reached regarding the role of LIR-1 in NK cell differentiation and function.

The effect of UL18 on $CD8^+$ T cell function is also under debate. Most groups report UL18-mediated inhibition of LIR-1^+ $CD8^+$ T cells, although one group reported UL18-mediated activation[111,112,113]. For these reasons, measuring LIR-1 expression on NK cells from CMV-infected $NKG2C^{null}$ individuals could prove interesting as LIR-1 expression is upregulated on NK cells in CMV infection and its function on NK and T cells is disputed. We propose that interactions between UL18 and other immune cells, such as DCs, may be skewing recent reports and leading to these discrepancies.

Activating KIRs have also been proposed as "alternate" drivers of adaptive NK cell expansions in CMV infection. Chiesa *et al.* investigated KIR expression among three $NKG2C^{null}$, CMV-infected donors post-allogeneic hematopoetic stem cell transplant and found preferential expression of activating KIRs among the most rapidly expanded NK cell population during recovery[85]. Beziat *et al.* analyzed 200 donor "KIR-omes" and found preference for the expression of activating KIR2DS4, 2DS2, and 3DS1 among CMV-infected healthy hosts, although this cohort was not NKG2C-deficient[71].

These results are intriguing, but analyzing the role of KIR expression in driving the expansion of adaptive NK cells is incomplete without concurrently genotyping donors for HLA class I in order to identify which NK cells have undergone education. Various

groups have found that CMV infection drives the expansion of inhibitory KIR^+ NK cells in the presence of their cognate HLA class I ligand[71,112].

The reasons for which CD2, LIR-1, and activating or educated KIRs are selectively upregulated among differentiated NK cells in CMV infection remain undetermined. The CMV-specific assay we have begun optimizing measures NK cell-mediated cytotoxicity against ^{51}Cr-labeled, CMV AD169-infected human lung fibroblast (MRC-5) cells after incubation with anti-CMV antibodies. Upon optimization of this assay, the addition of anti-CD2, -LIR-1 and -KIR antibodies in blocking assays might yield interesting results on relative levels of killing observed between groups. Furthermore, phenotyping NK cells post incubation with CMV-infected MRC-5s could offer novel insights into the expression patterns of CD2, LIR-1, and KIRs on responding NK cells among $NKG2C^{null}$ and matched individuals. Most importantly, the interaction between NKG2C and HLA-E is supported in this assay, which could better expose any underlying differences in NK cell function between $NKG2C^{null}$ and NKG2C-expressing donors.

There is reason to believe that differentiated NK cells could be harnessed for the targeted killing of unhealthy cells in various chronic infections. For example, $Fc\varepsilon R1\gamma^-$ NK cells have been associated with improved outcomes in hepatitis C virus (HCV) infections[114]. The observed equivalency in $Fc\varepsilon R1\gamma$ loss among NK cells between groups, along with the fact that we have shown that CMV-negative individuals have considerable (>5%) $Fc\varepsilon R1\gamma^-$ NK cells (data not shown) begs the question of a mechanism behind its loss. This demonstrates that $Fc\varepsilon R1\gamma$ loss can occur independently of CMV infection and independent of NKG2C expression. Various cytokines or stimulation through regulatory

receptors could be potential mechanisms behind this intracellular modification, which could be harnessed to create NK cells with enhanced effector function.

Differentiated NK cells are currently being considered for therapeutic purposes beyond chronic infections[1]. Similar to HIV infection, cellular transformation is also associated with MHC downregulation, which results in T cell evasion, and conversely, in NK cell activation. In recent years, adaptive NK cells have been associated with more effective lysis of cancer cells[115]. Indeed, numerous NK cell-based cancer immunotherapies are currently under investigation[116,117]. One form of NK cell-mediated tumor cell lysis involves ADCC and requires tumor-specific antibodies (mAbs)[118]. Differentiated NK cells have inherently superior ADCC, and could be used in concert with antigen-specific mAbs to improve killing efficacy in a multitude of settings.

No other group has characterized NKG2C^{null} NK cells in the context of CMV/HIV co-infection. Our results contradict the widespread assumption that NKG2C expression is heavily involved in driving NK cell differentiation in CMV infection, as our NKG2C^{null} donors were equivalent to matched controls in phenotype (aim 1) and function (aim 2). NK cells from NKG2C^{null} donors evidently have alternative routes of differentiation and this area of research is currently under exploration. Uncovering alternate routes of CMV-driven NK cell differentiation is critical for understanding novel ways by which we can harness these cells for therapeutic purposes in chronic infections and cancer.

5. References

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