Innate Adaptation: The Influence of Human Cytomegalovirus on Natural Killer Cells

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Abstract

During their lifetime, over half the world's population will experience chronic viral infection or cancer, both of which involve evasion of host immunity. We have within us immune cells called natural killer (NK) cells that constantly survey our tissues to detect and eliminate transformed and infected cells. Thus, they have a critical role in preventing cancer and containing virus infection. Although most viruses only disrupt our lives transiently, if at all, some viruses establish life-long persistent infection. Human cytomegalovirus (HCMV) is a common herpesvirus infecting most of the adult population and although relatively innocuous in healthy individuals, HCMV infection or reactivation has an enormous impact on the human immune system, poses serious health risks to the immunocompromised, and is an important cofactor driving ongoing immune activation in people living with HIV (PLWH). One outcome of HCMV infection is emergence of a stable differentiated population of phenotypically and functionally adapted NK cells exhibiting a form of memory. While mechanisms that create adapted NK cells *in vivo* remain enigmatic, exposure to HCMV is the one common factor underlying their presence. Exploring basic molecular mechanisms governing NK cell-mediated immunity can inform cell-based treatment strategies against virus infection or cancer. As chronic HIV-1 infection amplifies HCMV-driven accumulation of adaptive NK cells, we studied whether NK cell adaptation to HCMV infection functionally impacts their natural

and antibody-dependent cytotoxic functions in this setting. Although factors present during HCMV infection augmented NK cell activity, we found no evidence that NK cells acquire superior cytotoxic function or capacity for interferon-γ secretion in response to target cells following adaptation to HCMV infection. However, HCMV-driven NK cell adaptation in HIV-1 infection paralleled increased expression of TIGIT, an inhibitory immune checkpoint receptor, on NK cells. As chronic virus infection contributes to effector cell dysfunction, punctuated by increased expression of inhibitory immune checkpoint receptors, it is important to unravel the mechanisms by which viruses affect regular NK cell functions to either prevent dysfunction or introduce disease-appropriate mediators to invigorate NK cell responses. Understanding basic molecular mechanisms governing NK cell-mediated immunity will inform new strategies to optimize our immune system capacities.

General Summary

Over the course of our lifetime, we are exposed to many different types of virus infections, and our own healthy cells can transform into cancer cells. Although many viruses only cause transient, readily resolved infection, some viruses, such as human cytomegalovirus (HCMV) and human immunodeficiency virus (HIV)-1, establish life-long persistent infection, promote inflammation and impose great medical and socioeconomic hardship.

Natural killer (NK) cells identify and destroy cancer cells and fight virus infection. Exposure to HCMV causes NK cells to adapt towards specialization for recognizing infected cells coated with antibodies. This thesis explores how the pressures of HCMV and HCMV/HIV-1 co-infection affect adaptive NK cell formation and function, and whether we can harness these adapted NK cells against HIV-1 infection in combination with checkpoint inhibitors commonly used for cancer therapy. Greater insights into the basic mechanisms and biology that drives NK cell adaptation will facilitate development of new strategies for therapeutic targets to control a broad range of infections and malignancies.

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Table of Contents

List of Tables

CHAPTER 3

CHAPTER 4

List of Figures

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

List of Appendices

List of Abbreviations and Symbols

°C	degrees Celsius
%	percentage
α	alpha
Ab	antibody
ADAM	a disintegrin and metalloproteinase
ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	acquired immunodeficiency syndrome
aKIR	activating killer immunoglobulin-like receptor
ATCC	American Type Culture Collection
β	beta
bnAb	broadly neutralizing antibody
BSA	bovine serum albumin
C	cytosine
cART	combination antiretroviral therapy
CBMC	cord blood mononuclear cells
CD	cluster of differentiation
CLR	C-type lectin-like receptor
CMV	cytomegalovirus
cmvIL-10	CMV-encoded hIL-10 homologue
CNS	conserved non-coding sequence
Cr	Chromium
CTLA-4	cytotoxic T lymphocyte-associated protein 4
DC	dendritic cell
DDR	DNA damage response
dH2O	deionised molecular biology grade water
DNAM-1	DNAX accessory molecule-1
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EOMES	eomesodermin
ERK	extracellular signal-related kinases
E:T	effector to target ratio
Fab	antigen binding fragment
Fc	fragment crystallizable region
FcεRIγ (FcRγ)	Fc-receptor common gamma chain
FcγRII	CD32

FcγRIIIa	CD16
FcR	Fc receptor
FCS	fetal calf serum
γ	gamma
g	gram(s)
g	gravitational force
h	hour
HBV	hepatitis B virus
HCl	hydrochloric acid
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hIL-10	human IL-10
hIL-10R	human IL-10 receptor
HIV-1	human immunodeficiency virus type 1
HLA	human leukocyte antigen
IC	isotype control
IF	immunofluorescence
IFAR	interferon alpha receptor
IFN	interferon
Ig	immunoglobulin
iKIR	inhibitory killer immunoglobulin-like receptor
IL	interleukin
ILC	innate lymphoid cell
ILC1	group 1 ILC
IQR	interquartile range
ITAM	immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
IU	international units
K	lysine
k	kilo
kb	kilobase
KIR	killer immunoglobulin-like receptor
L	litre
LAcmvIL-10	latency-associated cmvIL-10
LAG-3	lymphocyte-activation gene 3
LILR	leukocyte immunoglobulin-like receptor
M	molar
mAb	monoclonal antibody
MCMV	murine cytomegalovirus
MFI	mean fluorescence intensity

MHC	major histocompatibility complex
min	minute
mL	millilitre
MMP	matrix metalloproteinase
MOI	multiplicity of infection
n	nano
nAb	neutralizing antibody
NaCl	sodium chloride
$Na_2{}^{51}CrO_4$	sodium chromate
Nef	negative factor
NCR	natural cytotoxicity receptor
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor kappa light chain enhancer of activated B cells
NK	natural killer
NKG	natural killer group
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD-1	programmed cell death protein 1
pH	potential of hydrogen
pDC	plasmacytoid DC
PHA-P	phytohemagglutinin-P
PLWH	people living with HIV
PLZF	promyelocytic leukemia zinc finger
PVR	poliovirus receptor
PVRIG	PVR-related Ig domain
P/S	penicillin/streptomycin
R	arginine
RNA	ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute medium 1640
SAP	SLAM-associated protein
SCID	severe combined immunodeficiency
SD	standard deviation
SEM	standard error of the mean
SIV	simian immunodeficiency virus
SLAM	signaling lymphocytic activation molecule
STAT	signal transducer and activator of transcription
Syk	spleen tyrosine kinase
T	thymine
TACTILE	T cell-activated increased late expression
T-bet	T-box transcription factor
TCR	T cell receptor

T_{FH}	follicular T helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
Tim-3	T cell immunoglobulin mucin-3
TLR	toll-like receptor
TMD	transmembrane domain
TNF-α	tumour necrosis factor alpha
μ	micro (10^{-6})
μL	micro litre
UL	unique long
US	unstructural
UV	ultraviolet
V2	variable loop 2
V_f	final volume
Vpr	viral protein R
Vpu	viral protein U
vSC8	recombinant vaccinia virus expressing β-galactosidase
ζ	zeta
Zap70	zeta-chain-associated protein kinase 70
Zbtb32	zinc finger transcription factor

Co-authorship Statement

I designed and conducted the experiments contained within this thesis with one exception: Supernatant samples from infected cells were analyzed by Luminex by a collaborator and data from this is included in Figure 4.3.3 D. I planned these studies in consultation with Dr. Michael Grant and I analyzed/critically evaluated the data, constructed all figures and images (created with BioRender.com) contained herein and am the original primary author of all material included in this thesis. Dr. Michael Grant revised each manuscript prior to their final publication. Works cited within this thesis include:

Chapter 1: Holder, K. A., Comeau, E. M., Grant, M. D. (2018) Origins of Natural Killer cell Memory: Special Creation or Adaptive Evolution. *Immunology*.

Holder, K. A. and Grant, M. D. (2020) TIGIT Blockade: A Multipronged Approach to Target the HIV Reservoir. *Frontiers in Cellular and Infection Microbiology*.

Chapter 2: Holder, K. A. and Grant, M. D. (2019) Human Cytomegalovirus IL-10 Augments NK cell Cytotoxicity. *Journal of Leukocyte Biology*.

Chapter 3: Holder, K. A., Lajoie, J., Grant, M. D. (2018) Natural Killer cells Adapt to Cytomegalovirus Along a Functionally Static Phenotypic Spectrum in Human Immunodeficiency Virus Infection. *Frontiers in Immunology*.

Chapter 4: Holder, K.A., Burt, K., Grant, M.D. (2021) TIGIT Blockade Enhances NK cell Cytotoxicity Against Autologous HIV-1-infected $CD4^{pos}$ T cells. *Clinical and Translational Immunology*.

1 Introduction and Overview

1.1 Innate Immunity

Archetypical classification of immunity divides our immune system into distinct innate and adaptive arms. Innate immunity encompasses all tissues, particularly barrier surfaces, providing rapid responses to pathogen exposure. To provide protection, both hematopoietic and non-hematopoietic cells can be activated after sensing host damage through germline-encoded receptors [1]. The generic response mounted by the innate immune arm juxtaposes the highly specific and exquisitely diverse collection of antigen-specific adaptive immune cells that retain lasting memory to previous infection [1, 2].

1.1.1 An Innate Effector, Natural Killer

Hematopoietic-derived innate immunity is diverse and encompasses both myeloid cells and innate lymphoid cells (ILCs) [3]. The positioning of ILCs in peripheral tissue and at barrier surfaces gives strategic advantage for innate defenses against pathogen exposure. Arising from common precursor bone marrow-derived leukocytes, ILCs are further divided into three defined subsets, categorized based on their expression of key transcription factors, cytokines and functional profiles [4]. Classified as interferon (IFN)-γ-producing group 1 ILCs (ILC1), natural killer (NK) cells express transcription factors eomesodermin (EOMES) and T-box transcription

factor (T-bet), orchestrate immune responses using regulatory cytokines and chemokines, and bear cytolytic capabilities [5].

Human NK cells typically constitute about 5 – 20% of peripheral blood mononuclear cells (PBMC) and are abundant in the bone marrow, liver, uterus, spleen, kidney, lung and secondary lymphoid tissues [6-8]. These potent effector lymphocytes use multiple mechanisms to swiftly respond to foreign or otherwise anomalous stimuli and are fundamental to our protection against bacteria, fungi and viruses [9-17]. Notably, their contribution to control of virus infection is highlighted by severe and often recurrent herpes simplex virus, varicella virus and papillomavirus infections in individuals deficient in NK cell numbers [11, 13, 18, 19].

1.2 Dynamics of Natural Killer cell Reactivity

1.2.1 'Missing Self' Recognition

The ability of NK cells to recognize infected cells and distinguish healthy 'self' from anomalous or 'missing-self' is enabled by integrating composite signals received from a diverse collection of stimulatory and inhibitory receptors and adhesion molecules [20, 21]. Rather than being dominated by a single antigen-specific receptor, NK cell activation is decided by the constellation of both stimulatory and inhibitory receptors recognizing cognate ligands expressed by the target cell. The meticulous regulation of NK cell recognition provides highly specific

responses using otherwise generalized pattern receptors to effectively discriminate differentially expressed cell surface ligands arising from infection or transformation.

NK cells have multiple detection systems to sense aberrant stimuli that offer flexibility and variety in their responses. NK cell functions are controlled by inhibitory receptors that recognize and bind major histocompatibility complex class I (MHC-I), expressed abundantly on normal cells and tissues to serve as markers of self. In humans, the three types of receptors that recognize human MHC or human leukocyte antigen (HLA), are receptors containing killer immunoglobulin (Ig)-like receptor (KIR) domains, leukocyte Ig-like receptor (LILR) and receptors with C-type lectin heterodimeric domains [22, 23]. By gauging minor alterations in MHC-I expression levels, NK cells can differentiate healthy self (abundant MHC-I) from missing-self (lowered/missing MHC-I) and dispatch unhealthy cells [20, 24]. These interactions, however, are also key requirements for the development of fully functional mature NK cells [25-28]. During maturation, NK cells are 'educated' to ensure they are tolerant to healthy peripheral cells and tissues, thereby preventing NK cell autoreactivity [29].

1.2.2 Self-Educated

NK cell education, often referred to as 'licensing' or 'arming', is a process whereby functionally immature NK cells mature predominantly through *cis* and *trans* interactions between HLA-I and NK cell-expressed receptors encoded within

the human *KIR* gene cluster on chromosome 19 [30-32]. This gene cluster encodes 14 polymorphic *KIR* genes (and two pseudogenes) to transduce signals through receptors with short (S) or long (L) cytoplasmic domains that activate (2DS1-5 and 3DS1) or inhibit (2DL1-3, 2DL5 and 3DL1-3) NK cell functions (Figure 1.1) [33-35]. With a long cytoplasmic domain, KIR2DL4 is the exception having the capacity to inhibit functions through an immunoreceptor tyrosine-based inhibitory motif (ITIM) or activate NK cells via signal relay through Fc-receptor common gamma chain (FcεRIγ or FcRγ) adaptor molecule which interacts with a charged asparagine residue in the same transmembrane domain (Figure 1.1) [36-40].

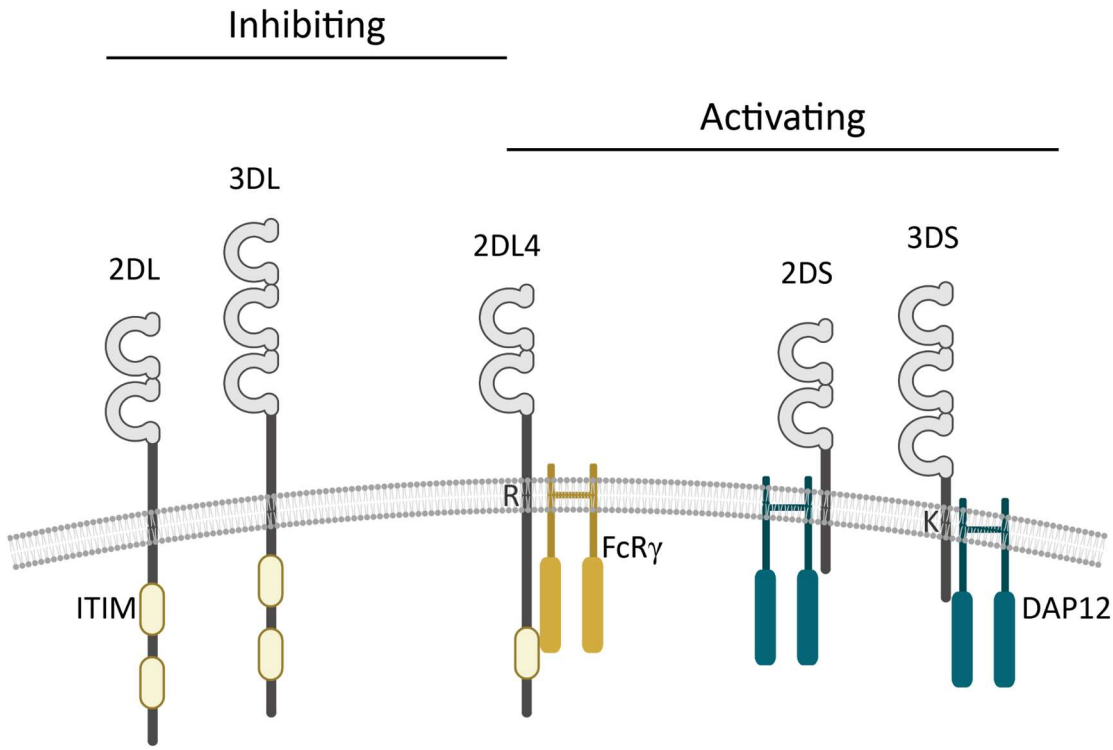

Figure 1.1| Differences between the KIR family members of receptors

The nomenclature for KIR receptors is based on the number of extracellular Ig domains (2D or 3D) and the length of the intracellular signaling domain. Generally, receptors with long (L) cytoplasmic domains contain at least one ITIM motif (sallow) and inhibit NK cell functions. Short (S) domains contain a basic amino acid residue (asparagine [R] or lysine [K]) that interacts with acidic residues in stimulatory FcRγ (mustard) and DAP12 (teal) adaptor subunits containing intracellular immunoreceptor tyrosine-based activator motif sequences (ITAMs). KIR2DL4 has a long cytoplasmic domain that contains an ITIM and a charged amino acid residue that can attract activating adaptor subunits.

As with HLA, KIR expression patterns are highly diverse. The extremely variable interindividual expression of *KIR* is determined by gene number, type, polymorphism and epigenetic regulation of its transcription [41, 42]. Stable NK cell inhibitory KIR (iKIR) expression depends on interactions between cognate HLA class I (HLA-I) complexes. These two loci are not genetically linked, thus, NK cells

may ‘audition’ the iKIR candidates for their HLA-I counterpart, expressing various iKIRs until an interaction with sufficient affinity to HLA-I occurs [43-47]. The combinatorial diversity of KIR and its dependence on instruction from ligands for its expression contribute to the broad pool of NK cells exhibiting varying degrees of functional specificities within each individual [48]. This diverse collection of NK cell clones expressing variegated combinations of inhibitory receptors is vital for rapid identification of differentially expressed class I HLA.

Typical NK cell education requires at least one self-recognizing inhibitory receptor and can include either KIR or the C-type lectin-like receptor (CLR) NKG2A [23, 28, 49]. NK cells that do not express at least one educating inhibitory receptor that binds HLA has the potential for auto-aggression. These NK cells have a very high threshold for reactivity due to the absence or downregulation of stimulatory pathways and circulate hyporesponsive under steady-state conditions [25, 27, 28]. Although seemingly paradoxical, the expression of educating inhibitory receptors recognizing self HLA is fundamental for generating highly cytotoxic NK cells. Strong inhibitory signals lower the threshold of NK cell reactivity, producing more robust effector NK cells [50, 51].

Educating NK cells to recognize and tolerate self is a quantitative process. NK cell function is not simply binary ‘on’ or ‘off’ but is tuned for reactivity. This rheostat model of NK cell education adjusts NK cell responses up or down quantitatively depending on frequency and strength of inhibitory receptor engagement with HLA

[50-52]. Although NK cells may stop auditioning inhibitory receptors once sufficient interaction with self HLA-I is found, NK cells can express two or more types of educating inhibitory receptors and respond more frequently and stronger to contextual stimuli compared with those expressing only one type of educating receptor [44, 53, 54]. While primary education gives NK cells license to actively survey tissues and organs, their education is not fixed. NK cells can receive further education as their environment dictates in order to fine tune their functional responses against broad ranges of malignant or pathogen-infected cells [50, 55]. Expression of self-inhibitory ligands educate NK cells to recognize missing self and tightly regulate their responses, however, co-expression of ligands that engage stimulatory receptors is imperative for effector responses.

1.2.3 Activating Receptors

NK cell stimulation occurs when interactions between distinct cellular and/or foreign ligands and germline-encoded activating receptors exceed inhibitory thresholds. Ligands can include self, altered and induced self (peptide-bound HLA complexes and stress ligands), virus-encoded proteins and cell-bound IgG antibodies [21, 56, 57]. Receptors recognizing these ligands include activating KIRs (aKIRs), CD16 (FcγRIIIa), activating receptors from the signaling lymphocytic activation molecule (SLAM) family, natural cytotoxicity receptors (NCRs), which include NKp46 (NCR1 or CD335), NKp44 (NCR2 or CD336) and NKp30 (NCR3 or

CD337), CLRs (NKG2D and heterodimeric CD94:NKG2C/E/H), and nectin/nectin-like binding family of receptors (DNAM-1 or CD226) [58-60]. Unlike B cells and T cells, NK cells express not one dominant stimulating receptor, but a diverse collection. Lacking signaling motifs, activating receptors with short cytoplasmic stalk domains necessitate the recruitment and association with signaling adaptor proteins, further adding to the regulatory layers controlling NK cell functions.

CD16 and NCRs utilize adaptor proteins that are constitutively expressed by mature NK cells. These include: (i) FcRγ; (ii) CD3ζ disulfide-bonded hetero- or homodimers; and (iii) DAP12 disulfide-bonded homodimers [61]. Each adaptor molecule contains ITAMs with FcRγ and DAP12 containing a single ITAM per chain and CD3ζ possessing three tyrosine-containing residues that upon phosphorylation, provide binding sites for Syk and/or Zap70 tyrosine kinases to trigger downstream signaling cascades [61]. Other activating receptors interact with alternative signaling adaptor proteins initiating downstream signaling cascades through tyrosine kinases. For instance, NKG2D homodimers interact with two DAP10 homodimers to form a hexamer and recruit phosphatidylinositol-3 kinase (PI3K) to the phosphorylated tyrosine in its conserved signaling motif (YINM) [62, 63]. The SLAM member, 2B4 (CD244), recruits tyrosine kinase Fyn to its SLAM-associated protein (SAP) adaptor molecules [61], whereas DNAX accessory molecule-1 (DNAM-1) signals via Grb2 coupling with its immunoreceptor tyrosine tail (ITT)-like motif [64]. Common convergent pathways from signals received from divergent

activating receptors provide complementary phosphorylation and synergistic NK cell activation [65, 66].

1.2.4 Receptor Pairs

NK cells use an integrated model of signaling, transmitting net signals to decide the fate of target cells. Together with soluble factors, a wide array of inhibitory receptors and activating/costimulatory receptors regulate NK cell function. Adding to their functional complexity, NK cells express paired receptors. These are groups of receptors that recognize the same ligands but can have diverse affinities and opposing functional consequences [67]. This includes the CLRs CD94:NKG2A and CD94:NKG2C (recognizing nonclassical HLA-E), discussed in more detail in Section 1.3.4 in the context of human cytomegalovirus (HCMV) infection, the KIR family (recognizing HLA-I), discussed above, and the family of nectin and nectin-like receptors, which are introduced here and expanded upon further in the context of human immunodeficiency virus type 1 (HIV-1) infection in Section 1.5.

While some receptor pairs have evolved together, other groups include receptors that are not genetically linked. DNAM-1 belongs to a larger family of nectin and nectin-like receptors that all recognize the same group of ligands yet are genetically distal [68, 69]. Its main counter-receptor, T cell immunoreceptor with Ig and ITIM domains (TIGIT), is expressed on NK cells and multiple T cell subsets [70-

75]. After interaction with either of its shared ligands, poliovirus receptor (PVR or CD155 or Necl-5) or PVRL2 (CD112 or nectin-2), TIGIT inhibits activation of T cell or NK cell effector functions [71, 72, 76]. Like TIGIT, TACTILE (CD96) and PVR-related Ig domain (PVRIG or CD112R) bind PVR and PVRL2, respectively, whereas the costimulatory DNAM-1 (CD226) competes with both TIGIT and TACTILE for PVR engagement and with PVRIG for PVRL2 binding (Figure 1.2) [77-81].

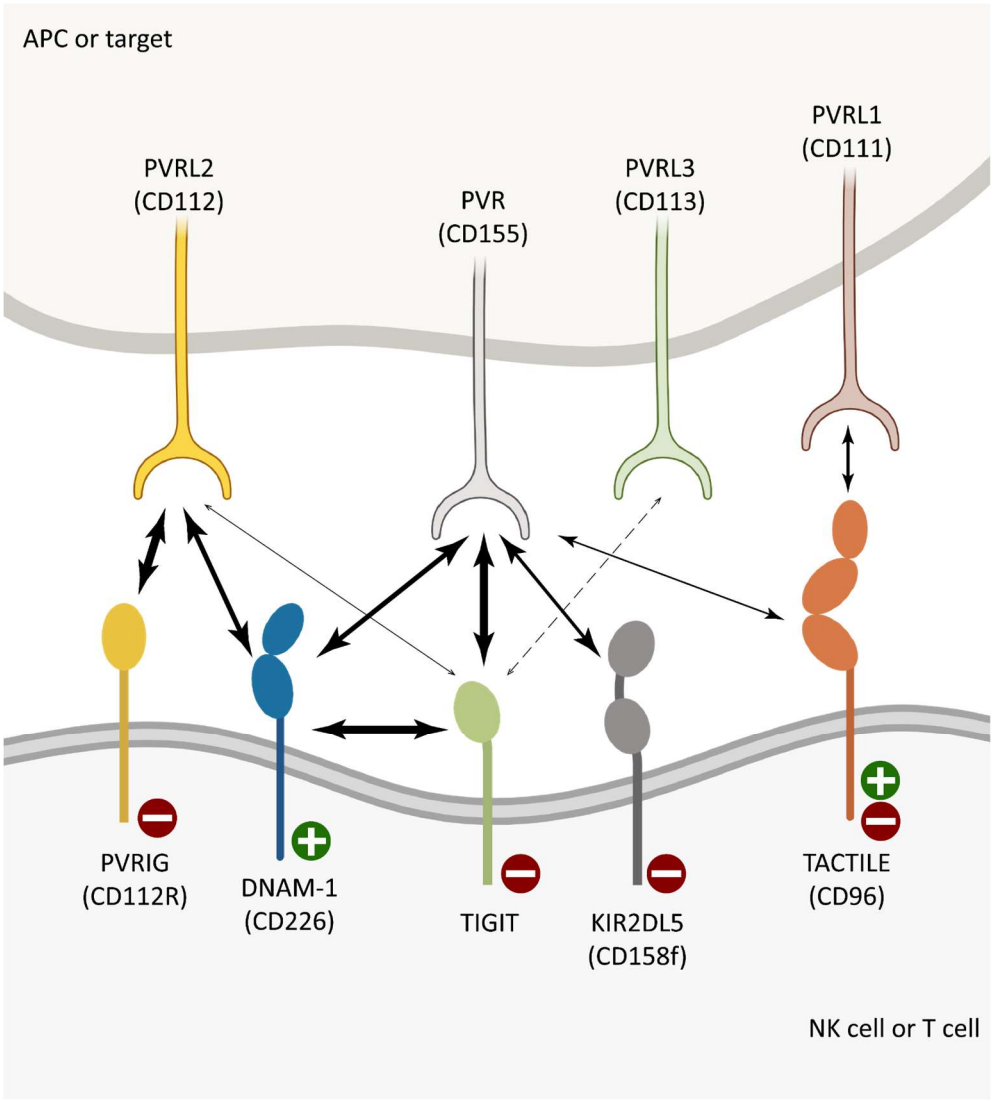

Figure 1.2| The TIGIT/DNAM-1 immune checkpoint axis

Interactions between inhibiting (⊖) and activating (⊕) T cell or NK cell receptors belonging to the nectin or nectin-like family of receptors and their corresponding family of ligands are depicted. Strong interactions such as those between TIGIT and PVR or DNAM-1 in *cis* or PVRIG and PVRL2 are illustrated with heavy arrows. There is no clear consensus regarding whether TIGIT binds PVRL3 (dotted arrow) and it is unclear whether TIGIT/PVRL2 interactions are physiologically relevant *in vivo* [71, 72, 82]. DNAM-1 interacts with both PVR and PVRL2 to counter inhibition, yet does so with lower affinity than either TIGIT or PVRIG. TACTILE preferentially interacts with PVRL1 over PVR [83]. The affinity of KIR2DL5 for PVR binding is currently unknown, as is whether any other nectin or nectin-like ligand or receptor can serve as its binding partner.

The inhibitory PVRIG receptor is expressed on activated NK cells (Figure 1.2), however, there is a lack of conclusive evidence in human NK cell studies as to whether TACTILE negatively or positively regulates activation [82, 84, 85]. Although PVR is a common ligand for TIGIT, TACTILE and DNAM-1, the binding affinities vastly differ, with TIGIT having a greater affinity for PVR than either DNAM-1 or TACTILE (Figure 1.2) [71]. This domination TIGIT has over DNAM-1 for ligand binding favours effector cell inhibition over effector cell costimulation, thereby dampening immune responses. Another means by which TIGIT controls NK cell activation is by interfering with DNAM-1 homodimerization by forming a heterodimer with DNAM-1 in *cis* (Figure 1.2) [86]. The intracellular TIGIT:DNAM-1 complex prevents effective intercellular DNAM-1/ligand interactions to reduce effector cell costimulation.

In 2019, the inhibitory KIR2DL5 receptor expressed on NK cells and CD8 T cells, was identified as a binding partner for PVR, adding another facet to this already complex regulatory pathway [87, 88]. The genes encoding KIR2DL5 (*KIR2DL5A* and *KIR2DL5*) are highly polymorphic [89, 90]. Less than 10% of $CD56^{dim}$ NK cells and a very small fraction of the $CD8^{pos}$ T cells of carriers express the most common allele, 2DL5A*001, which is detectable by monoclonal antibody (mAb) UP-R1 [87, 91]. An accurate measure of KIR2DL5 prevalence in the wider population is currently unavailable as it is unknown whether this is the only allele expressed or whether polymorphisms arising in other alleles alter epitopes recognized by UP-R1 [91, 92].

1.2.5 Effector Functions

Endowed with a finely tuned arsenal of inhibiting and activating receptors, NK cells are trained to provide protective immunity. There are two ways by which NK cells carry out their effector functions. NK cells are predominantly cytotoxic mediators and lyse irregular or infected cells by either directed release of secretory lysosomes containing perforin and granzyme or by death receptor recognition and induction of programmed cell death in the target cell [93, 94]. Both methods result in caspase cleavage and ordered cell death and are highly regulated to prevent bystander cell death. Secondly, NK cells can produce a wide array of immune modulating cytokines, including proinflammatory cytokines, in response to either receptor-ligand based activation or cytokine-induced activation [95-98]. This response regulates NK cell functions in *cis* and the functions of other innate and adaptive immune cells in *trans*. The NK cell response to cytokine stimulation is discussed in the context of HCMV infection in Sections 1.3.1 and 1.3.2.

NK cell activation is determined by the stimuli encountered, with each unique agonist having the potential to create a slightly different response. Multiple detection systems for sensing pathogens and tuned responses by use of overlapping intracellular signaling proteins and pathways give NK cells advantage with flexibility in the way they recognize and respond to ever-evolving strategies that viruses, bacteria and cancer employ to escape their recognition.

NK cells are endowed with a vast array of receptors, undergo an education process to ensure tolerance to self-tissue and give rise to heightened effector functions, and elicit robust responses through receptor- or cytokine-mediated stimulation. Given their plastic nature and propensity to hone their effector functions based on the ligands and cytokines to which they are exposed, the question arises as to whether our immune system can improve upon its natural killers. In the ideal environment, would an innate immune cell be receptive to further 'training' for better host immunity, unleashing their full breadth of immune cell functions. In short, can and do NK cells adapt?

1.3 Natural Killer cells Share Adaptive Immune Features

Our perception of NK cells has transformed dramatically since their identification in the 1970s [99-103]. In the mid-2000s, new research upended conventional understanding of NK cell biology when they were identified as being a highly diverse lymphocyte population with a capacity for immunological memory, a feature predominately associated with adaptive immunity [104]. Similar to the responses of B and T lymphocytes, the NK cell response to CMV and engagement in an immune response reshapes the baseline repertoire, giving rise to an adapted population with distinct phenotypic and functional features [105-107]. Although they share characteristics similar to memory B cells and T cells, NK cells adapt independent of antigen recognition and recombination events but rather through

somatically generated clonotypic receptors [2, 21]. However, selection mechanisms underlying dynamic remodeling of NK cell repertoires and NK cell maturation into memory cells remain mostly obscure. This section will address accumulating evidence for antigen-independent and -dependent NK cell maturation and memory formation including cytokine-induced memory, the NK cell response to murine (M)CMV and a description of human memory-like NK cell development in response to HCMV.

1.3.1 Cytokines are Essential for Adaptation

Factors fundamental for generating adapted NK cells arise during early CMV infection and likely precede physical interactions between receptor-ligand pairs. During this time, NK cells are primed by pro-inflammatory cytokines such as type I IFNs (IFN-α/β) and interleukin (IL)-12. These cytokines, initially produced by myeloid cells in response to CMV infection, stimulate NK cell antiviral cytokine production, induce their activation and initiate non-specific NK cell proliferation [108-110]. Interferon-α/β-induced IL-15 is also thought to contribute to non-specific NK cell proliferation during the early stages of CMV infection, providing stimulation critical to NK cell survival [95, 111]. Interactions between cytokines and their receptors encourage NK cell adaptation to CMV by transmitting signals through signal transducer and activator of transcription (STAT) mediators.

Engaging IFN-α and IL-12 receptors stimulates STAT1 and STAT4, crucial regulators of NK cell IFN-γ expression and cytolytic activity [112-116].

Murine models have provided detailed information on the role of cytokine-priming, accessory interactions and signaling pathways underlying formation of antigen-specific NK cell memory populations (Section 1.3.3). The Yokoyama lab initially described the prolonged effects of IL-12 on NK cell activation, however, the central role for IL-12 and NK cell memory was definitively illustrated by the inability of NK cells from IL-12R$^{k/o}$ mice to expand and provide protection against MCMV challenge [117-119]. IL-12 modulates NK cell adaptation, enhances NK cell responsiveness and causes inheritable modifications in NK cell progeny [119, 120]. Exposure to IFN-α/β and IL-12 during the later stages of NK cell adaptation also enables the proliferative burst observed after MCMV challenge, which is incited by a cytokine-specific increase in the expression of a zinc finger transcription factor (Zbtb32) [121, 122]. This early generic NK cell response to cytokines may be critical for priming NK cells to respond more effectively to virus-specific interactions during subsequent stages of CMV infection.

1.3.2 Cytokine-Dependent NK cell Memory

Cytokines provide useful stimulation for NK cell priming during CMV infection and subsequent proliferation and survival. However, NK cells have the ability to manifest a form of immunological memory simply from exposure to pro-

inflammatory cytokines in the absence of virus, hapten or other specific receptor/ligand engagement. The first indication that cytokines invoke NK cell adaptation followed an adoptive transfer study of murine splenic NK cells that were briefly activated *in vitro* with IL-12, IL-15 and IL-18 before administration to naïve recipients [119]. Cytokine-primed NK cells proliferated in their new host and generated a stable population with augmented IFN-γ responses upon re-exposure to IL-12, IL-15 and IL-18, or upon activating receptor engagement [119]. Heightened IFN-γ responses were maintained up to 12 weeks, and cytokine-induced NK cell memory was demonstrable as a heritable property, passed to daughter populations with no previous exposure to cytokines *in vitro* [119, 120]. Similar effects were noted with human NK cells, where *ex vivo* IL-12, IL-15 and IL-18 restimulation induced robust IFN-γ production in NK cells previously exposed to the same cytokine milieu *in vitro* [123].

It remains to be established whether the cytokine-induced alterations to NK cell functions are as durable as in HCMV-driven NK cell adaptation. Another intriguing question is whether immunomodulatory cytokines produced during viral infection contribute to *in vivo* NK cell adaptation. Commonly produced cytokines, such as IFN-α/β, or in the case of HCMV, a viral homologue to IL-10, could act in concert with signaling through various NK cell receptors to promote adaptation.

1.3.3 NK cell Adaptation to Murine Cytomegalovirus

The response against MCMV is the first and best characterized example of antigen-driven activation and maturation of NK cells into memory cells [124]. The MCMV model clearly illustrates the two key aspects of immunological memory previously attributed exclusively to lymphocytes bearing clonotypic receptors: selective expansion of NK cells bearing antigen-specific receptors, and more efficient secondary responses against the same antigen. This model draws parallels to T cell memory development, extending from the need for costimulatory receptor engagement and cytokine stimulation to the critical contraction phase following antigen-driven NK cell expansion.

Classical genetics mapped MCMV resistance to the Ly49H activating receptor on murine NK cells [125]. This receptor interacts with MCMV-encoded m157 glycoprotein to stimulate expansion of Ly49H-bearing NK cells that help control primary infection and provide enhanced protection against subsequent challenge (Figure 1.3) [125-127]. The key feature in murine NK cell memory responses to MCMV infection is a specific physical interaction between the MCMV m157 glycoprotein and Ly49H activating receptors that triggers selective proliferation and differentiation into a long-lived memory cell population with enhanced protective capacity [126-129]. Mouse NK cells lacking Ly49H fail to proliferate in response to MCMV during later stages of infection [130]. The interaction between Ly49H and m157 induces a 2-3-fold increase in NK cell numbers during the first week after

MCMV infection and this expansion remains detectable at least 70 days post infection [124, 130]. NK cell responses against MCMV display the key defining features of adaptive immune memory exhibited by B and T lymphocytes.

Antigen-specific NK cell expansion initiated by m157:Ly49H interactions relies on directed signaling through adaptor proteins (Figure 1.3) [131]. Although Ly49H receptors transmit signals through either DAP10 or DAP12, Orr *et al*. showed that signaling through both adaptor proteins is necessary for optimal NK cell adaptation [131]. In the absence of either DAP10 or DAP12 expression, NK cells still mediate resistance to MCMV infection, however, deleting either of these two proteins reduces surface Ly49H expression, NK cell proliferation and IFN-γ production in response to MCMV [131]. Signal transmission through either of these adaptor proteins after m157:Ly49H interactions may represent a redundancy mechanism whereby adequate memory-like NK cell responses are maintained in the absence of either DAP10 or DAP12 expression. However, if both DAP10 and DAP12 are absent, Ly49H expression is completely suppressed and mice are susceptible to MCMV challenge [131].

Analogous to the activation of naive B and T cells, costimulatory receptor engagement is a necessary component of NK cell adaptation. Upon infection, MCMV induces PVR and/or CD112 expression on monocytes or DCs (Figure 1.3) [132]. Engagement of NK cell-expressed DNAM-1 by CD112 and/or PVR drives signal transduction and promotes NK cell differentiation after initial and subsequent

MCMV challenge [133]. This process generates a pool of memory NK cells expressing high levels of maturation markers (CD11b, Ly6C, and KLRG1) and of Ly49H [133]. It is unknown whether other costimulatory molecules are involved in the activation, proliferation, or persistence of MCMV-specific memory NK cells.

During the first week of MCMV infection, NK cells are exposed to non-specific cytokines, viral proteins or virus-induced host ligands that support NK cell proliferation and expansion. Following activation and differentiation towards enhanced effector function, the next phase of memory NK cell generation begins as the NK cell pool contracts into an elite collection of highly functional mature NK cells [134]. The cells that survive persist as the long-lived memory NK cells that proliferate, expand and contract in response to repeated MCMV challenge and provide greater protection against successive MCMV infection than their naïve counterparts [124]. This model has provided detailed information on the role of cytokine-priming, accessory interactions and signaling pathways underlying formation of antigen-specific NK cell memory populations, ultimately broadening our perspective on immunological memory to include cells restricted to recognition of antigens with germline receptors.

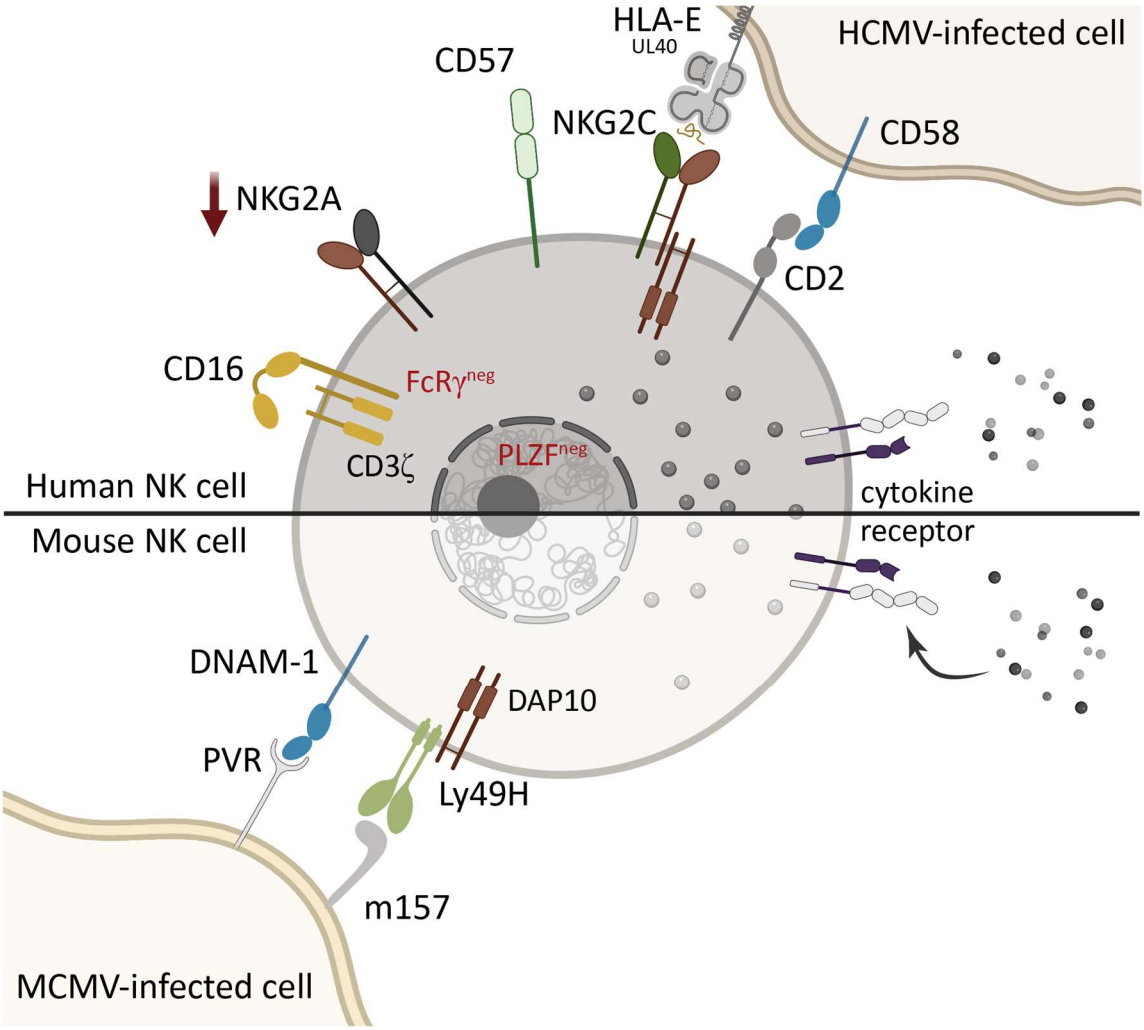

Figure 1.3| Adaptation of NK cells in response to CMV infection

NK cell memory for MCMV (bottom panel) is generated in response to interactions between MCMV-encoded m157 protein and the activating Ly49H NK cell receptor. The 'secondary signal' transmitted by DNAM-1 interactions with cognate ligands (PVR depicted here) and also cytokine (IL-12 and type I IFN) signaling are critical requirements for NK cell differentiation and proliferation. Individuals seropositive for HCMV (top panel) have increased fractions of circulating NK cells expressing CD57 and NKG2C with reduced levels of natural cytotoxicity receptors (not shown). Increased NKG2C expression on adapted NK cells is paired with the loss of NKG2A. The leader sequence of HCMV-encoded UL40 stabilizes HLA-E to favour NKG2C interactions. NK cells adapted to HCMV can be further identified by the loss of promyelocytic leukemia zinc finger protein (PLZF) transcription factor and the FcRγ signaling adaptor subunit (replaced with CD3ζ).

1.3.4 NK cell Adaptation to Human Cytomegalovirus

Diversity within NK cells is thought to have arisen in part by pressure from what has been described as an evolving 'arms race' between pathogens and the host immune system [135]. Despite evidence that NK cells control multiple types of herpesvirus infections, only HCMV infection is known to have a dramatic effect on the composition of the NK cell repertoire [13]. This is true in the case of both MCMV and HCMV, but despite superficial similarities, the operative mechanism for NK cell adaptation to murine and human CMV appears mostly unrelated.

In 2004, Guma *et al*. first reported that infection with HCMV leaves a durable imprint on the human NK cell repertoire [106]. This imprint is reflected in an increased frequency of NK cells expressing CD57 and activating receptor NKG2C, together with high levels of CD16 (Figure 1.3). Expression of activating and self-specific KIRs is higher on these NK cells, while levels of NCRs and NKG2A are reduced [106, 136-142]. There is substantial overlap between the population of NK cells co-expressing CD57 and NKG2C with NK cells that have reduced expression of the FcRγ signaling protein, Syk kinase, PLZF transcription factor and Ewing's sarcoma's/FLI-1 activated transcript-2 (EAT-2) [143, 144]. While accumulation of these cells was initially associated with several other viral infections, it later became clear that HCMV infection is the one constant required for marked expansion of $CD57^{pos}$ NK cells expressing NKG2C [107, 140, 141]. This association between selective expansion of NK cells bearing the activating receptor NKG2C and HCMV

infection inspired consideration that NKG2C might be analogous to Ly49H with its specific recognition of an HCMV protein or peptide driving expansion of NKG2C^{pos} NK cells (Figure 1.3). Lending credence to this idea, in some *in vitro* HCMV infection/NK cell co-culture systems, CD57^{pos}NKG2C^{pos} NK cell expansion is blocked by antibodies against either HLA-E or NKG2C [145]. Both NKG2C and its inhibitory counterpart, NKG2A, bind to HLA-E, which in turn is modulated by HCMV infection [146]. Expression of HLA-E is generally stabilized by peptides derived from class I molecules and its function as an inhibitory or activating ligand for NKG2A or NKG2C receptors, respectively, is critically dependent on the peptide presented (Figure 1.3) [147-150]. Although peptides derived from HCMV UL40 protein (*i.e.,* VMAPRTLIL) bind and stabilize HLA-E, expression of HCMV UL40 is dispensable for NKG2C^{pos} NK cell expansion *in vitro* (Figure 1.3) [136, 151, 152]. In contrast, the US2-US11 genes crucially contribute to *in vitro* NKG2C-driven NK cell expansion, indicating a role for class I HLA molecules [136, 153].

The largely NKG2C^{pos} phenotype of adapted NK cells in HCMV infection supports the possibility that NKG2C plays a direct role. However, in co-culture systems without exogenous cytokines, NK cells from HCMV-infected individuals selectively proliferate and release IFN-γ in response to HCMV-infected fibroblasts with little apparent role for NKG2C [154]. In addition, NK cells from individuals lacking the NKG2C gene respond similarly to HCMV infection with accelerated maturation, and co-express aKIRs (KIR2DS1, KIR2DS2, KIR2DS4 and KIR3DS1) to

a similar extent as NK cells from NKG2C^{pos} donors [155]. Therefore, at the very least, NKG2C-mediated interactions are not the only driving force behind the impact of HCMV on the NK cell repertoire [156]. An extensive study of adaptive NK cell responses in NKG2C-bearing and NKG2C^{null} individuals suggests CD2 costimulation may be a critical component in effector potency, irrespective of NKG2C expression [156]. Paralleling the 'second signal' or DNAM-1 co-activation of adaptive murine NK cells, human CD2 on NK cells interacts with CD58 on target cells (Figure 1.3) [157]. Increased CD2 expression on *in vitro*-expanded adaptive NK cells favours increased IFN-γ and TNF-α production and is critical for robust antibody-dependent responses [156, 157]. However, the extent to which CD2 engagement contributes to adaptive NK cell formation *in vivo*, or whether other ligands for CD2 provoke the same response, remains unresolved.

1.3.5 Functional Implications of Adapted NK cells

Intracellular mechanisms accompanying NK cell adaptation to HCMV infection became clearer with the identification of NK cells deficient for FcRγ, as emergence of adaptive NK cells in HCMV-infected individuals is closely associated with loss of this transmembrane signaling adaptor protein (Figure 1.3) [158, 159]. While CD57 and NKG2C are mostly co-expressed on adapted NK cells, some FcRγ^{neg} NK cells lack NKG2C and retain low levels of NKG2A [144, 159]. The FcRγ adaptor protein associates with NKp30, NKp46 and/or CD16 receptors as a homodimer or

heterodimer with CD3ζ to signal through ITAMs [61, 160]. Loss of NK cell FcRγ expression in response to HCMV parallels development of adaptive NK cells with low levels of NKp30 and NKp46 and impaired cytokine and cytotoxic responses through NCRs [158, 159]. In contrast, the loss of FcRγ has limited impact on CD16 expression and some data associate this loss with broadly enhanced antibody-dependent cytokine production and cytotoxic potential [159]. Lee *et al*. and Schlums *et al*. independently showed that HCMV-dependent loss of NK cell FcRγ relates to decreased expression of the PLZF transcription factor and DAB2 [143, 144]. Loss of PLZF, in concert with epigenetic hypermethylation of the FcRγ, Syk and EAT-2 promoter regions to which PLZF binds, reduces FcRγ, and in some instances Syk and EAT-2 transcription, producing a pattern that reliably identifies adaptive NK cells (Figure 1.3) [143, 144]. The differential methylation pattern observed in adaptive NK cells represents a shift from the epigenetic profile of conventional NK cells towards that of $CD8^{pos}$ cytotoxic T cells [143].

Ancillary to the epigenetic modifications that create a population of NK cells purportedly more capable for ADCC, partial demethylation of the *IFNG* locus gives rise to daughter cells with heritable DNA modifications and an enhanced capacity for IFN-γ production [161, 162]. Epigenetic modifications of both the IFNG promoter and conserved non-coding sequence (CNS)-1, located 4 kbp upstream of the human IFNG promoter, fixes $NKG2C^{hi}$ NK cells with strong and stable IFN-γ responses [161-164]. During NK cell differentiation, hypomethylated CNS-1

encourages binding of T-bet, STAT4, NF-κB and NFAT to enhance downstream *IFNG* transcription after stimulation through activating NK cell receptors, particularly NKG2C [162-165]. The epigenetic imprint that HCMV leaves on the NK cell repertoire contributes to a highly specialized collective readily able to produce IFN-γ in response to appropriate stimuli.

While NKG2C is an effective surrogate marker for NK cell adaptation to HCMV infection, its role in either *in vivo* selection or protection against HCMV has not been confirmed. In fact, there is only one reported example of a T cell-deficient child where HCMV replication was reduced *in vivo* coincident with the emergence of a predominantly NKG2C^{pos} NK cell subset with high CD16 expression [166]. The most notable functional feature attributed to the NK cells responding to HCMV infection is an enhanced capacity for antibody-dependent activation [159]. However, effective suppression of HCMV by the adapted NK cell population through antibody-dependent or other mechanisms has not yet been proven. Many individuals infected with HCMV do not have large CD57^{pos}NKG2C^{pos} NK cell populations and low numbers of these cells is not associated with reduced containment [106, 144, 155, 156, 167]. Expression of NK cell receptors, adaptor subunits and transcription factors vary considerably in HCMV-seropositive individuals with high frequency of CD57^{pos}NKG2C^{pos} NK cells, suggesting that shaping the NK cell response to HCMV involves many factors. Whether CD16, NKG2C, activating or inhibiting KIR, the absence of inhibitory receptors such as

NKG2A, epigenetic remodeling, or all the above endow NK cells with a selective advantage for expansion and persistence in HCMV-infected individuals remains to be clarified.

In the absence of demonstrable specific interactions between activating NK cell receptors and HCMV proteins, the question remains as to why HCMV has such a dramatic effect on the NK cell repertoire. The consistency and robustness of the NK cell response to HCMV suggests either a strong adaptive advantage to the host or that HCMV has evolved to shape the NK cell repertoire to its own advantage.

1.3.6 An Eternal Experience with HCMV

Codivergence of herpesviruses and our ancient ancestors for millennia influences both host and virus evolution [168-170]. The HCMV β-herpesvirus is one of the world's most ubiquitous viral infections with seroprevalence (circulating IgG antibodies) ranging from 45 – 100% depending on age, geographic location, and socioeconomic status [169, 171]. Its vast genome makes HCMV one of the largest and most complex viruses to infect humans, with more than 80% of its gene products being dispensable for replication [172].

Latency and reactivation are common features of all herpesviruses. Through the nature of its latent reservoir and mode of reactivation, HCMV may disseminate more broadly than other herpesviruses and adopt a wider cellular host range, greater variety of infection states and clinical manifestation [173-177]. Inflammation and

persistent antigen stimulation promote HCMV reactivation, thus, it has evolved many strategies to support the chronic cycling of immune activation and inflammation in favour of its replication. HCMV modulates immune inflammation by inducing host cytokines and chemokines (*e.g.* IL-1β, IL-6, IL-8, IFN-α/β, CCL5 and CXCL14) and expressing virus-encoded cytokine and chemokine homologues (*e.g.* cmvIL-10, cmvCXC-1 and cmvCXC-2) and virus-encoded receptors (*e.g.* US28) that act to 'sponge up' chemokines integral to host defense [178-187]. In its orchestration, however, HCMV may also be establishing conditions to support NK cell adaptation in response to the overt pressure our immune system faces counteracting this virus.

HCMV infection is largely asymptomatic with infrequent subclinical reactivation events having an inconsequential impact in immunocompetent hosts. Considering the striking influence HCMV exerts on the NK cell compartment alone, HCMV infection in younger healthy people may provide heterologous immunity against infection with other viruses and lends an explanation for our historical tolerance of its high prevalence [188-191]. Unfortunately, acute or persistent HCMV infection in individuals with immature, weakened or deficient immune systems causes complications and contributes to age-related morbidities and mortality [192-197]. Thus, although perhaps considered to enhance immune fitness in youth, the once 'beneficial' biological features associated with HCMV diminish with age,

becoming more detrimental to human health and follows the theory of antagonistic pleiotropy [198].

1.4 An Inflammatory Duet: HCMV and HIV Accentuate Immune Activation

HCMV infection proves particularly disadvantageous in people living with HIV (PLWH). Combination antiretroviral therapy (cART) has dramatically reduced HIV-1 disease progression, acquired immunodeficiency syndrome (AIDS)-related events and end-stage organ failure, however, despite HIV-1 suppression, coinfection with HCMV increases the incidence of systemic inflammation, cardiovascular disease, immune senescence, morbidity and mortality in PLWH [199-203]. The NK cell response to HCMV infection, discussed in Section 1.3, is markedly accentuated in conditions of immune deficiency, immune suppression or ongoing immune reconstitution, including cART suppression of HIV-1 [140, 142, 166, 204]. With a seroprevalence of 80 – 100%, HCMV is concentrated in PLWH and it is common for coinfected individuals to have large NK cell fractions expressing CD57 and NKG2C [204-207].

Smoldering subclinical HCMV replication in PLWH, in combination with HCMV reactivation events promote inflammatory environments contributing to NK cell adaption [118, 208, 209]. The same setting of chronic inflammation and immune activation that supports HCMV replication also favours HIV-1 persistence [210-212]. HIV-1 alone is associated with generalized immune activation and,

although cART markedly diminishes it, inflammation in PLWH remains abnormally elevated [213, 214]. As distinct viruses, HCMV and HIV-1 each potentiate inflammation to create environments favourable for their replication. This phenomenon presents a doubly detrimental scenario in PLWH coinfected with HCMV. Sustained inflammation and immune activation from both HCMV and HIV-1 create a symbiosis whereby HCMV not only drives inflammation to ensure its own survival and reservoir seeding, but also stimulates low levels of HIV-1 replication, enabling maintenance or inflation of HIV-1 reservoirs. In turn, HIV-1-mediated immune activation will promote the inverse effect, fueling an unwelcome negative feedback loop. Subclinical HCMV infection is implicated in contributing to HIV-1 reservoir seeding as both cART-naïve and treated individuals have increased HIV-1 DNA levels compared to PLWH who are HCMV seronegative [209-211]. This constant cycling drives immune exhaustion, accelerates aging and gives rise to a higher incidence of morbidity and mortality in HCMV seropositive PLWH [199-203].

1.5 The Obstinate HIV-1 Proviral Reservoir

Current cART reduces HIV-1 replication to levels where the amount of viral ribonucleic acid (RNA) in the bloodstream falls below current limits of detection. In most cases, maintenance of undetectable viral loads requires strict adherence to therapy [215-217]. Despite their efficacy, complete eradication of HIV-1 is

unattainable with current cART regimes. During early infection, HIV-1 establishes proviral reservoirs, concealing itself within various cell types in different anatomical niches [218, 219]. In this largely dormant state, the HIV-1 reservoir is invisible to the immune system and insensitive to cART [220]. As a consequence of this widespread thorough concealment, if cART is interrupted, HIV-1 reactivates and produces replication-competent viruses capable of nascent infection [217, 221]. Organs and tissues such as the gut and lymph nodes are key sites enriched for cells harbouring HIV-1 provirus [219]. Although various types of cells including macrophages, monocytes and astrocytes can serve as HIV-1 reservoirs, the predominant cell type containing HIV-1 provirus and, thus, the predominant source of viral replication with withdrawal of cART, are $CD4^{pos}$ T cells [217, 222]. Seeding itself in long-lived memory $CD4^{pos}$ T cells during acute and ongoing infection allows HIV-1 to persist indefinitely, despite consistent and effective cART suppression.

1.5.1 HIV-1 Treatment Strategies Target the Reservoir

In the absence of cART, activation of the resting $CD4^{pos}$ T cells harbouring HIV-1 proviruses drives HIV-1 out from latency, replenishes the reservoir and promotes disease progression. Cure of the "Berlin patient" in 2008 and the "London patient" in 2019 with HIV-1-resistant bone marrow transplants provides conceptual proof that HIV-1 can be eradicated in those already living with the virus [223, 224]. Although application of this approach is not feasible for most PLWH, other

elimination strategies are under investigation. These can include 'block and lock' or gene editing, both of which aim to fix latent proviral HIV-1 in a permanent inactive state with either drug therapy or *in situ* HIV-1 genome editing. Conversely, a 'kick/shock and kill' approach focuses on purging the latent HIV-1 reservoir by forced HIV-1 activation from reservoir cells, thereby exposing it to the immune system and/or cART [225-232].

To completely cure HIV-1 infection by the 'kick/shock and kill' approach, two currently unattainable objectives must be met. Firstly, viral reactivation needs to occur in all latently infected cells bearing replication competent viral genomes. Secondly, those cells in which HIV-1 reactivates must be eliminated efficiently enough to prevent spread to uninfected cells. The second goal requires enhanced antiviral immune function, likely combined with novel pharmacologic strategies. Direct reservoir cytolysis by T cell and specific antibody-dependent NK cell mechanisms is a key element of this goal. Incomplete purging of the latent HIV-1 reservoir, although not an absolute cure, may be sufficient to reduce or even remove dependence upon cART for suppression of HIV-1 replication and yield a functional cure for HIV-1 infection.

1.5.2 Checkpoint Inhibitors Enhance Effector Responses

Considering the role that the immune system will play, similarities between cancer and chronic viral infection imply that administration of checkpoint inhibitors

can benefit immune-based HIV-1 treatment strategies. Like cancer, chronic viral infection often progresses to a stage where effector cell functions fundamental for its control are severely impaired [233, 234]. Following activation, T cells upregulate inhibitory receptors such as CTLA-4 and PD-1 to limit T cell responses and prevent immune pathology arising from unregulated responses [233]. In settings of chronic infection with persistent microbial replication, T cell function is dysregulated by sustained high expression of these inhibitory checkpoint receptors [235, 236]. Checkpoint inhibitors targeting different inhibitory receptors on immune cells or their corresponding ligands are transforming cancer therapy and many are relevant to immunotherapy for HIV-1 infection.

One hallmark of chronic HIV-1 infection is disruption of normal lymphocyte functions, leading to signs and symptoms of immune dysfunction. This dysfunctional profile is illustrated by increased expression of multiple inhibitory immune checkpoint molecules including PD-1, CTLA-4, TIGIT, Tim-3 and LAG-3 on $CD8^{pos}$ T cells and in some instances, on NK cells [71, 81, 237]. There is convincing evidence of a central role for TIGIT in control of $CD8^{pos}$ T cell maturation and exhaustion [86]. However, considering its parallel regulation of NK cell functions, targeting TIGIT with checkpoint inhibitors may have even greater implications for bolstering antiviral immunity than targeting PD-1, CTLA-4 or Tim-3. Of all lymphocyte subsets, NK cells have the highest fraction of cells constitutively expressing TIGIT [70]. Between 20 – 90% of resting NK cells express TIGIT and levels

are increased by acute and chronic viral infections or cancers [70, 86, 238, 239]. Targeting TIGIT is an especially attractive approach to incorporate into HIV-1 cure strategies as it impacts multiple functions of multiple types of effector cells.

1.5.3 TIGIT Regulates Effector cells in HIV-1 Infection

Expression of TIGIT is broadly dysregulated on both $CD8^{pos}$ T cells and NK cells in HIV-1 infection. An increased fraction of $CD8^{pos}$ T cells expressing TIGIT arises despite early initiation of effective cART [240, 241]. The high potential impact of targeting TIGIT as a therapeutic strategy to invigorate effector cell responses against HIV-1 is emphasized by TIGIT expression on a large fraction of $CD8^{pos}$ T cells and almost all HIV-1-specific $CD8^{pos}$ T cells in PLWH [240, 241]. Cells expressing TIGIT proliferated less and mounted weaker antiviral cytokine responses compared with their $TIGIT^{neg}$ $CD8^{pos}$ T cell counterparts, indicating a prominent role for TIGIT as a negative regulator of HIV-1-specific $CD8^{pos}$ T cell immunity [240]. Interrupting TIGIT signaling using therapeutic mAb blockade rescued $CD8^{pos}$ T cell IFN-γ responses and increased cytotoxicity [86, 240]. In parallel with increased TIGIT on $CD8^{pos}$ T cells, its costimulatory counterpart, DNAM-1, is often downregulated, further contributing to T cell exhaustion [241, 242]. This 'one-two punch' increases inhibitory intercellular TIGIT/PVR interactions and *cis* TIGIT/DNAM-1 heterodimers further restrict the potential for productive costimulation mediated by DNAM-1/PVR interactions (Figure 1.2).

Similar to the relationship seen with $CD8^{pos}$ T cells, higher levels of TIGIT on NK cells correlate with HIV-1 disease progression [243]. Although TIGIT blockade can rescue NK cell function against cancer, further evidence illustrating the potential benefits of targeting the TIGIT axis in the context of HIV-1 infection is needed [239]. While TIGIT expression is increased on NK cells from cART-naïve PLWH, cART may return TIGIT expression to similar levels as that of healthy controls [243, 244]. In untreated PLWH, NK cells expressing higher amounts of TIGIT were less likely to degranulate and produce IFN-γ in response to cytokine stimuli than those that did not express TIGIT. In this case, baseline NK cell function was rescued by mAb against TIGIT [243]. In another study in which NK cells were activated for 3 days with IL-2, blockade of TIGIT provided no benefits to NK cells responding against *in vitro* HIV-1 infected autologous primary $CD4^{pos}$ T cells [244]. In the setting of active HIV-1 infection, TIGIT expression is increased on subsets of NK cells co-expressing DNAM-1 [243, 244]. Combining viral reactivation strategies with effector cell reinvigoration by preventing TIGIT interactions with either its ligand or DNAM-1 should promote cytolysis of infected cells (Figure 1.4). More evidence is needed to delineate the cytotoxic potential of these cells. Expression of TIGIT on $CD8^{pos}$ T cells and NK cells suggests that TIGIT-specific mAb therapy could synergistically unleash both types of antiviral effector cells to target active HIV-1 infection more robustly.

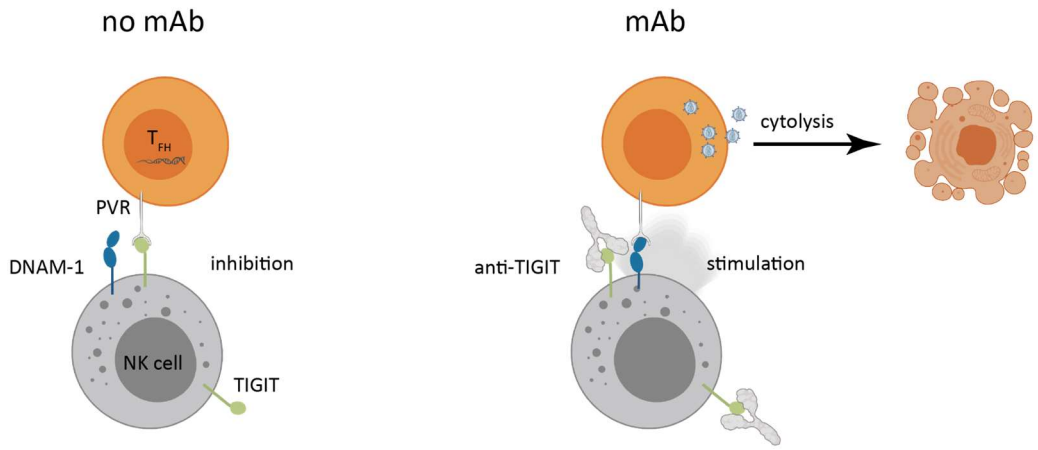

Figure 1.4| Hypothetical outcome of using TIGIT blockade to target HIV-1 reservoirs

Increased PVR expression on $CD4^{pos}$ T cells [*e.g.*, lymph node follicular helper T (T_{FH}) cells] can contribute to NK cell dysregulation by engaging TIGIT and inhibiting effector functions ('no mAb', left panel). Combined $CD4^{pos}$ T cell reservoir activation and TIGIT mAb (right panel) could create a scenario where previously latent HIV-1 actively replicates, introducing targets for NK cell recognition. Reservoir cytolysis is promoted in this scenario by preventing inhibitory TIGIT interactions and allowing DNAM-1 (co)stimulation.

1.5.4 A Ligand for TIGIT is Enriched on HIV-1 Reservoir cells

Although expression levels of many inhibitory checkpoint molecules increase on multiple types of effector cells during HIV-1 infection, inhibition relies on the interactions between these receptors and their cognate ligands. The predominant ligand for TIGIT and DNAM-1 is PVR (Figure 1.2), which is expressed on monocytes, dendritic cells, T cells and other cell types including tumor cells and HIV-1-infected cells [132, 240, 245, 246]. Originally identified in 1989 as a receptor for poliovirus, PVR belongs to a larger family of adhesion molecules that facilitate cell adhesion and migration, while over-expression of PVR in transformed cells promotes proliferation [245, 247]. Stimulated T cells have increased total PVR protein and cell

surface expression levels, with preferential PVR expression on proliferating T cells in the S or G_2/M cell cycle phase [248]. Increased cellular PVR expression occurs after the DNA damage response (DDR) pathway is induced [248]. Although activated primary $CD4^{pos}$ T cells express PVR, whether or not HIV-1 influences PVR expression on circulating primary $CD4^{+}$ T cells remains controversial [249].

During infection, expression of HIV-1-encoded Vpr helps promote cell cycle arrest in G_2 via the DDR pathway [250]. Through this same Vpr-dependent mechanism, PVR was reported to be upregulated on the surface of HIV-1-infected Jurkat T cells, yet expression of Nef and/or Vpu reduced surface-expressed PVR on both Jurkat and primary $CD4^{pos}$ T cells [251-253]. Another study reported no role for HIV-1-specific modulation of PVR expression on primary $CD4^{pos}$ T cells [249]. These studies used various *in vitro* systems with $CD4^{pos}$ T cell lines or *ex vivo* $CD4^{pos}$ T cells from healthy controls infected with different laboratory passaged HIV-1 strains. In all cases, PVR expression was assessed on all infected T cells, yet *in vitro*-infected $CD4^{pos}$ T cells can be subsequently distinctly grouped into either $CD4^{pos}$ or $CD4^{neg}$ cells [254]. In so doing, Tremblay-McLean *et al*. found that surface PVR expression is reduced on infected $CD4^{neg}$ T cells compared with infected $CD4^{pos}$ T cells [254]. This could indicate that if HIV-1 does regulate PVR expression *in vivo*, productively infected or reservoir T_{FH} cells that maintain their expression of CD4 may have a different PVR expression profile than their $CD4^{neg}$ T cell counterparts.

Investigation of *ex vivo* PVR expression on $CD4^{pos}$ T cells from PLWH has been limited. Very low levels of PVR expression on circulating $CD4^{pos}$ T cells combined with the relative inaccessibility of lymph node sections from PLWH make informed assessment of PVR expression problematic [243, 244]. Upregulation of PVR can occur on $CD4^{pos}$ T cells in HIV-1 infection, especially on lymph node T_{FH} $CD4^{pos}$ T cells, which are the major site of HIV-1 reservoir concentration [241, 255, 256]. Further, within the lymph nodes from PLWH, PVR is expressed on both germinal center $CD3^{pos}$ cells and interdigitating follicular DCs [242]. This compact compartment comprised of cells expressing PVR in proximity to $CD4^{pos}$ T cells enriched in HIV-1 provirus could exploit higher localized TIGIT expression on $CD8^{pos}$ T cells and NK cells to limit effector cell functions as they transit through lymph nodes. As NK cell and $CD8^{pos}$ T cell expression of TIGIT increases with acute HIV-1 infection, introducing mAb therapy to overcome the higher affinity TIGIT/PVR inhibitory interaction in favour of DNAM-1/PVR-mediated activation is a rational strategy to address lingering HIV-1 infection [243]. In this event, PVR expressed on reservoir $CD4^{pos}$ T cells would render them more susceptible targets for DNAM-1-expressing NK cells (Figure 1.4).

1.6 The Potential to Harness NK cells

HCMV has a broad impact on human immunity. This introduction has extensively discussed the influence of HCMV infection on the NK cell repertoire and

has highlighted an important interface between NK cells and their control of virus infection. Many questions remain regarding what factors force NK cell adaptation in the face of HCMV infection and whether we can harness their inherent and adaptive functional capacity against a wide range of cancer and infection. Understanding basic molecular mechanisms governing NK cell-mediated immunity informs new treatment strategies to harness these powerful killers for our benefit and future health.

Given the impact HCMV infection has on NK cells, we examined NK cell function along the continuum of NK cell adaptation progressing from (i) short term effects of virokines and cytokines and (ii) long term effects reflected in HCMV adaptation, including altered inhibitory receptor expression with longer term changes. My research identified soluble factors present during HCMV infection that rapidly increase NK cells killing activity: an HCMV-encoded human IL-10 homologue (cmvIL-10) and host-derived type I IFN. Although sustained exposure to these cytokines, in combination with other (un)known factors, may skew NK differentiation in HCMV infection, this question was outside our scope and was not assessed as part of this project. Adaptation to HCMV infection has long been believed to enhance NK cell antibody-dependent IFN-γ production [159], however, studies assessing direct NK cell cytotoxicity of adapted NK cells are lacking.

Considering the exaggerated impact of HCMV infection on the NK cell repertoire in PLWH, we focused on this cohort to measure the potential for NK cells

from PLWH to respond to and mount antiviral responses in the presence of cytokines that would be more abundantly present in the setting of chronic inflammation. As the NKG2C-expressing NK cell compartment is amplified in chronic HIV-1 infection, we evaluated the functional and phenotypic impact of HCMV-driven NK cell adaptation in HIV-1 infection by comparing three groups of subjects distinguished by either HCMV-seronegative status or by high versus low percentages of NKG2C-expressing NK cells. Despite a clear hierarchy of adaptation to HCMV in our study groups, CD16-mediated NK cell degranulation, IFN-γ production or ADCC did not differ significantly between groups. Further, TIGIT was present on a large fraction of NK and $CD8^{pos}$ T cells and its level was associated with HCMV status. Engaging TIGIT inhibited HIV-1-specific ADCC, suggesting TIGIT is an appropriate target for invigorating ADCC against $CD4^{pos}$ T cells expressing PVR induced by HIV-1 infection.

Investigating how basic NK cell biology is altered in the setting of chronic infection informs how we can exploit their innate functions to control infection. This research demonstrated that cytokines or mAbs can release the brakes on NK cells to counter inhibition and favour virus control or eradication.

2 Human Cytomegalovirus IL-10 Augments NK cell Cytotoxicity

2.1 Abstract

Human cytomegalovirus (HCMV) persistently infects most of the adult population with periods of productive and latent infection differentially orchestrated by multiple HCMV-encoded gene products. One HCMV gene (UL111a) encodes cmvIL-10, a virokine homologous to human IL (hIL)-10. Although the effects of cmvIL-10 on most human lymphocyte subsets have been extensively studied, its impact on NK cell function was unreported prior to this study. We investigated effects of short-term cmvIL-10 exposure on human NK cells and found it substantially enhanced NK cell cytotoxicity through natural cytotoxicity receptors NKp30 and NKp46 as well as through C-type lectin-like receptors NKG2C and NKG2D. Antibody-dependent cell-mediated cytotoxicity triggered through CD16 also increased significantly with short-term cmvIL-10 exposure. These effects of cmvIL-10 on NK cell cytotoxicity were rapid, dose dependent, neutralized by polyclonal anti-cmvIL-10 or monoclonal anti-IL-10 receptor (IL-10R) antibodies and independent of increased perforin synthesis or up-regulation of activating receptors. A low percentage (0.5 – 5.4%; n = 12) of NK cells expressed IL-10R and the impact of cmvIL-10 on NK cell degranulation following CD16 stimulation directly correlated with this percentage (P = 0.0218). Short-term exposure of human NK cells to cmvIL-10 did not introduce phenotypic changes

reminiscent of NK adaptation to HCMV infection *in vivo*. Determining how expression of a viral protein that activates NK cells contributes to their function *in vivo* will increase understanding of HCMV infection and NK cell biology.

2.2 Introduction

Human cytomegalovirus (HCMV) is a common β-herpesvirus that infects most of the adult population, remains predominantly dormant after primary infection and is relatively innocuous in healthy adults [196]. Although initially defined by innate anti-tumour activity, natural killer (NK) cells have since been implicated in immunity against viruses, especially herpesviruses [11, 13, 24]. Infection with HCMV drives development of a discrete natural killer (NK) cell population exhibiting phenotypic and functional adaptations [106, 140, 141]. The NK cells adapted to HCMV infection selectively display receptor constellations and intracellular signaling pathways reconfigured to enhance antibody-dependent activation [257].

Cytokines, such as interleukin (IL)-12 are produced early during infection [145]. Exposure to cytokine cocktails of IL-12, IL-15 and IL-18 demonstrated that combinations of priming stimuli, independent of activating/inhibitory receptor engagement, induce NK cell memory-like responses [123]. Both human- and virus-encoded cytokines are produced during HCMV infection. The HCMV *UL111a* gene encodes a homologue of human (h)IL-10, cmvIL-10, expressed during active infection as a full length variant and during viral latency as a truncated, latency-associated (LA) splice variant [181, 182, 258, 259]. With only 27% sequence identity to hIL-10, cmvIL-10 is more divergent than Epstein-Barr virus and Orf parapoxvirus homologues, but retains specificity for the hIL-10 receptor (hIL-10R) [181, 182,

260-263]. The influence of cmvIL-10 on human monocytes, macrophages, B cells, dendritic cells and on T cell priming has been extensively studied, but its impact on NK cell function was previously unreported [264-270]. Therefore, we investigated whether short-term exposure to cmvIL-10 affects NK cell functions and whether any functional changes that occur are reflected in phenotypic changes.

In response to HCMV infection, the human NK cell repertoire redistributes into an adapted population of cells displaying the CD57 maturation marker, C-type lectin-like activating NKG2C receptor, high levels of the CD16 low affinity IgG receptor (FcγRIIIa), and low to non-detectable levels of inhibitory NKG2A receptors [257]. Adapted NK cells downregulate NKp30 and NKp46 natural cytotoxicity receptors (NCR), but readily undergo antibody-dependent activation [158, 159]. Altered expression of intracellular signaling moieties and transcription factors complements these changes to surface receptor repertoires. This generalized reconfiguration of cell surface receptors and emergence of NK cells with features classically associated with adaptive immunity demonstrates that formative interactions between NK cells and HCMV occur over the course of chronic infection [257].

Despite the clear phenotypic and functional differences between HCMV-adapted and non-adapted NK cells, mechanisms underlying memory-like NK cell generation remain obscure. Using peripheral blood mononuclear cells (PBMC) as a source of NK cells, we found that exposure to recombinant full-length cmvIL-10 in

5 h assays significantly enhanced NK cell cytotoxicity through multiple activating receptors, independent of previous exposure to HCMV. A small percentage of peripheral blood NK cells expressed IL-10R and the cmvIL-10-induced increase in magnitude of NK cell degranulation in response to CD16 stimulation correlated directly with this percentage. Adding soluble IL-10Rα partially inhibited the effect of cmvIL-10 on NK cells, while adding anti-cmvIL-10 antibodies or blocking IL-10R with monoclonal antibody (mAb) neutralized the effect. Enhancement of NK cell cytotoxicity occurred in the absence of any significant increase in activation markers, perforin synthesis, or NCR, NKG2C/D or CD16 surface expression levels. Taken together, these data indicate that short-term exposure to a low concentration of cmvIL-10 enhances NK cell cytotoxicity mediated through multiple receptors without concurrent changes in expression levels.

2.3 Materials and Methods

2.3.1 Sample Collection and PBMC Isolation

This study was carried out in accordance with recommendations of the Canadian Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans. The protocol was approved by the Health Research Ethics Authority of Newfoundland and Labrador, Canada. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Whole blood was collected from healthy donors and PBMC isolated as described [271]. Where indicated, NK cells were enriched by negative selection using EasySep Human NK Cell Enrichment Cocktail (STEMCELL Technologies, Vancouver, BC, Canada) as per manufacturer's instructions.

2.3.2 Cell Culture

K562 (ATCC CCL 243), P815 (ATCC TIB-64), and HLA-B27-transfected C1R (C1R-B27; Dr. Kelly McDonald, University of Manitoba, Winnipeg, MB, Canada) cell lines were propagated in lymphocyte medium consisting of RPMI-1640 with 10% FCS, 200 IU/mL penicillin/streptomycin, 0.01 M HEPES, 1% L-glutamine (all from Invitrogen, Carlsbad, CA, USA) and 2.0×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) at 37°C with 5% CO_2. Cells were maintained in log phase for ^{51}Cr labeling and cytotoxicity assays.

2.3.3 NK cell Cytotoxicity

Recombinant hIL-10, recombinant cmvIL-10 and polyclonal anticmvIL-10 (AF117; all R&D Systems, Minneapolis, MN, USA) were used in 5 h ^{51}Cr release assays. K562 or C1R-B27 cells were labeled for 90 min with 100 µCi $Na_2{}^{51}CrO_4$ (PerkinElmer, Akron, OH, USA) then washed 4 times in PBS with 1% FCS. C1R-B27 cells were incubated 30 min with 1 µg/mL anti-HLA-I mAb (W6/32, ATCC #HB-95) to sensitize cells to ADCC. Effector cells were incubated for 5 h with ^{51}Cr labeled K562 or C1R-B27 target cells at various effector to target (E:T) ratios and ^{51}Cr release in supernatants measured on a Wallac 1480 Wizard gamma counter. Percent specific lysis was calculated by (experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(maximum ^{51}Cr release – spontaneous ^{51}Cr release) × 100.

2.3.4 Flow Cytometry and NK cell Activation Assay

PBMC or enriched NK cells were stained with directly conjugated mAb (clones in parentheses) against human CD3 (BW264/56), CD56 (REA196), or hIL-10R (REA239) from Miltenyi Biotec (San Diego, CA, USA). To detect functional activity of NK cells by flow cytometry, PBMC were stimulated with 0.5 µg anti-CD16 mAb (LEAF™ 3G8; BioLegend) per 5×10^5 PBMC in the presence of vehicle or 10 ng/mL cmvIL-10 and labeled with 0.25 µg/10^6 PBMC of anti-CD107a (H4A3; BioLegend) for 5 h (Vf = 500 µL) to detect NK cell degranulation. Cytokine secretion was inhibited by adding brefeldin A (Sigma-Aldrich) 1 h after the start of incubation

to a final concentration of 10 μg/mL (V_f = 500 μL). Cells were fixed and permeabilized using the Inside Stain Kit (Miltenyi Biotec) as per manufacturer's instructions and stained with directly conjugated mAb against human IFN-γ (4S.B3) from eBioscience (San Diego, CA, USA). Data were acquired using either CytoFLEX or MoFlo Astrios EQ flow cytometers and analyses and illustration performed using Kaluza software (all Beckman Coulter, Brea, CA, USA).

2.3.5 Redirected NK cell Cytotoxicity

NK cell receptor-mediated cytotoxicity was measured over 5 h using ^{51}Cr labeled P815 cells and 3 μg/mL soluble IgG isotype control (Ag8) or mAb against human NKp30 (210845), NKG2C (134591), NKG2D (149810) from R&D Systems, NKp44 (9E2), or NKp46 (P44-8) from BioLegend in the presence of 10 ng/mL cmvIL-10 or vehicle control (V_f = 300 μL). Percent specific lysis was calculated as above.

2.3.6 Functional hIL-10R Blocking Assays

Carrier-free recombinant hIL-10Rα protein (R&D Systems) was serially diluted and its ability to block the effect of cmvIL-10 on NK cell cytotoxicity against K562 cells measured as above with the following additions: Increasing concentrations of hIL-10Rα were pre-incubated 30 min on ice with cmvIL-10 before transferring an amount corresponding to a final cmvIL-10 concentration of 10 ng/mL to wells of a microtiter plate containing freshly-isolated PBMC and ^{51}Cr

labeled K562 cells at E:T 30:1 (V_f = 300 µL) for 5 h. In other experiments, PBMC were pretreated for 30 min with serial dilutions of purified mAb against hIL-10Rα (LEAF™ 3F9; BioLegend) after which 10 ng/mL of either cmvIL-10 or hIL-10 and ^{51}Cr labeled K562 cells were added at E:T 30:1 and cytotoxic activity measured over 5 h. Percent specific lysis was calculated as above.

2.3.7 Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5 with 2-sided *P*-values < 0.05 considered significant. Normality of data distributions were assessed with Shapiro-Wilk and D'Agostino and Pearson tests. Differences in means or medians between groups were compared by Student's *t*-tests or Mann-Whitney *U*-test as appropriate. For paired analyses, Student's paired *t*-test was used when data were normally distributed, and nonparametric Wilcoxon signed rank test otherwise.

2.4 Results and Discussion

2.4.1 cmvIL-10 and hIL-10 Increase NK cell Cytotoxicity

Detectable amounts of cmvIL-10 arise in culture within 3 d of infection with AD169 or Toledo HCMV strains [264]. To test the immunomodulatory properties of this virokine on NK cells, we measured NK cell cytotoxicity against classical targets in the presence of recombinant cmvIL-10. Over the course of 5 h, the presence of cmvIL-10 increased NK cell lysis of K562 cells by up to 55% (Figure 2.1 A, solid line). Titration of cmvIL-10 from 50 ng/mL to 500 pg/mL indicated that 10 ng/mL stimulated maximal increases in NK cell killing, therefore, we chose this concentration for subsequent experiments. This concentration is comparable to cmvIL-10 concentrations reported in HCMV AD169-conditioned cell culture medium (0.8–4 ng/mL) [264]. Circulating concentrations of 31–547 pg/mL cmvIL-10 have been detected in human serum, implying considerably higher localized cmvIL-10 concentrations at sites of active HCMV replication [272]. To confirm this effect on cytotoxicity was specifically mediated by cmvIL-10, NK cell killing of K562 cells with 10 ng/mL cmvIL-10 was measured in the presence of increasing concentrations of neutralizing antibody against cmvIL-10. At 2 μg/mL, polyclonal anti-cmvIL-10 antibodies abrogated the cmvIL-10-dependent increase in NK cell lysis of K562 cells (Figure 2.1 B). These results demonstrate dose-dependent

enhancement of NK cytotoxicity by cmvIL-10 that is neutralized by cmvIL-10-specific antibodies.

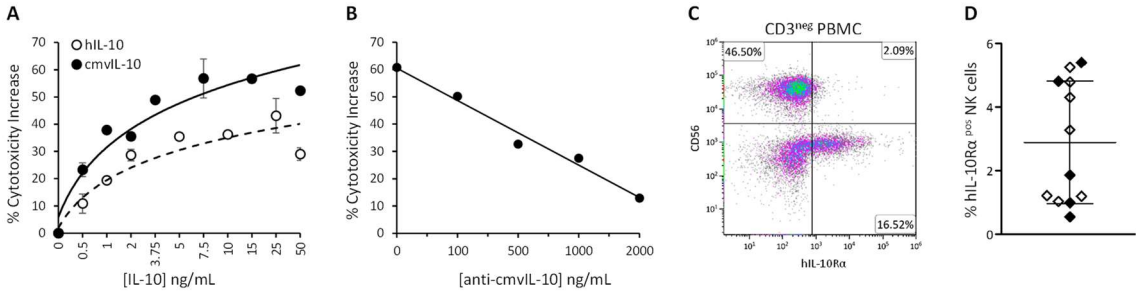

Figure 2.1| Effect of cmvIL-10 on NK cell cytotoxicity

Freshly isolated PBMC from an HCMV-seronegative donor were incubated for 5 h with ^{51}Cr-labeled K562 cells in the presence of (A) increasing concentrations of recombinant cmvIL-10 (dark circles, solid line), hIL-10 (open circles, dashed line), or (B) 10 ng/mL cmvIL-10 with increasing concentrations of neutralizing antibodies against cmvIL-10. Percent increase in specific lysis of K562 target cells was calculated as ([% specific lysis with cmvIL-10 – % specific lysis control]/ % specific lysis control) × 100. Assays in (A, B) were performed in duplicate; n = 2. (C) Representative flow cytometry plot of hIL-10Rα expression on CD3^{neg} PBMC (left panel) with summary graph (right panel) depicting hIL-10Rα expression on CD56^{pos}CD3^{neg} PBMC for 12 donors with mean and SD shown. Subjects seropositive for HCMV are represented with filled black diamonds.

As cmvIL-10 binds the hIL-10R, we compared the NK cell response to hIL-10 under identical conditions. Previous studies examining NK cell responses to hIL-10 used combinations of hIL-10 and other exogenous cytokines, such as IL-15, with overnight exposure prior to cytotoxicity assays [273]. We observed a comparable dose-dependent increase in NK cell cytotoxicity with exposure to increasing concentrations of hIL-10 alone over 5 h cytotoxicity assays (Figure 2.1 A, dashed line). In short-term culture with no other exogenous cytokines, NK cell cytotoxicity rose 35% in response to hIL-10, closely mirroring the effect of cmvIL-10. Despite

robust increases in NK cell cytotoxicity from human and cmvIL-10, we found that only a small fraction (2.89 ± 1.92% mean ± SD; n = 12) of the total resting NK cell population expressed hIL-10R detectable by flow cytometry (Figure 2.1 C, D). As hIL-10R expression level on individual NK cells was also low, it was not feasible to assess its distribution on distinct NK cell subsets.

2.4.2 Recombinant cmvIL-10 Enhances Cytotoxicity of Enriched NK cells

As most hematopoietic cells can express the hIL-10R and the frequency of NK cells expressing this receptor is low [274], we enriched NK cells to ~90% purity (Figure 2.2 A) to address whether cmvIL-10 acted directly on NK cells or through other PBMC expressing hIL-10R. After treatment with recombinant cmvIL-10, purified NK cell cytotoxicity against K562 cells increased to a similar extent as with PBMC treatment at all E:T ratios tested (Figure 2.2 B). At the 5:1 E:T ratio, cmvIL-10 had the greatest relative impact, causing an 82 ± 38% increase (mean ± SD; n = 6; P = 0.0313, Wilcoxon signed rank test) in NK cell cytotoxicity against K562 targets. This demonstrates that cmvIL-10 stimulates NK cells independent of immune accessory cells present in PBMC. Neutralizing antibodies against cmvIL-10 abolished the cmvIL-10 treatment-induced increase in purified NK cell cytotoxicity (Figure 2.2 C), confirming our initial observation with PBMC (Figure 2.1 B). These data illustrate that recombinant cmvIL-10 rapidly and specifically increases NK cell cytotoxicity, independent of any other major PBMC subset.

After establishing the rapid and specific effect of cmvIL-10 on NK cell cytotoxicity with 6 purified NK cell samples, the generalizability of this result was tested with PBMC from 9 additional donors. On average, cmvIL-10 caused a 25 ± 15% (mean ± SD; n = 10; $P < 0.0001$, Student's paired *t*-test) increase in NK cell lysis of K562 cells (Figure 2.2 D). To determine whether augmentation of NK cell cytotoxicity by cmvIL-10 extended to other forms of cytotoxicity, we tested its effect on ADCC using a previously described anti-HLA-I-opsonized C1R-B27 assay [271, 275]. Over 5 h incubation, exposure to cmvIL-10 stimulated a 36.5 ± 15.5% (mean ± SD; n = 8; $P = 0.0033$ Student's paired *t*-test) increase in ADCC (Figure 2.2 E). These data illustrate that, in addition to enhancing natural cytotoxicity, short-term exposure to recombinant cmvIL-10 robustly increases levels of NK cell ADCC.

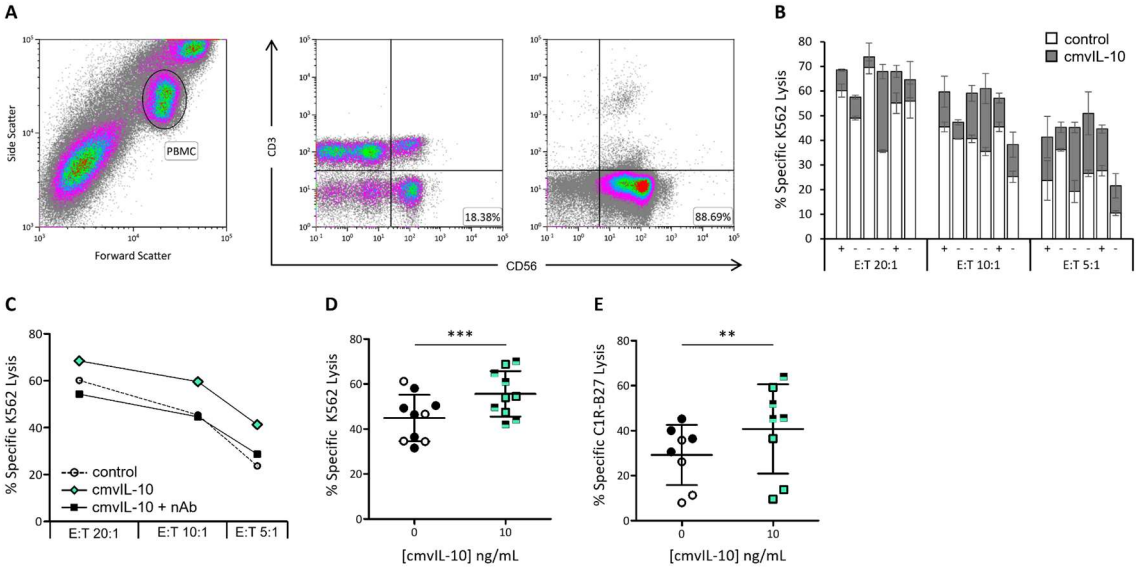

Figure 2.2| Generalized effect of cmvIL-10 on NK cell natural cytotoxicity and ADCC

(A) Representative flow cytometry plots showing PBMC scatter (left plot), NK cell percentage in starting PBMC population (middle plot) and NK cell percentage in enriched population (right plot). (B) NK cells enriched from fresh PBMC by negative selection were incubated for 5 h at the indicated E:T ratios with ^{51}Cr-labeled K562 cells with and without 10 ng/mL cmvIL-10 and (C) with 10 ng/mL cmvIL-10 and 2 µg/mL of neutralizing anti-cmvIL-10 antibody (nAb) for a representative HCMV seropositive donor. Assays were performed in duplicate; n = 6. Cytotoxicity by freshly isolated PBMC incubated with or without 10 ng/mL cmvIL-10 against (D) K562 cells (n = 10) or (E) anti-HLA-I-coated CIR-B27 cells (n = 8) was measured over 5 h at an E:T ratio of 60:1. In (B), HCMV status is indicated below the x-axis for each individual donor, with "+" and "-" denoting HCMV^{pos} and HCMV^{neg} individuals, respectively. Those seropositive for HCMV are represented with filled black circles or colored squares half-shaded black in (D, E). Assays were performed in triplicate and error bars represent mean with SD. Student's paired *t*-test was performed to test the probability of a difference between conditions. **p = 0.0033, ***p < 0.0001.

2.4.3 cmvIL-10-Induced Increase in NK cell Degranulation Correlates with the Percentage of NK cells Expressing hIL-10R

To assess the impact of cmvIL-10 on NK cell function at the effector cell level, we tested whether cmvIL-10 increased the number of degranulating NK cells following stimulation. The representative flow plot in Figure 2.3 A illustrates the gating strategy used to assess $CD107a^{pos}$ or IFN-γ^{pos} NK cells within the entire NK cell population after stimulation through CD16. By measuring CD107a expression following anti-CD16 stimulation with and without cmvIL-10, we sought to assess whether cmvIL-10 exposure inflated the number of killer cells triggered or increased the efficiency of cells poised to degranulate. Despite no statistically significant change in either CD107a expression or IFN-γ production after cmvIL-10 exposure (Figure 2.3 B, C), we noted a modest increase in CD107a expression for a number of donors. The absolute increase in percentage of NK cells expressing CD107a after cmvIL-10 exposure significantly correlated ($p = 0.0218$) with the percentage of NK cells expressing hIL-10R (Figure 2.3 D). This direct and significant correlation between the increase in fraction of NK cells expressing CD107a and fraction of NK cells expressing hIL-10R suggests that cmvIL-10 enhances NK cell cytotoxicity through binding the hIL-10R.

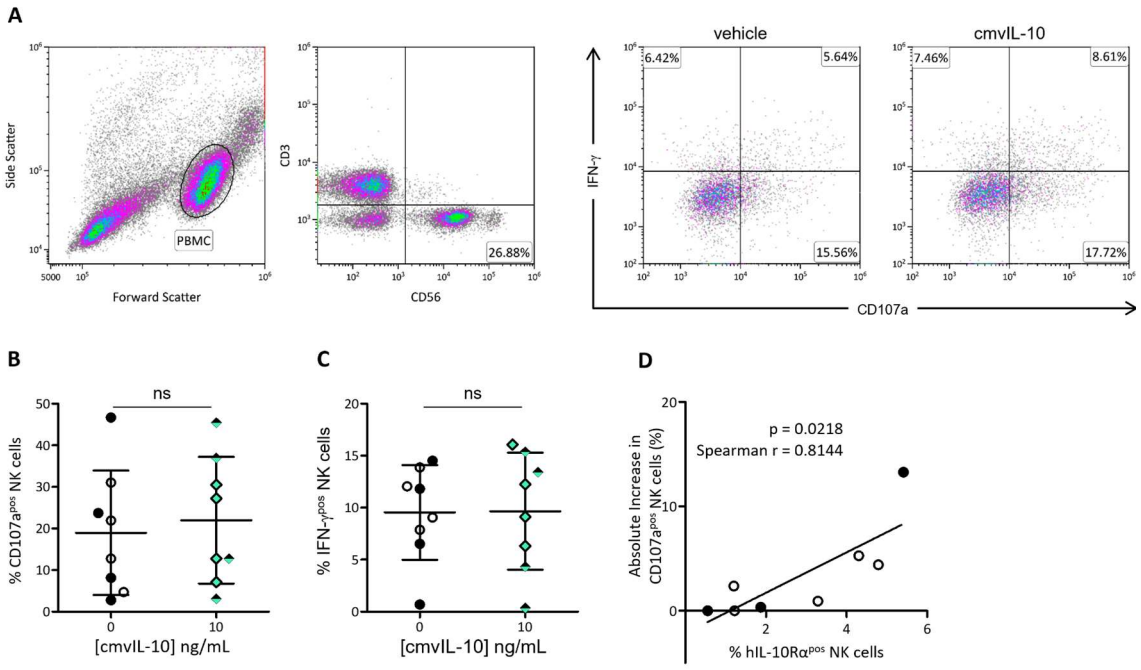

Figure 2.3| CD16-dependent NK cell degranulation, IFN-γ production with cmv-IL-10

The gating strategy used to measure percentages of NK cells expressing CD107a and/or IFN-γ with either control or cmvIL-10 conditions for an HCMV-seronegative donor is shown in (A). The effect of cmvIL-10 on percentage of the total NK cell population (B) degranulating ($CD107a^{pos}$) or (C) producing IFN-γ was measured (n = 8). Figure (D) shows the correlation between the percentage of NK cells expressing hIL-10R and absolute increase in percentage of NK cells expressing CD107a after CD16 stimulation and exposure to cmvIL-10 compared to CD16 stimulation in the absence of cmvIL-10 (n = 8). The correlation coefficient (r) and probability of a significant correlation are shown on the graph. Subjects seropositive for HCMV are represented in with filled black circles or coloured diamonds half-shaded black. Error bars in (B) represent median with IQR and comparison between conditions carried out with Wilcoxon signed-rank test. Bars in (C) represent mean ± SD and Student's paired *t*-test was performed.

2.4.4 Blocking hIL-10R Prevents cmvIL-10-Mediated Increases in NK Cytotoxicity

Using cmvIL-10-specific antibodies, we showed that the increased NK cell cytotoxicity observed in previous experiments was due to cmvIL-10. We also showed that the hIL-10R is expressed in low amounts on only a small fraction of the total

NK cell population. Despite binding to the hIL-10R, cmvIL-10 has relatively low homology to hIL-10, therefore, we carried out a series of experiments to confirm that cmvIL-10 acts on NK cells through the hIL-10R [181]. We first added increasing concentrations of exogenous soluble rhIL-10Rα protein (the ligand-specific subunit of the hIL-10R complex) to cytotoxicity assays to compete with cell surface hIL-10R for binding to cmvIL-10. A representative plot in Figure 2.4 A shows the effect of 10 ng/mL cmvIL-10 on NK cell cytotoxicity was only partially neutralized at up to very high relative concentrations (8 μg/mL) of the rhIL-10Rα protein. We next introduced a mAb against hIL-10Rα to directly block hIL-10Rs. Anti-hIL-10Rα dampened cmvIL-10-induced enhancement of NK cell responses against K562 cells in a dose dependent fashion, neutralizing the effect at or above 5 μg/mL (Figure 2.4 B). As higher concentrations of anti-hIL-10Rα or the IgG_1 isotype control can affect NK cell cytotoxicity in the absence of cmvIL-10, we chose 5 μg/mL to test its neutralization capacity with more subjects. At this concentration, anti-hIL-10Rα completely prevented the action of cmvIL-10 for most individuals tested, and neutralized cmvIL-10 activity by at least 60% in all cases (Figure 2.4 C). This demonstrates that cmvIL-10 increases NK cell cytotoxicity through binding to the hIL-10R. In similar experiments, anti-hIL-10Rα blocked the effect of hIL-10 on NK cell cytotoxicity, albeit at lower concentrations than needed to block the effect of cmvIL-10 (Figure 2.4 D).

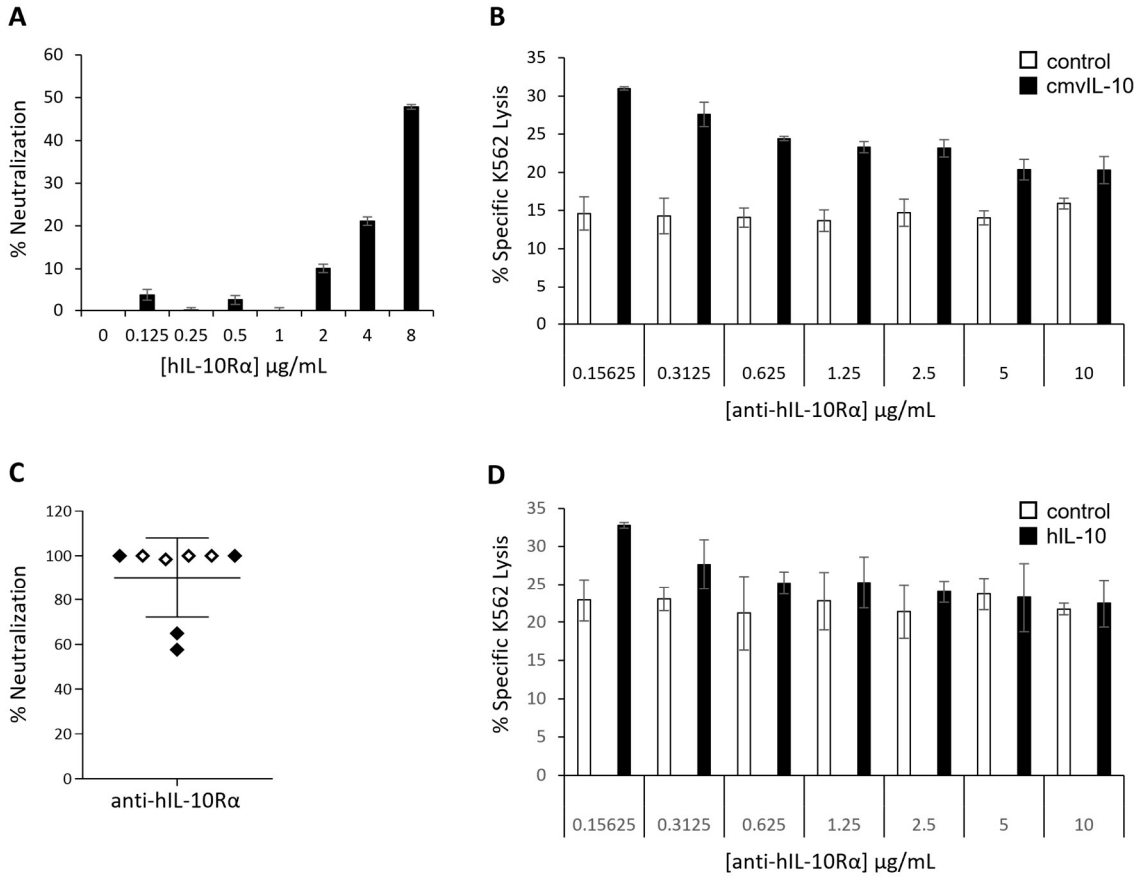

Figure 2.4| Role of hIL-10R in cmvIL-10-enhanced NK cell cytotoxicity

The interaction between cmvIL-10 and the hIL-10R was explored by introducing increasing concentrations of either (A) soluble hIL-10Rα protein to compete with cell surface hIL-10R for binding cmvIL-10 or (B) mAb against hIL-10Rα to block the hIL-10R under control conditions (white bars) or with 10 ng/mL cmvIL-10 (black bars). The extent to which 5 µg/mL anti-hIL-10Rα prevented cmvIL-10-specific increases in NK cell lysis of K562 cells is summarized for nine individual donors (performed in triplicate) in (C) with those seropositive for HCMV represented with filled black diamonds. Neutralization of 10 ng/mL hIL-10 by anti-hIL-10Rα is shown for comparison in (D). Bars in plots (A, B, and D) represent mean ± SEM of three replicates for an HCMV-seropositive donor. Percent neutralization of specific K562 cell lysis was calculated as [(% increase in specific lysis with IC – % increase in specific lysis with anti-hIL-10Rα) / % increase in specific lysis with IC] × 100 from paired data for each donor.

2.4.5 Recombinant cmvIL-10 Enhances NK cell Activation Through Multiple Receptors

Considering that cmvIL-10 increased general and antibody-dependent cytotoxicity through NCRs and CD16, respectively, we assessed the impact on NK cytotoxicity triggered by various individual activating receptors. To do this, we used a redirected lysis assay in which murine FcR-expressing P815 cells orient mAb specific for activating receptors to enable NK cell activation selectively through that receptor. Activating receptors probed in this manner were NKG2C, NKG2D, NKp30, NKp44, and NKp46. A representative summary of target lysis by natural cytotoxicity, ADCC, and redirected killing through individual activating receptors is shown in Figure 2.5 A. Using an IgG isotype control antibody, there was little P815 cell lysis and no significant difference from control conditions when cmvIL-10 was present at 10 ng/mL (Figure 2.5 B). By introducing mAb against individual NK cell activating receptors, we observed that cmvIL-10 significantly increased P815 cell lysis through NKG2C (Figure 2.5 C), NKG2D (Figure 2.5 D), and the NCRs, NKp30 and NKp46, but not NKp44 (Figure 2.5 E-G).

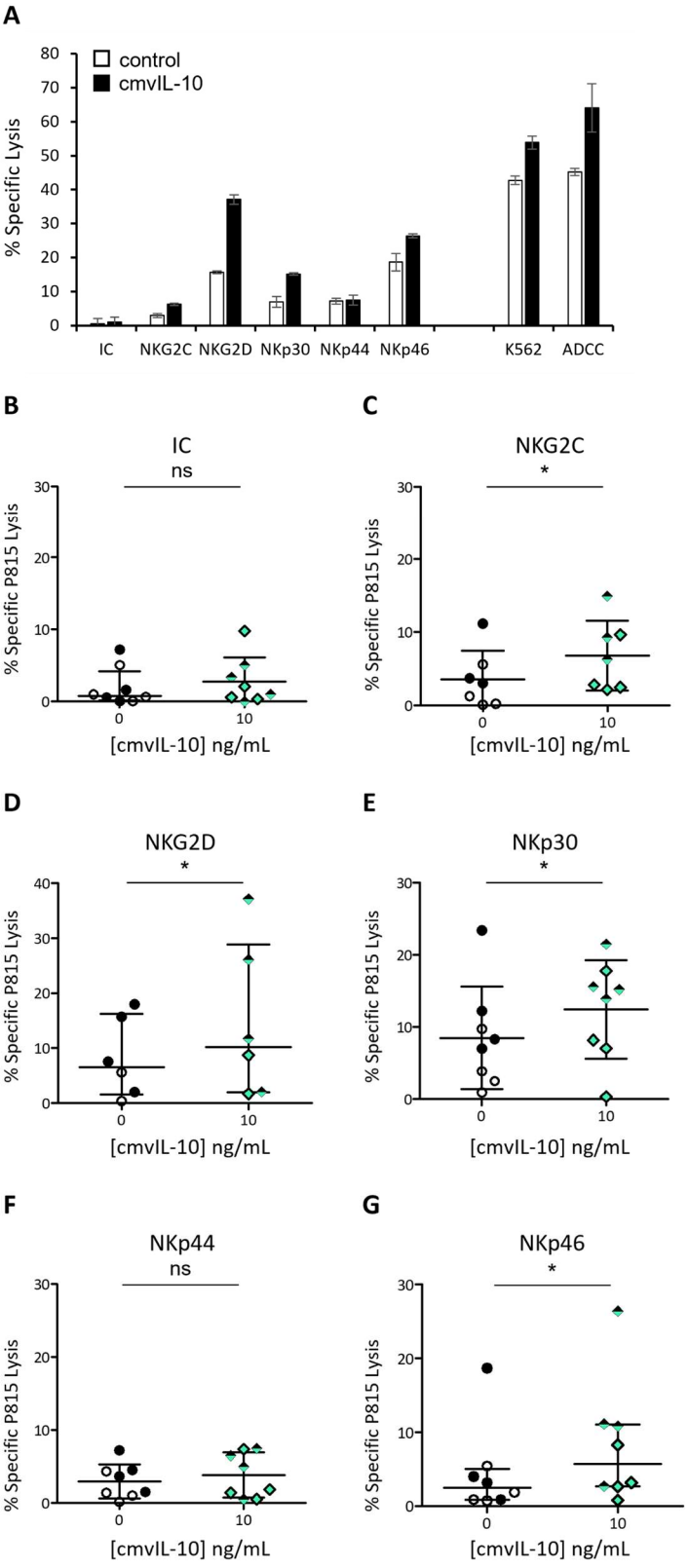

Figure 2.5| Effects of cmvIL-10 on cytotoxicity mediated through major NK cell activating receptors

(A) Levels of natural cytotoxicity, ADCC and redirected lysis of P815 cells in the presence of NK receptor-specific mAb are shown for a representative donor with or without 10 ng/mL cmvIL-10 present during 5 h assays. Redirected P815 cell lysis was measured with 3 μg/mL (B) isotype control, (C) anti-NKG2C, (D) anti-NKG2D, (E) anti-NKp30, (F) anti-NKp44 or (G) anti-NKp46 mAbs. Bars in summary plot (A) represent mean ± standard error of the mean (SEM) of three replicates. Assays represented in plots B-G were performed in triplicate for 6–8 individual donors. Subjects seropositive for HCMV are represented with filled black circles or colored diamonds half-shaded black. Error bars for anti-NKp30 (p = 0.0236) and anti-NKp44 plots represent mean ± SD and the probability of a difference between conditions was assessed by Student's paired *t*-test. All other bars represent median with interquartile range (IQR) with the probability of a difference between conditions assessed by Wilcoxon signed rank test. *p = 0.0313 (NKG2D); *p = 0.0156 (NKG2C and NKp46).

Whereas the effect of cmvIL-10 on natural cytotoxicity and ADCC against optimally sensitized targets was robust, the impact on cytotoxicity mediated through individual receptors in mAb-redirected lysis assays was relatively modest. This reflects low intrinsic levels of cytotoxicity mediated by most donors' NK cells through individual NKp30, NKp46, NKG2C, and NKG2D receptors as well as differential receptor expression levels across the population. Expression of NKp44 is restricted to activated NK cells, therefore, without additional stimulation, cmvIL-10 should not and did not enhance killing triggered through NKp44 [276]. cmvIL-10 rapidly sensitizes NK cell cytotoxicity through most NCRs and activating CLRs, suggesting cmvIL-10 has a general effect of lowering the threshold for NK cell activation.

Expression of extracellular receptors investigated in redirected cytotoxicity experiments was assessed after 5 h in the presence or absence of 10 ng/mL cmvIL-10. Although cmvIL-10 had a robust and consistent influence on NK cell cytotoxicity, short-term exposure caused no phenotypic alterations in NKG2C, NKG2D, NKp30, NKp46, or CD16 levels, perforin synthesis or in the degree of CD69 or TNF-α up-regulation with any of six HCMV-seronegative donors tested (data not shown). Previous studies demonstrated PLZF downmodulation arises from adaptation to HCMV infection, however, short-term treatment with cmvIL-10 did not alter PLZF expression in these same donors (data not shown) [143, 144]. Consistent levels of perforin expression indicate that cmvIL-10 does not stimulate

NK cell perforin synthesis or release in the absence of appropriate physical stimuli during short-term exposure (data not shown). As various other cytokines are produced during HCMV infection, examining the collective impact of cmvIL-10 and other cytokines on NK cell function, phenotype and adaptation over short- and longer-term culture periods in combination with physical stimuli should follow.

Here we showed that cmvIL-10 acts through the hIL-10R to rapidly potentiate NK cell cytotoxicity against different targets through multiple NK cell activating receptors. A small fraction of peripheral blood NK cells express hIL-10R at low levels, yet NK cell enrichment did not reduce the effect of cmvIL-10. Further, the percentage of NK cells expressing hIL-10R correlated with the modest increase in NK cells degranulating (measured by cell surface CD107a expression) after stimulation with CD16 and cmvIL-10. We cannot definitively exclude the possibility that cmvIL-10 acts through an innate lymphoid cell subset or other cells not reduced in prevalence by the NK cell enrichment procedure, but the simplest explanation for our results is that cmvIL-10 acts directly on human NK cells.

There are no previous reports of cmvIL-10 augmenting NK cell cytotoxicity, however, hIL-10-mediated NK cell stimulation has been documented [277-279]. During long-term culture, or in combination with IL-15 or IL-18, hIL-10 modestly augmented NK cell cytotoxicity [273, 279, 280]. Consistent with this functional enhancement, Mocellin *et al*. also showed that hIL-10 increased transcription of genes involved in NK cell activation, cytotoxicity, and migration, again consistent

with a direct effect of hIL-10R engagement on NK cells [281]. Here, we further demonstrated that NK cells are sensitive to hIL-10 stimulation alone over short-term assays in the absence of other exogenous cytokines. While full-length cmvIL-10 stimulated NK cell cytotoxicity in this study, additional experiments investigating the effects of truncated LAcmvIL-10 are warranted as cmvIL-10 and LAcmvIL-10 can have biologically different effects and roles [266].

NK cells provide rapid defense against viruses, particularly herpesviruses. The relevance of their interactions is highlighted by evolution of numerous viral ligands for NK cell inhibitory receptors and by emergence of a distinct memory-like NK cell population following HCMV infection. In this study, we report a contrasting instance in which a viral product, cmvIL-10, rapidly and independently augments NK cell cytotoxicity through most activating receptors. Determining if and how this activity benefits HCMV persistence or contributes to NK cell function *in vivo* could inform strategies to more effectively engage NK cells against viral infections and cancer.

2.5 Conclusions

Before this study, there were no previous reports of cmvIL-10 augmenting NK cell cytotoxicity. As various other cytokines are produced during HCMV infection, examining the collective impact of cmvIL-10 and other cytokines on NK cell function, phenotype and adaptation over short- and longer-term culture periods in combination with physical stimuli should follow.

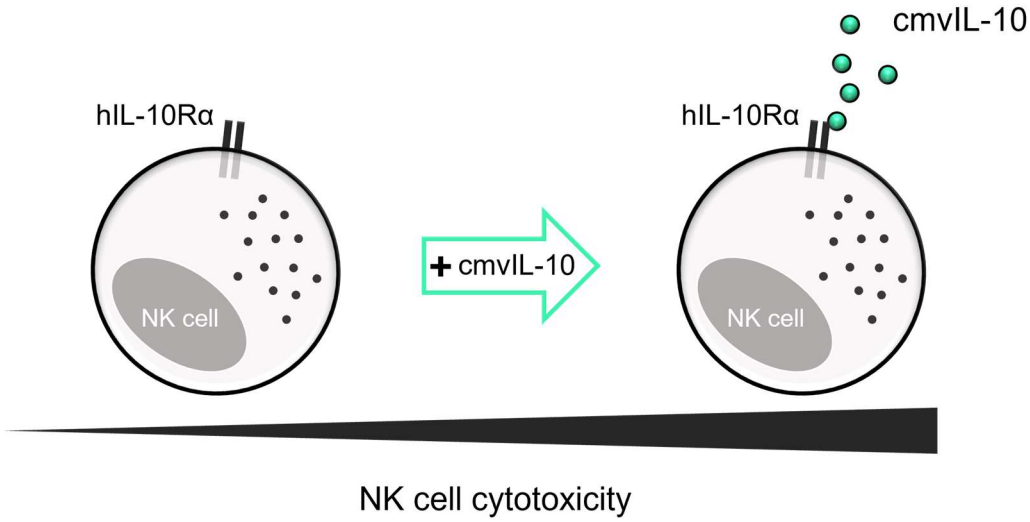

Figure 2.6| cmvIL-10 interacts with NK cell IL-10R and augments cytotoxicity

NK cells expressing hIL-10R exhibit modest increases in degranulation (measured by cell surface CD107a expression) after exposure to cmvIL-10 and CD16 stimulation.

3 Natural Killer Cells Adapt to HCMV Along a Functionally Static Phenotypic Spectrum in HIV-1 Infection

3.1 Abstract

Events related to HCMV infection drive accumulation of functionally enhanced $CD57^{pos}NKG2C^{pos}$ adapted NK cells. We investigated NK cell adaptation to HCMV along a proposed continuum progressing from acute activation through maturation and memory formation towards functional exhaustion. Acute exposure to conditioned medium collected 24 h after HCMV infection (HCMVsn) increased NK cell cytotoxicity for all HCMV-seronegative and seropositive donors tested, with mean 38 and 29% boosts in natural and antibody-dependent cell-mediated cytotoxicity (ADCC), respectively. Increases in NK cell cytotoxicity were completely abrogated by blocking type I interferon (IFN) receptors and equivalent responses occurred with exposure to IFN-α2 alone at the same concentration present in HCMVsn. To study longer term effects of HCMV infection, we focused on three groups of people living with HIV (PLWH) distinguished as HCMV-seronegative or HCMV-seropositive with either high (> 20%) or low (< 6%) fractions of their NK cells expressing NKG2C. The NK cells of all three PLWH groups responded to HCMVsn and IFN-α2 in a manner similar to the NK cells of either HCMV-seronegative or seropositive controls. Neither HCMV status, nor the extent of

phenotypic evidence of adaptation to HCMV infection significantly affected mean levels of ADCC or CD16-mediated NK cell degranulation and IFN-γ production compared between the PLWH groups. Levels of IFN-γ production correlated significantly with the fraction of NK cells lacking FcεRIγ (FcRγ), but not with the fraction of NK cells expressing NKG2C. There was negligible expression of exhaustion markers LAG-3 and PD-1 on NK cells in any of the groups and no significant difference between groups in the fraction of NK cells expressing Tim-3. The fraction of NK cells expressing Tim-3 was unaffected by CD16 stimulation. Relative to the total NK cell population, responses of Tim-3-expressing cells to CD16 stimulation were variably compromised in HCMV seronegative and seropositive groups. In general, NK cell function in response to signaling through CD16 was well preserved in HIV-1 infection and although HCMV had a clear effect on NK cell FcRγ and NKG2C expression, there was little evidence that the level of adaptation to HCMV infection affected CD16-dependent NK cell signaling in HIV-1 infection.

3.2 Introduction

Natural killer (NK) cells provide defense against malignancy and infection by recognizing certain alterations in affected cells and responding accordingly. Activation of NK cells against altered host cells is regulated by integration of positive and negative signals received through members of a relatively small set of germline encoded receptors. While NK cells recognize altered host cells and mediate effector functions without prior exposure to the altered cells, NK cell functional competence depends upon previous selective engagement of an inhibitory NK cell receptor with a class I human histocompatibility-linked antigen (HLA) [25]. This developmental education process enables NK cells to mediate cytotoxicity and/or produce cytokines when encountering altered host cells, while ensuring appropriate levels of self-tolerance [52, 282-284]. It also illustrates that NK cell function is not constitutive upon lineage determination, but dependent upon subsequent relay of specific signals. Selective NK cell education at this stage raises the possibility of further maturation within select subsets under conditions associated with infections or malignancy. Such NK cell maturation clearly takes place in murine cytomegalovirus (MCMV) infection. In this setting, cytokine production and specific receptor ligand interactions between Ly49H and MCMV m157 drive a subset of NK cells expressing the Ly49H activating receptor to selectively expand, persist at elevated levels and provide protection against subsequent MCMV infection [124-127, 285, 286].

The NK cell response to MCMV infection is the prototype system within which elements required to generate memory NK cells were first identified [118, 119, 128, 129, 287, 288]. Infection with human (H)CMV drives selective expansion of NK cells expressing the C-type lectin-like activating receptor NKG2C, prompting speculation that NKG2C on human NK cells functions in an analogous way to Ly49H on murine NK cells [106, 141, 289, 290]. In several HCMV-infected cell culture systems, selective expansion of NKG2C-expressing NK cells depends upon the presence of certain cytokines and interaction between NKG2C and HLA-E complexed with particular peptides [17, 145]. Peptides derived from the HCMV UL40 protein enable selective proliferation of NKG2C-expressing NK cells, including those from HCMV seronegative individuals [152]. The NKG2C-expressing NK cells expanded *in vivo* or *in vitro* by exposure to HCMV acquire phenotypic changes that impact their capacity for effector function [158, 159, 291]. This differentiation produces $CD57^{pos}$ NK cells with increased CD16 expression, lower levels of the associated FcεRIγ (FcRγ) adaptor protein, reduced natural cytotoxicity receptor (NCR) expression, and epigenetic changes in cytokine promoter regions [143, 144, 158, 159, 257]. The CD57/NKG2C-expressing NK cells are reportedly more responsive to stimulation through CD16, at least in terms of antibody-dependent cytokine production [158, 159, 291].

Aging, and various forms of immunological stress, including congenital, iatrogenic and HIV-1 infection, exacerbate HCMV-driven expansion of NKG2C-

expressing NK cells [140, 166, 204, 290, 292, 293]. It is common for HIV/HCMV coinfected individuals to have large NK cell fractions expressing CD57 and NKG2C, within which limitations to NK cell adaptation imposed by terminal differentiation or exhaustion might be evident [204]. Therefore, to assess NK cell function along a phenotypic spectrum of adaptation to HCMV infection, we studied healthy controls and PLWH displaying varying degrees of NK cell adaptation. This included a PLWH HCMV-seronegative group, an HIV/HCMV coinfected group with small fractions of $NKG2C^{pos}$ NK cells and an HIV/HCMV coinfected group with large fractions of $NKG2C^{pos}$ NK cells. Functional assessment began with exposure of NK cells from HCMV-seronegative controls to HCMV-related cytokines and extended across a wide range of NK cell adaption to HCMV infection as indicated by the accumulated fractions of phenotypically adapted NK cells.

3.3 Materials and Methods

3.3.1 Study Subjects and Sample Collection

This study was carried out in accordance with the recommendations of the Canadian Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans. The protocol was approved by the Health Research Ethics Authority of Newfoundland and Labrador, Canada. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Whole blood was collected with informed consent from healthy donors and peripheral blood mononuclear cells (PBMC) isolated by Ficoll-Paque (VWR, Mississauga, ON, Canada) density gradient centrifugation were suspended in lymphocyte medium consisting of RPMI-1640 supplemented with 10% fetal calf serum (FCS), 200 IU/mL penicillin/streptomycin (P/S), 0.01 M HEPES, 1% L-glutamine (all from Invitrogen, Carlsbad, CA, USA) and 2.0×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). PLWH recruited through the Newfoundland and Labrador Provincial HIV Clinic provided informed consent for whole blood collection, immunological studies, and researcher access to medical laboratory records. Freshly isolated PBMC were resuspended in freezing medium composed of lymphocyte medium supplemented to 20% FCS with 10% dimethyl sulfoxide and cooled at 1°C/min overnight to –80°C. Frozen PBMC were then maintained in liquid nitrogen until analysis. Cryopreserved PBMC were recovered overnight in lymphocyte medium at 37°C, 5% CO_2. Humoral and $CD8^{pos}$

T cell responses against HCMV were measured previously as described and data included with general characteristics of the PLWH study subjects [204, 294].

3.3.2 Generation of HCMVsn

MRC-5 cells (from Dr. Jules Doré, Memorial University of Newfoundland, St. John's, NL, Canada) were seeded at 1.25×10^5 cells per well in a 24-well plate and grown in DMEM supplemented with 10% FCS, 1% L-glutamine, 1% P/S, and 1 mM sodium pyruvate (Invitrogen) at 37°C with 5% CO_2. Forty-eight hours after seeding, MRC-5 cells were infected with either HCMV AD169 (Dr. Karen Biron, NIH HIV Reagent Program, Division of AIDS, NIAID, NIH) at multiplicity of infection (MOI) 0.025 for 1 h or a recombinant vaccinia virus expressing β-galactosidase (vSC8) at MOI 0.2 (Dr. Bernie Moss, NIH HIV Reagent Program, Division of AIDS, NIAID, NIH) or left untreated [295]. Conditioned medium from uninfected (CONsn) and AD169-infected (HCMVsn) MRC-5 cells was harvested and medium replaced in 24 h increments post infection for a total of 120 h. Conditioned medium from vSC8-infected MRC-5 cells (vSC8sn) was harvested and medium replaced in 24 h increments post infection for a total of 72 h. Supernatants were clarified for 10 min at 400 *g* to remove cell debris, frozen at –80°C in single-use aliquots and recovered on ice. Inactivated HCMVsn was generated by collecting conditioned medium from MRC-5 cells that were exposed to the same amount of ultraviolet (UV)-irradiated AD169 (41 watts for 1 h at 30 cm).

3.3.3 Cytotoxicity and Redirected Lysis Assays

K562 (ATCC® CCL 243™), P815 (ATCC® TIB-64™), and HLA-B27-transfected C1R (C1R-B27; Dr. Kelly McDonald, University of Manitoba, Winnipeg, MB, Canada) cell lines were propagated in lymphocyte medium at 37°C with 5% CO_2 and maintained in log phase for ^{51}Cr labeling and cytotoxicity assays. K562, C1R-B27, or P815 cells were labeled for 90 min with 100 µCi $Na_2{}^{51}CrO_4$ (PerkinElmer, Akron, OH, USA). C1R-B27 cells were incubated 30 min with 1 µg/mL monoclonal antibody (mAb) against pan HLA-I (W6/32, ATCC® HB-95™) to sensitize cells to antibody-dependent cellular cytotoxicity (ADCC). For interferon (IFN)-α/β receptor blocking experiments, PBMC were pretreated 30 min with 5 µg/mL of mAb (clone MMHAR-2) against the IFN-α/β receptor chain 2 (IFNAR) prior to their addition in a cytotoxicity assay as described [296]. When indicated, purified active recombinant human IFN-α2 (US Biological, Salem, MA, USA) was used at a final concentration of 20 pg/mL. Receptor-triggered cytotoxicity was measured by adding 3 µg/mL soluble IgG isotype control (Ag8) or mAb (clones in parentheses) against human NKp30 (210845), NKG2C (134591), or NKG2D (149810) from R&D Systems (Minneapolis, MN, USA) and NKp44 (9E2) or NKp46 (P44-8) from BioLegend (San Diego, CA, USA). PBMC were incubated in microtiter plates in a final volume of 300 µL for 5 h with 5×10^3 ^{51}Cr-labeled K562, C1R-B27, or P815 target cells/well at various effector to target (E:T) ratios with either CONsn, vSC8sn or HCMVsn at a final dilution of 1 in 5. ^{51}Cr release was measured in 125 µL supernatants collected from each well on

a Wallac 1480 Wizard gamma counter. Control wells for spontaneous lysis contained target cells in medium alone while target cells in maximum lysis wells were treated with 1 N HCl. Percent specific lysis was calculated by (experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(maximum ^{51}Cr release – spontaneous ^{51}Cr release) × 100.

3.3.4 Macromolecular Clearance of Conditioned Media

Intact HCMV particles were cleared from conditioned media by treating CONsn or HCMVsn with 0.06 μg/mL monoclonal anti-gB (Dr. Lucy Rasmussen, NIH AIDS Reagent Program) for 2 h at 4°C in a tube rotator followed by 30 min incubation with 4 × 10^6 sheep anti-mouse IgG Dynabeads® (ThermoFisher) per milliliter of conditioned media. Beads and virus particles were pelleted for 1 min at 1,000 *g* after which time supernatants were decanted and stored at –70°C for functional experiments. To remove insoluble materials from conditioned media, samples were ultracentrifuged using a Sorvall TH-641 rotor at 100,000 *g* for 16 h at 4°C.

3.3.5 Multiplex Array

Concentrations of cytokines and chemokines within culture medium collected from MRC-5 cells untreated or infected with AD169 or vSC8 (see above) were measured by Milliplex (Millipore, Merck KGaA, Darmstadt, Germany) according to the manufacturer instructions and analyzed on the BioPlex-200 (Bio-

Rad, Mississauga, ON, Canada). Samples were run in duplicate and incubated overnight to improve the sensitivity of detection as previously described [297].

3.3.6 Flow Cytometry

Human PBMC were stained using directly conjugated mAb against human CD3 (BW264/56), CD56 (REA196), CD57 (TB03), from Miltenyi Biotec (San Diego, CA, USA), Tim-3 (F38-2E2) from BioLegend and NKG2C (134591) from R&D Systems. Cells were stimulated with 1 µg anti-CD16 mAb (3G8; BioLegend) per 10^6 PBMC and prepared for intracellular staining by adding brefeldin A (Sigma-Aldrich) 1 h after the start of incubation to a final concentration of 10 µg/mL and continuing the incubation for an additional 4 h. NK cell degranulation was detected by introducing directly conjugated anti-CD107a mAb (H4A3; BioLegend) at 0.25 µg per 10^6 PBMC at the time of brefeldin A addition. Cells were fixed and permeabilized after 5 h incubation using the Inside Stain Kit (Miltenyi Biotec) as per manufacturer's instructions and then stained with directly conjugated polyclonal Ab against human FcRγ from MilliporeSigma (Burlington, MA, USA) and anti-human IFN-γ mAb (4S.B3) from eBioscience (San Diego, CA, USA). Non-viable cells were excluded by fixable live/dead stain (Invitrogen) as per manufacturer's instructions. Data were acquired using a MoFlo Astrios EQ flow cytometer and data analyses and illustration performed using Kaluza software (both Beckman Coulter, Brea, CA, USA).

3.3.7 Statistical Analysis

Statistical analyses were performed using GraphPad Prism software version 5 with two-sided *P*-values < 0.05 considered significant. Normality of data distributions were assessed with Kolmogorov–Smirnov, Shapiro–Wilk and D'Agostino and Pearson tests. Student's paired or unpaired *t*-tests were used for group comparisons when data were normally distributed and non-parametric Wilcoxon paired signed-rank test or Mann–Whitney *U*-tests used otherwise as appropriate.

3.4 Results

3.4.1 Soluble Factors Released Early in HCMV Infection Augment NK Cell Cytotoxicity

Natural killer cells are considered important effectors during herpesvirus infections. To examine how NK cells respond to factors released early in HCMV infection, we collected supernatant from uninfected (CONsn) and AD169-infected (HCMVsn) MRC-5 fibroblasts over 24 h intervals up to 120 h post infection. Supernatant was collected from vSC8-infected (vSC8sn) MRC-5 fibroblasts over 24 h intervals up to 72 h post infection. We then exposed NK cells to these supernatants in the presence of K562 target cells in 5 h ^{51}Cr release assays to assess the effects of acute exposure on NK cell cytotoxicity. The vSC8sn collected from any of the three time points had no effect on NK cell cytotoxicity against K562 cells (data not shown, n = 3) compared to CONsn. In contrast, HCMVsn collected within the first 24 h after infection increased NK cell target killing by 50% relative to cytotoxicity in the presence of CONsn (Figure 3.1 A). While this property of HCMVsn persisted up to 48 h post infection with conditioned media collected over a cumulative 48 h (data not shown), its production peaked during the first 24 h of infection as exposure to HCMVsn collected between 24 and 48 h post infection had little effect on NK cell activity (Figure 3.1 A). Increased NK cell cytotoxicity following exposure to HCMVsn collected at 24 h occurred for all 12 donors tested, with a mean 38% boost in natural

cytotoxicity (Figure 3.1 B) and 29% increase in NK cell ADCC (Figure 3.1 C). An equal number of HCMV-seropositive and seronegative donors, differentiated by symbol shading, are shown in Figure 3.1, with no apparent differences in responsiveness to stimulation with HCMVsn related to previous HCMV exposure. Factor(s) present in HCMVsn and primarily produced the first 24 h after infection cause a rapid significant increase in NK cell natural cytotoxicity and ADCC.

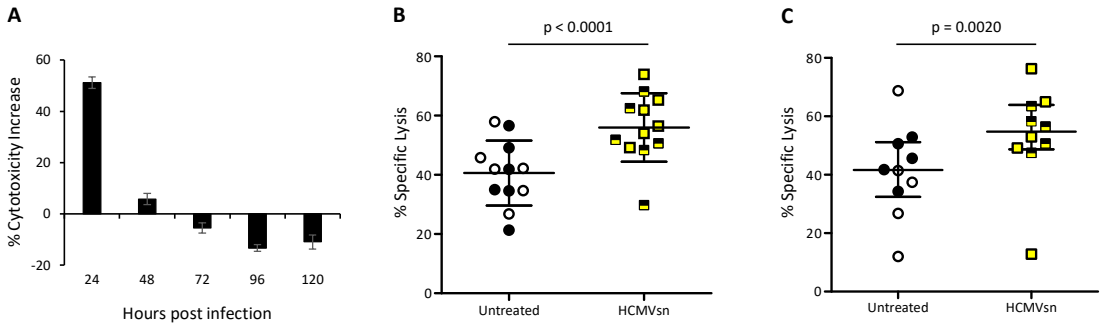

Figure 3.1| Effect of HCMVsn on NK cell cytotoxic responses of healthy controls

(A) CONsn or HCMVsn was collected at 24 h intervals up to 120 h post infection, diluted 1:5 and effects on NK cytotoxicity tested using fresh PBMC from healthy controls in 5 h ^{51}Cr release assays. NK cell cytotoxicity with either CONsn or HCMVsn against (B) K562 cells (n = 12) or (C) anti-HLA-I-coated C1R-B27 cells (n = 10) was measured over 5 h at E:T 60:1. Subjects seropositive for HCMV are represented with filled black circles or yellow squares half-shaded black. Error bars in (A) represent mean ± standard deviation (SD) of two replicates, (B) mean ± SD with Student's paired *t*-test used for comparisons between conditions and (C) median with interquartile range (IQR) with Wilcoxon signed-rank test used to estimate the probability of a difference between conditions. Percent cytotoxicity increase in (A) was calculated as [(% specific lysis with HCMVsn – % specific lysis with CONsn) / % specific lysis with CONsn] × 100 from paired data performed in duplicate.

3.4.2 HCMVsn Augments Cytotoxicity Triggered Through Multiple NK Cell Activating Receptors

As HCMVsn increased both NK cell natural cytotoxicity and CD16-dependent responses, we investigated the effect of HCMVsn on cytotoxicity through other common NK cell activating receptors. We probed NKG2C, NKG2D, NKp30, NKp44, and NKp46 using a redirected lysis assay in which murine FcR-expressing P815 cells orient mAb specific for particular activating receptors to enable selective NK cell activation. A representative summary of target cell lysis by natural cytotoxicity, ADCC and redirected killing through individual activating receptors is shown in Figure 3.2 A. Using an IgG isotype control antibody, there was little P815 cell lysis and no significant difference from control conditions when HCMVsn was present during the 5 h assay (Figure 3.2 B). By introducing mAb against individual NK cell activating receptors, we observed that HCMVsn significantly increased P815 cell lysis through NKG2C (Figure 3.2 C), NKG2D (Figure 3.2 D), and the NCRs NKp30 and NKp46, but not NKp44 (Figures 3.2 E–G). These data demonstrate that HCMVsn sensitizes NK cells for cytotoxicity triggered through CD16, most NCRs and through activating CLRs. As with the increased killing of K562 and ADCC, there was no apparent difference in the responses of NK cells from HCMV-seropositive and seronegative subjects discriminated on the graphs by different symbols.

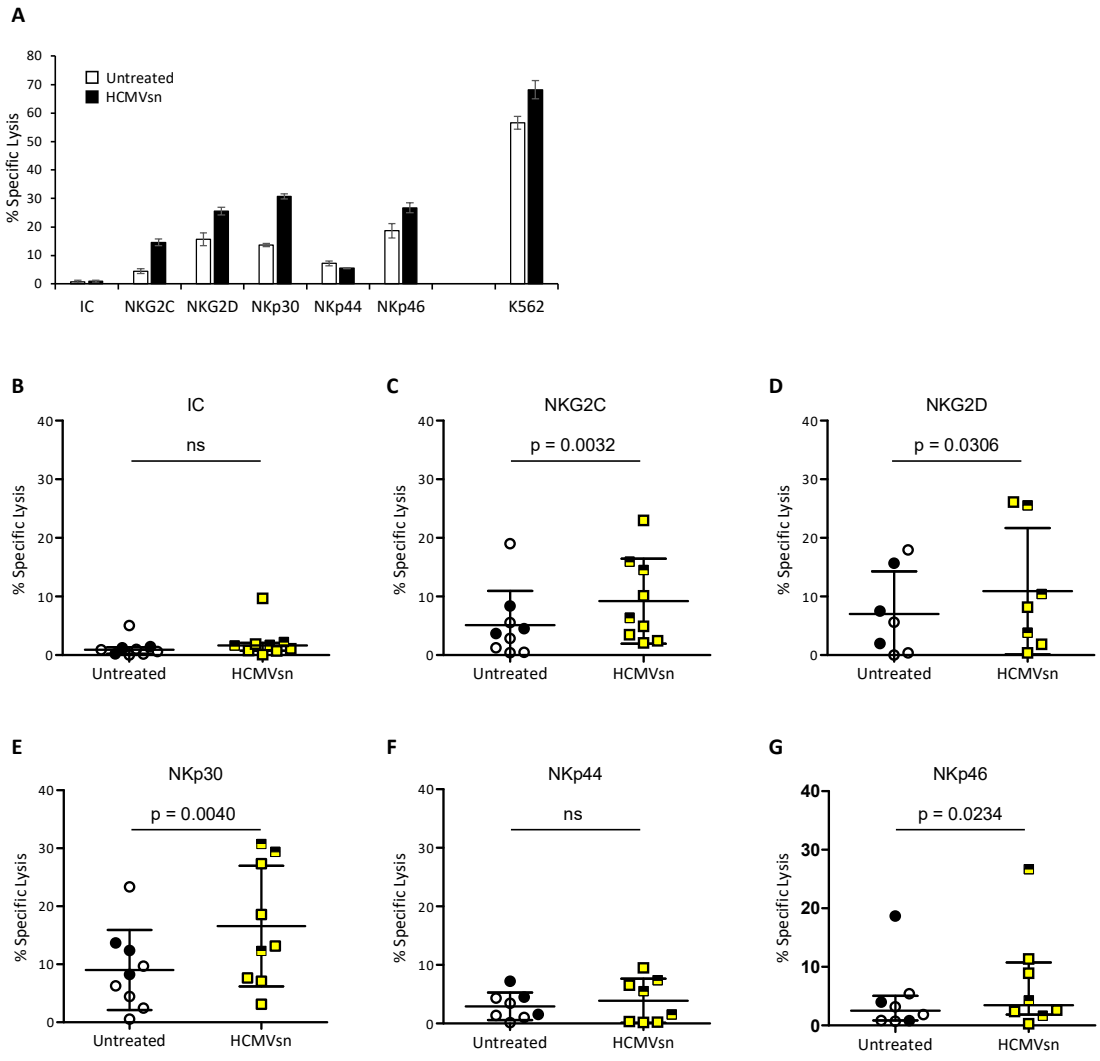

Figure 3.2| Effect of HCMVsn on NK cell cytotoxicity directed through different receptors

(A) Natural cytotoxicity (K562 cells), and redirected lysis of P815 cells in the presence of NK receptor-specific mAb for a representative subject with or without HCMVsn was measured over 5 h. Error bars represent mean ± standard error of the mean with three replicates. Redirected P815 cell lysis was measured at E:T 30:1 with 3 μg/mL (B) isotype control (IC), (C) anti-NKG2C, (D) anti-NKG2D, (E) anti-NKp30, (F) anti-NKp44, or (G) anti-NKp46 mAbs. Assays represented in plots (B–G) were performed in triplicate for 7–9 individuals. Subjects seropositive for HCMV are represented with filled black circles or yellow squares half-shaded black. Error bars for isotype control and anti-NKp46 plots represent median with IQR and the probability of a difference between conditions was assessed by Wilcoxon signed-rank test. All other bars represent mean ± SD with comparison between conditions by Student's paired *t*-test.

3.4.3 IFN-α Produced During HCMV Infection Enhances NK Cytotoxicity

While a cell-free component of HCMVsn increased NK cell pan cytotoxic function, it was unclear whether this effect was mediated through macromolecular interactions with different NK cell activating receptors or by cytokines. Augmented NK cell cytotoxicity was sustained following clearance of macromolecular material by HCMVsn ultracentrifugation (Figure 3.3 A) or following selective removal of HCMV particles by magnetic beads and a mAb against HCMV glycoprotein gB (Figure 3.3 B). Inactivating HCMV before its addition to MRC-5 cells (UV HCMVsn) prevented the increase in NK cell cytotoxicity observed with replication competent HCMVsn (Figure 3.3 C), indicating that a soluble product from HCMV-infected cells increases NK cell cytotoxicity through multiple activating receptors.

To identify potential factors present in HCMVsn that increase NK cell cytotoxicity, we measured analytes using a bead-based multiplex assay. Luminex analysis of HCMVsn revealed increased concentrations of monocyte chemoattractant protein and macrophage colony stimulating factor (data not shown), IL-6, IL-8, IL-15, IFN-α2, and IFN-β compared to CONsn (Figure 3.3 D left panel). Modest concentrations of biologically active IL-15 (10 pg/mL; data not shown) were also detected. Analysis of vSC8sn revealed increased concentrations of IL-6, IL-8, and IL-15 with a marginal increase in IFN-β at the 48 h time point (Figure 3.3 D right panel). Blocking stimulation through IFNAR alone with a specific mAb fully prevented any increase in NK cell activity in response to HCMVsn (Figure 3.3

E). This blocking effect was specific for type I IFNs as increased NK cell activity in response to IL-2 was maintained in the presence of the same amount of mAb against IFNAR (data not shown). HCMV-infected fibroblasts produced both IFN-α2 and IFN-β 80 and 450 pg/mL, respectively, data not shown) during the first 24 h of infection. To determine whether the amount of IFN-α2 detected in HCMVsn during the first 24 h of infection could alone mediate the same increases in NK cell cytotoxicity as HCMVsn, we compared NK cell natural and antibody-dependent cytotoxic activity in response to HCMVsn or purified recombinant IFN-α2. Exposure to similar concentrations of IFN-α2 (20 pg/mL) as added with HCMVsn diluted 1 in 5 in ^{51}Cr release assays increased NK cell natural cytotoxicity (Figure 3.3 F) and ADCC (Figure 3.3 G) to an extent indistinguishable from the increases mediated by HCMVsn. Thus, stimulation through the type I IFN receptor alone rapidly sensitized NK cells to natural and antibody-dependent cytotoxicity and the acute increase in natural cytotoxicity and ADCC for NK cells from healthy controls mediated by soluble factors in HCMVsn is replicated with IFN-α2 alone at levels present in HCMVsn.

The increase in NK cell cytotoxicity following exposure to HCMVsn occurred with both HCMV^{pos} and HCMV^{neg} healthy donors indicating that the NK cell response to cytokines produced by HCMV-infected cells is independent of previous sensitization from *in vivo* HCMV infection (Figures 3.1, 3.2). To investigate such responses along a continuum of NK cell adaptation to HCMV infection, we tested

effects of HCMVsn and IFN-α2 with PLWH whose NK cells spanned a broad range of adaptation in response to HCMV infection.

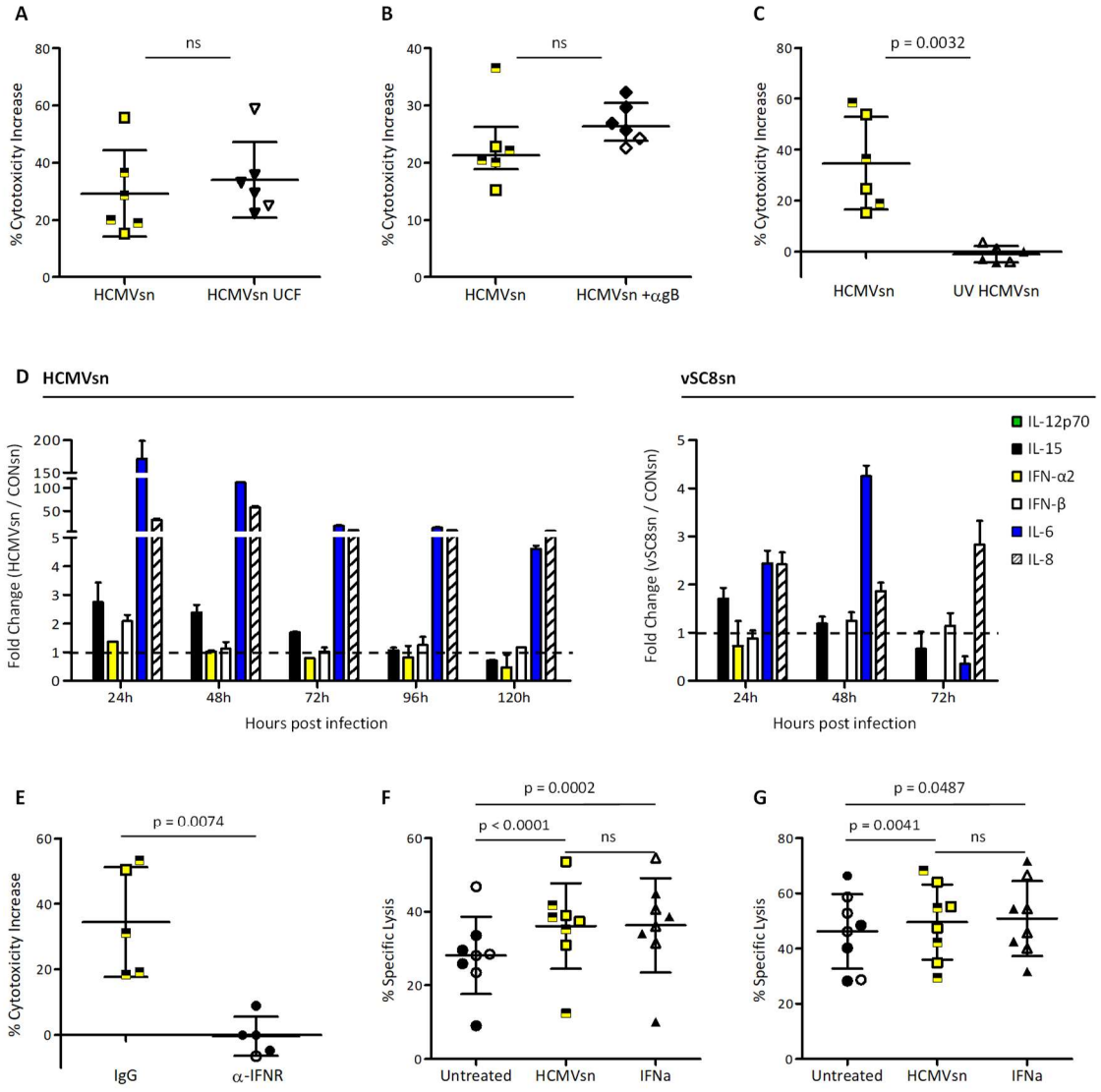

Figure 3.3| Identification of cytokines produced by HCMV-infected MRC-5 cells

The effect of HCMVsn on NK cell cytotoxicity was assessed by ^{51}Cr release using K562 target cells at E:T 30:1 after removal (A) of insoluble materials by ultracentrifugation (HCMVsn UCF) or (B) HCMV particles with monoclonal anti-gB and goat anti-mouse IgG Dynabeads. (C) Ultraviolet-light inactivated virus was used to generate UV HCMVsn and its effect on NK cell cytotoxicity was

measured as above (n = 6). Subjects seropositive for HCMV are represented with filled black circles or triangles and yellow squares half-shaded black. Error bars in (A, C) represent mean ± SD with conditions compared using Student's paired *t*-test and (B) median with IQR and conditions compared by Wilcoxon signed-rank test. (D) Luminex analysis of HCMVsn collected at 24 h intervals over 120 h (left panel) or vSC8sn collected at 24 h intervals over 72 h (right panel) compared IL-6, IL-8, IL-15, IL-12p70, IFN-α2, and IFN-β concentrations. Data shown represents fold change in the amount of cytokine detected in HCMVsn or vSC8sn/CONsn performed in duplicate ± SD. (E) PBMC (n = 5) were pretreated with 5 μg/ml of IgG control or anti-IFNAR before incubation with CONsn or HCMVsn in a 5 h ^{51}Cr release assay. Bars represent mean ± SD performed in duplicate with conditions compared using Student's paired *t*-test. The effect of 20 pg/mL recombinant IFN-α2 was compared to that of HCMVsn on NK cell (F) natural cytotoxicity and (G) ADCC (n = 8). Bars represent mean ± SD, performed in triplicate with conditions compared using Student's paired *t*-test. Data in (A–C, E–G) represent percent cytotoxicity increase or percent specific lysis, respectively, calculated as described in the legend for Figure 4.3.1.

3.4.4 HCMVsn and IFN-α2 Enhance NK Cell ADCC in PLWH

After establishing that NK cells from healthy donors respond rapidly, robustly and independent of HCMV serostatus to IFN-α2 produced by HCMV-infected fibroblasts, we next studied NK cell responses from HCMV^{neg} and HCMV^{pos} individuals within a study cohort of PLWH. Since adapted NK cells exhibit enhanced CD16-mediated effector responses, we measured the impact of HCMVsn and IFN-α2 on NK cell ADCC in HIV-1 infection and compared baseline levels of ADCC between three groups reflecting a broad range of NK cell adaptation to HCMV infection. General characteristics of the individuals and comparisons between HCMV-seronegative and seropositive groups are shown in Table 3.1. We selected three groups of PLWH, two distinguished by high (> 20%) versus low (< 6%) fractions of NK cells expressing NKG2C and a third distinguished by seronegative HCMV status (Table 3.1). Nine of the 28 age- and sex-matched PLWH selected for this study were HCMV-seronegative. The efficacy of antiretroviral therapy was

roughly equivalent between groups as indicated by undetectable HIV-1 viral loads (< 50 copies HIV-1 RNA/mL plasma). Neither nadir, nor current $CD4^{pos}$ T cell counts, indicative of past disease progression and present immunological status, respectively, differed significantly between groups. All donors had a robust fraction of mature ($CD57^{pos}$) NK cells, independent of HCMV status or fraction of their NK cells expressing NKG2C. However, the percentage of NK cells expressing CD57 was significantly lower in the $NKG2C^{lo}$ group than in the $NKG2C^{hi}$ group ($p = 0.0133$, Mann–Whitney *U*-test), suggesting lesser overall NK cell maturation in the $NKG2C^{lo}$ group (Table 3.1). There were no significant differences in either humoral (anti-CMV IgG levels) or cellular (% $CD8^{pos}$ T cells specific for HCMV pp65 and IE-1) immune responses against HCMV between the $HCMV^{pos}$ groups with high or low fractions of NK cells expressing NKG2C.

Table 3.1| PLWH Study Subject Characteristics

	Age range	Sex	α-CMV IgG (OD)*	CMV-specific $CD8^{pos}$ T cells (%)†	$CD4^{pos}$ T cells‡	Nadir§	HIV VL‖	$NKG2C^{pos}$ NK cells (%)	$FcR\gamma^{neg}$ NK cells (%)	$CD57^{pos}$ NK cells (%)	$Tim\text{-}3^{pos}$ NK cells (%)
$HCMV^{neg}$	44–56	7♂	0.054	0.1	714	51	1.4	1.03	3.95	22.54	2.36
		2♀	0.055	0.1	640	286	2.3	2.68	22.19	11.89	8.22
			0.036	0.0	1320	81	1.3	2.84	7.07	54.79	5.31
			0.044	0.0	700	324	1.3	2.35	12.32	20.77	8.28
			0.029	0.1	966	154	1.3	2.99	7.63	57.54	0.53
			0.048	0.0	429	85	1.3	3.70	9.43	55.95	5.55
			0.080	0.0	667	154	1.3	4.41	20.72	54.68	4.95
			0.027	0.0	912	658	1.3	3.31	7.35	50.74	6.56
			0	0.0	833	490	1.3	2.37	30.07	61.86	0.40
x̄	51.8		0.040	0.0	798	254	1.4	2.85	13.41	43.42	4.68
$HCMV^{pos}$ $NKG2C^{lo}$	33–58	7♂	1.265	4.7	1085	314	1.3	1.96	61.14	41.84	3.58
		2♀	1.418	2.2	638	192	1.7	4.76	55.75	26.74	4.2
			1.324	5.0	946	245	1.3	1.46	12.60	54.96	6.61
			0.686	1.0	1026	229	1.3	0.30	6.74	37.88	3.8
			1.047	2.2	492	93	1.3	4.72	12.76	26.46	6.69
			1.567	1.6	624	6	1.3	0.47	25.97	32.04	5.64
			0.846	2.2	957	192	1.6	5.92	26.07	39.83	8.56
			0.767	0.7	725	660	1.3	1.72	19.23	58.60	8.37
			0.670	1.9	903	169	1.3	1.81	71.70	10.50	6.25
x̄	47.8		1.070	2.4	822	233	1.4	2.57	32.44	36.54	5.97
$HCMV^{pos}$ $NKG2C^{hi}$	36–60	8♂	1.552	1.6	588	25	1.3	46.25	46.25	42.97	6.82
		2♀	0.897	0.2	570	206	1.6	44.21	86.07	79.36	4.25
			1.489	1.0	285	16	ND	50.81	80.85	45.91	7.03
			0.993	0.8	760	380	1.3	41.07	47.98	68.19	8.34
			0.810	0.6	780	245	1.3	27.05	77.83	54.35	3.24
			0.723	0.4	760	63	1.3	24.4	30.63	40.37	9.68
			1.769	0.2	1254	276	1.3	27.61	44.42	39.01	7.02
			0.579	7.2	792	108	1.3	46.48	60.22	61.39	2.74
			1.275	2.3	748	407	1.3	42.66	42.07	66.01	6.05
			1.676	4.0	455	416	1.3	42.85	57.09	52.93	5.53
x̄	48.1		1.180	1.9	699	214	1.33	39.34	57.34	55.05	6.07

*Optical density (OD) by ELISA measuring α-CMV IgG (plasma diluted 1:500) against lysate from HCMV AD169-infected MRC-5 cells (34).
†Percentages of CMV-specific $CD8^{pos}$ T cells identified by stimulation with overlapping peptides from CMV pp65 and IE-1 proteins followed by detection of intracellular IFN-γ (34, 35).
‡Number of $CD4^{pos}$ T cells per microliter of peripheral blood at time of testing.
§Lowest recorded $CD4^{pos}$ T cell count per microliter of peripheral blood.
‖Log_{10} copies HIV RNA per milliliter of plasma at time of testing.
ND, not determined.

The HCMV^{neg} individuals and those with high or low fractions of NKG2C-expressing NK cells were grouped to assess responsiveness of NK cells from HCMV^{neg} and HCMV^{pos} PLWH to HCMV-related cytokines. The mean increase in ADCC mediated by HCMV-related cytokines (24%) was similar to that seen in the HIV-1-naïve group and was not significantly different from the mean 14% increase elicited by recombinant IFN-α2 alone (Figure 3.4 A). Neither HIV-1-infection nor adaptation to HCMV infection appeared to affect NK cell responsiveness with acute exposure to IFN-α2. Median levels of NK cell ADCC measured from PLWH were also unrelated to either HCMV serostatus or NK cell adaptation to HCMV infection (Figure 3.4 B). Adaptation to HCMV infection had little effect on the capacity of NK cells from PLWH to respond to acute IFN-α2 exposure or to mediate ADCC.

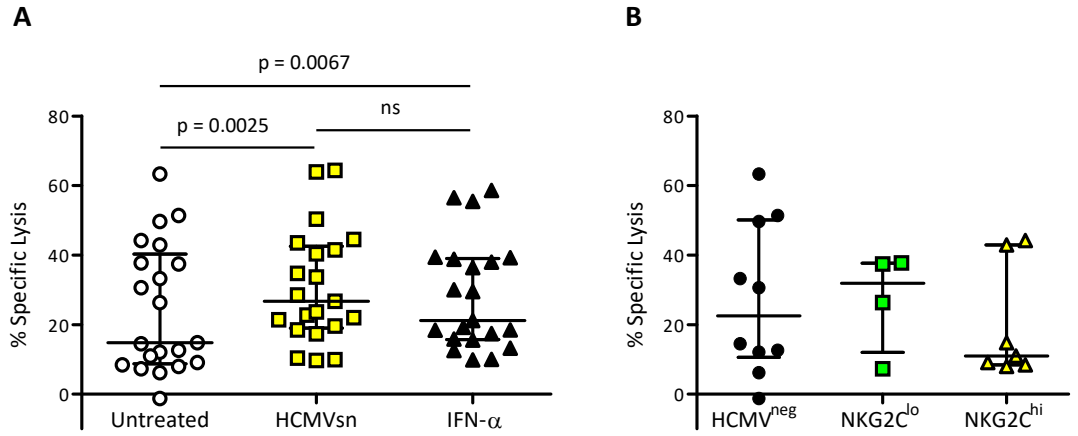

Figure 3.4| Effects of HCMVsn and IFN-α2 on ADCC of NK cells from PLWH

(A) ADCC within the PLWH group was measured using PBMC and W6/32 opsonized C1R-B27 target cells (E:T 30:1) in 5 h ^{51}Cr release assays in the presence of a 1:5 dilution of HCMVsn or 20 pg/mL recombinant human IFN-α2 and compared to untreated conditions (n = 21). (B) Baseline ADCC levels were compared between the three groups of PLWH distinguished by HCMV serostatus and fraction of NK cells expressing NKG2C. Error bars represent median with IQR, performed in duplicate with conditions compared using Wilcoxon signed-rank test.

3.4.5 Long-Term Adaptation of NK Cells from PLWH to HCMV Infection

To study longer-term effects of HCMV infection, including the possibility of progression to exhaustion, we compared features of the three groups of PLWH distinguished by high versus low NK cell fractions expressing NKG2C or by seronegative HCMV status (Table 3.1). The flow cytometry gating strategy for a representative HIV/HCMV coinfected donor is shown in Figure 3.5 A. Median NKG2C expression levels were similar between the HCMV^{neg} and NKG2C^{lo} groups (Figure 3.5 B), however, the influence of adaptation to HCMV within the NKG2C^{lo} group was evident from the lower mean fraction of NK cells expressing FcRγ (Figure 3.5 C) and strong correlation between loss of FcRγ expression and level of HCMV-

specific antibodies (Spearman r = 0.5539, *P* = 0.0022 Table 3.1). Loss of the FcRγ signaling adaptor subunit in parallel with expansion of NK cells expressing NKG2C is apparent from the progressive increase in percentage of FcRγ^{neg} NK cells across the three groups and by the significant correlation between fractions of NK cells expressing NKG2C and fractions lacking FcRγ (Figures 3.5 C, D). Despite this general progression and significant correlation, three individuals living with HIV-1 within the NKG2C^{lo} group with < 6% of their NK cells expressing NKG2C and > 50% lacking FcRγ illustrate that loss of FcRγ by NK cells is not inexorably linked to NKG2C expression (Figure 3.5 C, Table 3.1).

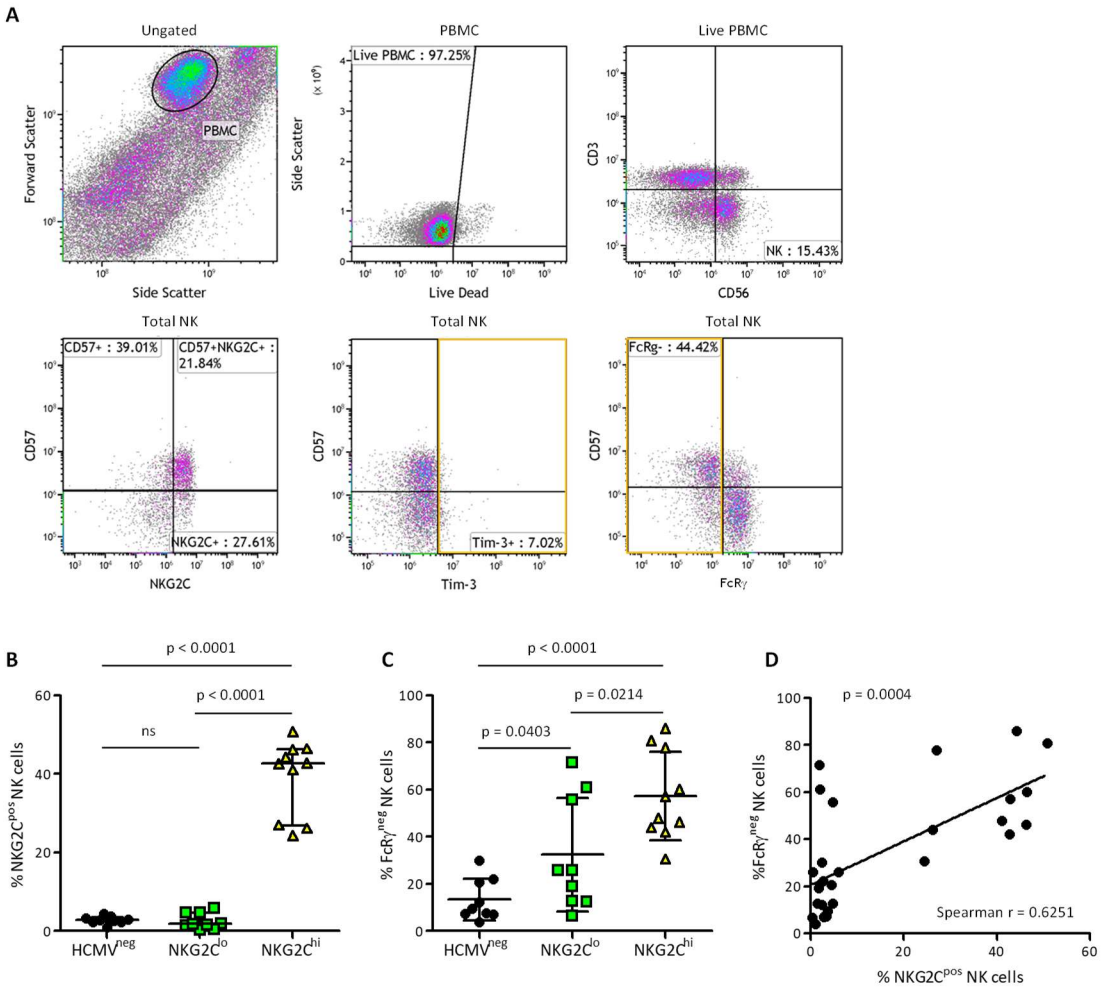

Figure 3.5| Phenotypic characterization of NK cells from PLWH with different levels of adaptation to HCMV infection

To identify the NK cell population, (A) PBMC were selected and dead cells excluded. NK cells were identified by expression of CD56 in the absence of CD3 and further characterized for percentage expressing NKG2C, CD57, Tim-3, and FcRγ in a representative plot. Summary plots depict the percentage of total NK cells that are positive for (B) NKG2C or (C) FcRγ. Individual donors were grouped based on HCMV serostatus ($HCMV^{neg}$) and fraction of NK cells expressing NKG2C within the $HCMV^{pos}$ donors ($NKG2C^{lo}$ or $NKG2C^{hi}$). Correlation between NK cell NKG2C and FcRγ expression was assessed in (D) with Spearman correlation coefficient (r) calculated and the probability of a significant correlation shown on the graph. Error bars in (B) for the $HCMV^{neg}$ group represent median with IQR, while all others represent mean ± SD. Groups were compared using Mann–Whitney *U*-tests.

3.4.6 Preservation of NK Cell Antibody-Dependent Effector Functions in PLWH

An association between NK cell adaptation to HCMV infection and increased capacity for antibody-dependent IFN-γ production was previously reported [161, 162]. Since ADCC responses against antibody-coated C1R-B27 cells by PLWH measured by ^{51}Cr release assays were similar, irrespective of HCMV status and NKG2C expression levels, we next measured and compared IFN-γ production and CD107a expression of NK cells in the three groups in response to CD16 stimulation.

Activation through CD16 induced robust NK cell IFN-γ responses (representative plots in Figure 3.6 A), the magnitude of which directly correlated with the percentage of NK cells lacking FcRγ (Figure 3.6 B). This is exemplified by one subject with over 80% NK cells lacking FcRγ and over 40% producing IFN-γ in response to stimulation through CD16 (Figure 3.6 B). In contrast, we found no significant correlation between the fraction of FcRγ^{neg} NK cells and fraction of NK cells expressing NKG2C or between NK cell FcRγ expression and degranulation, as measured by CD107a expression following CD16 stimulation. When we compared CD16-stimulated degranulation and IFN-γ production between HCMV^{neg}, NKG2C^{lo}, and NKG2C^{hi} groups, there were no significant differences in percentages of CD107a^{pos} NK cells (Figure 3.6 C), IFN-γ^{pos} NK cells (Figure 3.6 D) or polyfunctional NK cells doubly positive for CD107a and IFN-γ (Figure 3.6 E). All groups' NK cells responded robustly to CD16 stimulation, irrespective of NKG2C expression levels or

HCMV serostatus, illustrating HCMV-independent preservation of antibody-dependent NK cell responses in chronic HIV-1 infection.

To assess NK cell progression toward an exhausted phenotype, expression of PD-1, LAG-3, and Tim-3 was assessed on NK cells within the three defined groups. Levels of PD-1 and LAG-3 were low to undetectable on NK cells (data not shown), but substantial fractions of NK cells within each group expressed Tim-3 (Figure 3.5 A and Table 3.1)

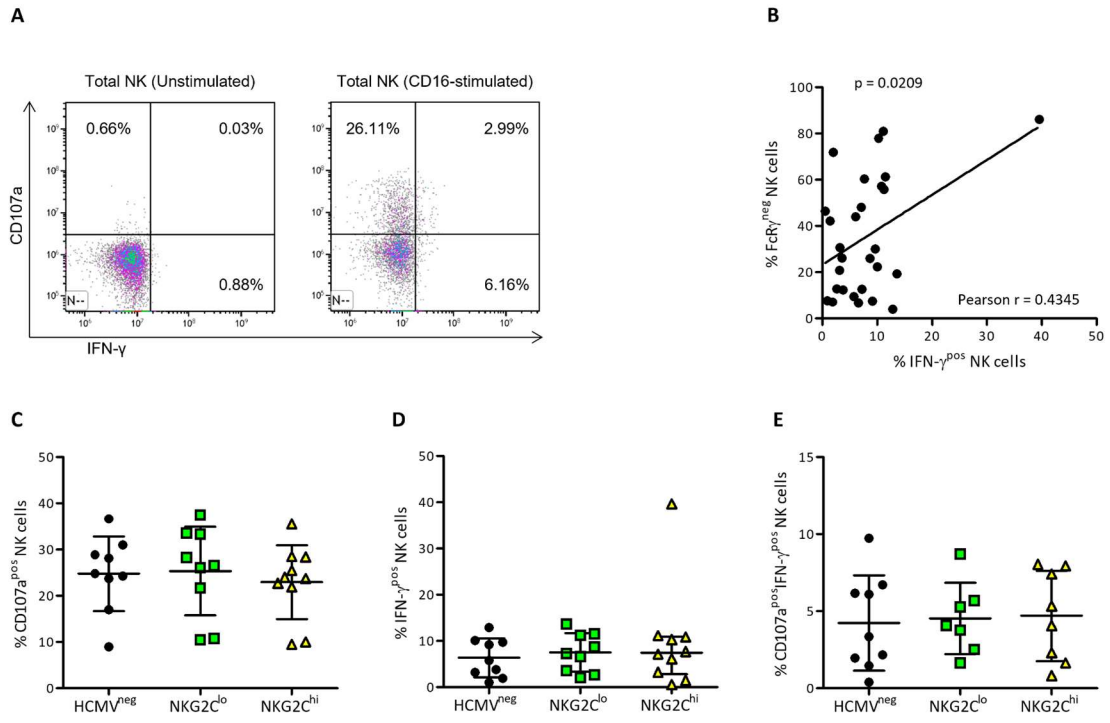

Figure 3.6| Effects of NK adaptation to HCMV infection on CD16-dependent degranulation and IFN-γ production in HIV-1 infection

NK cells were untreated or stimulated for 5 h with anti-CD16 (3G8) after which time IFN-γ production and CD107a expression was assessed by flow cytometry. Representative plots with gating on total NK cells as in Figure 3.5 show (A) IFN-γ and CD107a expression on resting (left panel) and CD16-stimulated NK cells (right panel). For each donor, percent NK cells positive for (B) IFN-γ was plotted versus percent FcRγ^{neg} cells and correlation between the magnitude of NK cell IFN-γ responses and fraction of NK cells lacking FcRγ was assessed using Pearson correlation coefficient (r) with the probability (p) of a significant correlation shown on the graph. The percentage of NK cells positive for (C) CD107a, (D) IFN-γ, or (E) doubly positive for CD107a and IFN-γ was measured for each donor (n = 7–10) and grouped as in Figure 3.5. Error bars represent mean ± SD where data was normally distributed and median with IQR in all other cases.

3.4.7 Antibody-Dependent Effector Functions of Tim-3^{pos} NK Cells From PLWH

As Tim-3 receptors were expressed at levels readily measurable by flow cytometry, we compared the extent of Tim-3 expression on NK cells within the three groups and the functional capacity of the Tim-3^{pos} subset. The percentage of NK cells expressing Tim-3 ranged from 0 – 10%, did not differ significantly between groups (Table 3.1) and CD16-stimulation did not induce NK cell Tim-3 expression (data not shown). The percentage of Tim-3^{pos} NK cells responding to CD16 stimulation with IFN-γ expression, CD107a expression, or both, was compared with the percentage of total NK cells responding likewise. Tim-3-expressing NK cells from HCMV^{neg} PLWH degranulated to a lesser extent than the general NK cell population but showed no apparent deficit in IFN-γ production (Figure 3.7 A).

In contrast, Tim-3^{pos} NK cells from HIV/HCMV coinfected donors responded similarly to the general NK cell population in terms of CD107a expression but were less likely to produce IFN-γ in response to stimulation through CD16 (Figure 3.7 B). The significance of Tim-3 expression on NK cells may vary with respect to the function studied and extent of adaptation to HCMV infection. There were no significant differences between HCMV^{neg}, NKG2C^{lo}, and NKG2C^{hi} groups in percentage of Tim-3^{pos} NK cells expressing CD107a or IFN-γ in response to stimulation through CD16 Figure 3.7 C.

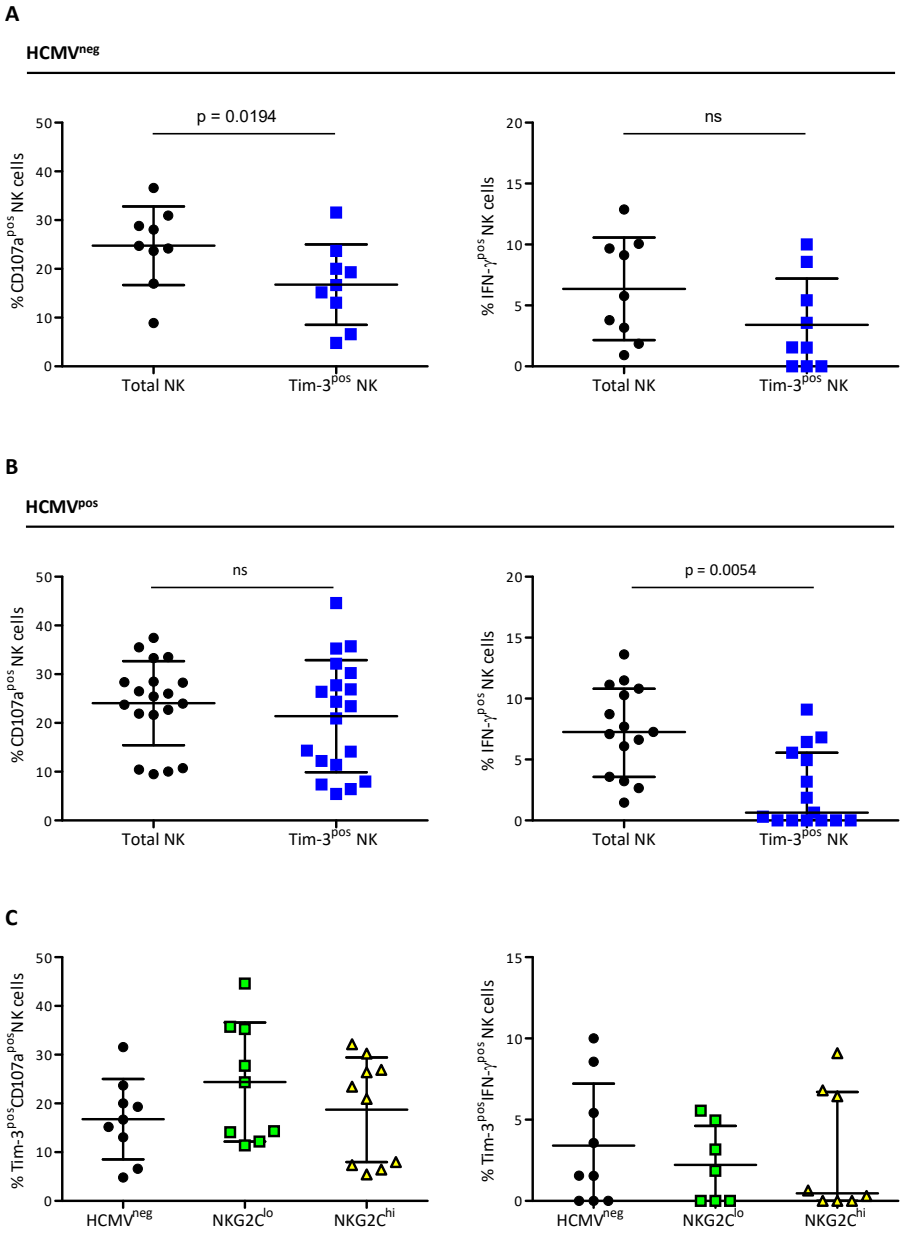

Figure 3.7| CD16-dependent degranulation and IFN-γ production by Tim-3^{pos} NK cells in HIV-1 infection

The percentage of cells expressing CD107a (left panels) or IFN-γ (right panels) within either the total or Tim-3^{pos} NK cell populations after CD16 stimulation was measured based on the gating strategy shown in Figure 3.5 and contrasted between (A) HCMV^{neg} (n = 9) and (B) HCMV^{pos} (n = 15–19) and between the (C) HCMV^{neg}, NKG2C^{lo}, and NKG2C^{hi} PLWH study groups. Error bars in (A) represent mean ± SD and comparison between conditions carried out with Student's paired *t*-test. Bars in (B) represent mean ± SD and Student's paired *t*-test performed (left panel) or median with IQR with significance calculated by Wilcoxon signed-rank test (right panel). Error bars in (C) represent mean ± SD where data was normally distributed and median with IQR in all other cases.

3.5 Discussion

Expansion of NKG2C-expressing NK cells was initially reported in the context of multiple viral infections, including hepatitis C virus, HIV-1, chikungunya, and hantavirus, but subsequent investigation identified HCMV infection as the critical common denominator [107, 298]. While only a small fraction of NK cells in healthy HCMV-seronegative individuals usually express NKG2C, HCMV infection triggers various levels of adaptation, resulting in more than 80% NKG2C^{pos} NK cells in extreme cases [204]. Therefore, we envision a continuum of NK cell adaptation to HCMV infection through which naïve NK cells respond to initial HCMV exposure, differentiate into mature effector cells and, under the influence of time, repeated exposure and other factors, progress toward terminal differentiation. In this study, we investigated NK cell function along this proposed continuum, beginning with exposure of naïve NK cells to cytokines present during acute HCMV infection and encompassing responses of NK cell populations reflecting broadly different degrees of adaptation to HCMV. To compare the quality of NK cell responses across the spectrum of adaptation to HCMV, we selected subjects from a well characterized cohort of PLWH within which there are HCMV-seronegative subjects, HCMV-seropositive subjects with small fractions of NKG2C^{pos} NK cells and HCMV-seropositive subjects with large fractions of NKG2C^{pos} NK cells [204]. This chosen study group allowed comparison of CD16-dependent NK cell functions over a broad

range of phenotypic adaptation to HCMV infection in the context of coinfection with HIV-1.

Acute exposure to conditioned media collected from HCMV-infected fibroblasts elevated NK cell cytotoxicity through NCRs, CLRs and CD16, irrespective of NK cell donor HIV-1 or HCMV status. While enhanced antibody-dependent effector function through CD16 is a characteristic of the NKG2C^{pos} NK cells adapted to HCMV infection, these adapted NK cells are also reported to downregulate NCRs [106]. Downregulation of NCRs is a common consequence of chronic viral infection, likely involving physical interactions and chronic cytokine stimulation. In combination with additional stimulation, pan increases in NCR-mediated cytotoxicity stimulated by HCMV-related cytokines may lead toward phenotypic alterations over periods of prolonged cytokine exposure. While IL-6, IL-8, and IL-15 were present in HCMVsn together with type I IFNs, these same cytokines were also elevated in vSC8sn, which did not increase NK cell cytotoxicity, indicating that at most, they may play some ancillary role in acute activation of NK cell cytotoxicity. Our data indicate that IFN-α2 is sufficient to effect the same increases in NK cell cytotoxicity as mediated by conditioned media from HCMV-infected fibroblasts. There were no significant differences between NK cell responses to purified recombinant IFN-α2 versus HCMVsn in either the healthy control cohort or the PLWH group. As type I IFNs are produced in a variety of viral infections that do not lead to expansion of NKG2C^{pos} NK cells, it is unlikely that acute exposure to IFN-α2

alone contributes significantly to the NK cell adaptation specific to HCMV infection. Infection with HCMV *in vivo* may result in different patterns of type I IFN production and cellular responses dependent upon the local environment, they type of cell infected and virus characteristics.

By selecting three groups of individuals with comparable features of HIV-1 infection, we were able to focus on the impact that different levels of adaptation to HCMV infection have on NK cell phenotype and function in this setting. Two HCMV-infected groups were selected for high versus low levels of NKG2C expression, with the low NKG2C expression group indistinguishable from the HCMV^{neg} group in this respect. Although one donor within the NKG2C^{lo} category had a CD57^{pos} NK cell fraction comparable to those in the NKG2C^{hi} group, the rest had low to moderate (10 – 27%) CD57^{pos} NK cell fractions. The influence of HCMV infection on FcRγ downregulation was the most apparent phenotypic aspect, displaying a clear hierarchy; the HCMV^{neg} group had the lowest fraction of FcRγ^{neg} NK cells followed sequentially by the NKG2C^{lo} and NKG2C^{hi} groups. A disconnect between NKG2C expression and loss of FcRγ was notable in three individuals with < 6% NKG2C^{pos} and more than 50% FcRγ^{neg} NK cells. Although it involves only three individuals in this case, the disconnect has also been noted in studies of NKG2C^{null} cohorts, where lack of NKG2C had little effect on HCMV-driven NK cell maturation [156, 299, 300]. Despite these three outliers, there was a strong overall correlation between the fraction of NK cells expressing NKG2C and fraction of FcRγ^{neg} NK cells.

Thus, NK cells from these outliers may express features similar to those from NKG2C^{null} subjects [156]. Evidently, loss of FcRγ is a more consistent indicator of NK cell differentiation and adaptation in response to HCMV infection than NKG2C expression.

Adaptation to HCMV infection through NKG2C^{pos} NK cell expansion and loss of FcRγ reportedly produces an NK cell population with superior CD16-mediated effector functions [158, 159, 291]. In the context of our PLWH study cohort, there was no evidence of this in cytotoxicity assays against antibody-coated target cells. Although we saw significant correlation between the size of the IFN-γ response and fraction of NK cells lacking FcRγ, there was also no significant difference between the groups in terms of the mean fraction of NK cells producing IFN-γ in response to CD16 signaling. Despite low levels of NKG2C^{pos}FcRγ^{neg} NK cells, the HCMV^{neg} group had CD16-mediated responses equally as robust as the other groups and the HCMV^{pos}NKG2C^{lo} group displayed no functional deficits relative to either of the other groups. This finding is somewhat unexpected in light of epigenetic remodeling of the NK cell IFNG locus following HCMV infection, with the adapted NK cell population reportedly exhibiting enhanced IFN-γ responses [161, 162]. Whether this apparent discrepancy reflects the impact of HIV-1 infection, different experimental methodology or peculiarities of the limited number of subjects tested in this study remains to be determined.

The extent of NK cell degranulation following CD16-stimulation was also not significantly different between groups and in contrast to IFN-γ production, we noted no significant correlation between the magnitude of CD107a responses and fraction of NK cells lacking FcRγ. Polyfunctional NK cell responses, indicated by dual expression of IFN-γ and CD107a also did not differ between groups. Thus, our data suggest that in the context of HIV-1 infection, phenotypic evidence of NK cell adaptation to HCMV infection does not equate with superior CD16-mediated effector functions. The finding here most consistent with previous studies using donors not infected with HIV-1 was the correlation between CD16-triggered IFN-γ production and loss of FcRγ adaptor subunits [158, 159]. Whether loss of FcRγ is a marker for other alterations or its absence plays a direct role in enhanced signaling through CD16, this aspect of NK cell adaptation to HCMV infection appears to be maintained in HIV-1 infection. We saw no relationship between FcRγ expression and either ADCC measured by ^{51}Cr release or CD16-triggered degranulation, lending credence to speculation that NK cell adaptation to HCMV affects CD16-mediated cytokine production more so than it affects cytotoxicity.

To investigate NK cell progression toward terminal differentiation, we assessed PD-1, LAG-3 and Tim-3 expression. We detected little to no PD-1 or LAG-3 in any of the three groups, yet a significant fraction of NK cells expressed Tim-3. Tim-3 expression levels did not differ between groups and were unaffected by CD16 stimulation. Although recent reports suggest enhanced function of Tim-3^{pos} NK

cells, we observed functional deficits in degranulation and IFN-γ production in the HCMV^{neg} and HCMV^{pos} groups, respectively, relative to the general NK cell population [301]. Although significant, these deficits were relatively slight and based on the responses of a small group of PLWH. Further study of the role Tim-3 has on NK cell functions in different settings is warranted.

In summary, type I IFNs produced during *in vitro* HCMV infection of fibroblasts increased NK cell cytotoxicity through multiple receptors. This increase in NK cytotoxicity occurred with NK cells from HCMV-seronegative and seropositive healthy controls. In the PLWH group, we saw a similar increase in ADCC that was unrelated to HCMV status or extent of NK cell adaptation to HCMV infection. Cytokine and degranulation responses mediated through CD16 were well preserved in the PLWH we studied, again unrelated to their HCMV status. Despite an inverse correlation overall between NK cell CD16-triggered IFN-γ production and FcRγ expression, the HCMV^{neg} PLWH group did not have a significantly lesser IFN-γ response than either of the groups with higher fractions of FcRγ^{neg} NK cells. There was no evidence that NK cell adaptation to HCMV affects degranulation responses or cytotoxicity triggered through CD16 in HIV-1 infection. While these findings suggest that HCMV-related NK cell adaptation has different or lesser functional consequence in HIV-1 infection, the clear effect of HCMV on FcRγ expression and the extreme levels of adaptation observed in terms of NKG2C expression illustrate the same selectivity operating with increased pressure. Immunological pressures

associated with HIV-1 infection may preserve or enhance NK cell function through compensatory pathways distinct from HCMV-driven adaptation.

3.6 Conclusions

Acute exposure to HCMVsn (IFN-α2) elevated NK cell cytotoxicity through NCRs, CLRs and CD16, irrespective of NK cell donor HCMV status. Compared with $HCMV^{neg}$ participants, $HCMV^{pos}$ individuals demonstrated no evidence of superior ADCC in cytotoxicity assays against antibody-coated target cells or CD16-mediated IFN-γ production. Although reports suggest enhanced function of Tim-3^{pos} NK cells, we observed functional deficits in Tim-3-expressing NK cells from PLWH. Adapted NK cells did not display a classical exhausted phenotype as there was little to low LAG-3, PD-1, and Tim-3 inhibitory immune checkpoint receptor expression, however, further study is needed to determine the relevance of the more recently discovered inhibitory immune checkpoint receptors.

4 TIGIT Blockade Enhances NK cell Cytotoxicity Against Autologous HIV-1-infected CD4^{pos} T cells

4.1 Abstract

During chronic human immunodeficiency virus type 1 (HIV-1) infection, inhibitory molecules upregulated on lymphocytes contribute to effector cell dysfunction and immune exhaustion. People living with HIV (PLWH) suffer greater risk for age-related morbidities, an issue magnified by human cytomegalovirus (HCMV) coinfection. As HCMV infection modifies natural killer (NK) cell properties and NK cells contribute to protection against HIV-1 infection, we considered the role of T cell immunoreceptor with immunoglobulin and intracellular tyrosine inhibitory motif domains (TIGIT) in NK cell-based HIV-1 immunotherapy and elimination strategies. We measured TIGIT expression on immune cell subsets of 95 PLWH and assessed its impact on NK cell function, including elimination of autologous CD4^{pos} T cells infected through reactivation of endogenous HIV-1. TIGIT was expressed on CD4^{pos} T cells, CD8^{pos} T cells and NK cells from PLWH. Although TIGIT levels on T cells correlated with HIV-1 disease progression, the extent of TIGIT expression on NK cells more closely paralleled adaptation to CMV. TIGIT interaction with its predominant ligand, poliovirus receptor (PVR), inhibits effector cell functions. Circulating CD4^{pos} T cells from PLWH more frequently express PVR compared with HIV-seronegative controls and PVR expression was enriched on

$CD4^{pos}$ T cells replicating HIV-1 *ex vivo*. Treatment with anti-TIGIT monoclonal antibodies increased NK cell HIV-1-specific antibody dependent cytotoxicity *in vitro* and *ex vivo*. Blocking TIGIT may be an effective strategy to invigorate antibody-dependent NK cell activity against HIV-1 activated in cellular reservoirs for cure or treatment strategies.

4.2 Introduction

Chronic viral infection imposes a persistent burden on human health. Human immunodeficiency virus type 1 (HIV-1) remains pandemic despite tremendous improvements to combination antiretroviral therapies (cART) that have extended the health and lifespan of people living with HIV (PLWH). Active HIV-1 infection drives immune dysfunction with generalized T cell and natural killer (NK) cell exhaustion in untreated PLWH. This dysfunctional immune phenotype is punctuated by increased expression of inhibitory immune checkpoint receptors, including PD-1, CTLA-4, Tim-3 and LAG-3 [233-235]. Early intervention with cART attenuates this expression, but levels remain elevated compared to people not living with HIV [235].

In this context, some checkpoint inhibitors currently used in cancer therapy have potential relevance in controlling viral infections, reducing viral load and revitalizing host immunity. T cell immunoreceptor with immunoglobulin (Ig) and intracellular tyrosine inhibitory motif domains (TIGIT) is differentially expressed on T cells and NK cells and negatively regulates effector function when engaged by its ligands, PVR (CD115) or PVRL2 (CD112) [71-73]. These ligands can also be recognized by killer cell Ig-like receptor (KIR)2DL5 and members of the nectin and nectin-like family of receptors that include DNAM-1 (CD226), TACTILE (CD96) and PVR-related Ig domain (PVRIG) [64, 77, 78, 88]. Broad dysregulation of TIGIT expression on $CD4^{pos}$ and $CD8^{pos}$ T cells in HIV-1 infection has been extensively

noted, however, a role for TIGIT in modulating NK cell function in HIV-1 infection remains controversial [240, 241, 243, 244, 302].

NK cells are among the first cells to respond to virus infection and as such, can contribute to control of HIV-1 [303-305]. NK cell function is scrupulously regulated through aggregate signals transmitted from interactions between distinct cellular and/or foreign ligands and activating and/or inhibitory receptors. Ligands can include self, altered and induced self, virus-encoded proteins and IgG antibodies bound to cells [56, 57, 306]. NK cells recognize cell-bound IgG antibodies specifically and independently through interactions with CD16 (FcγRIIIa) receptors and transmit signals through FcRγ and CD3ζ to perform antibody-dependent cell-mediated cytotoxicity (ADCC) [61]. In contrast, natural cytotoxicity is mediated by a wide array of activating receptors including receptors directly interacting with PVR (e.g. DNAM-1) or altered self (e.g. NKG2D) [58, 249, 307, 308]. Restraint over these mechanisms is imposed through inhibitory receptors, including inhibitory KIR, NKG2A and TIGIT [25-28, 309].

Previous reports outlined a role for HIV-1-based modulation of TIGIT expression on both $CD8^{pos}$ T cells and NK cells with NK cell TIGIT levels increasing with HIV-1 disease progression, as measured by $CD4^{pos}$ T cell nadir [241, 243, 244]. Dysregulated NK cell TIGIT expression in the context of chronic infection suggests targeting TIGIT could be an attractive option for HIV-1 immunotherapy and cure strategies, however, whether monoclonal antibodies (mAb) can improve effector

function for TIGIT-expressing NK cells and enhance antiviral responses *in vivo* remains unclear [243, 244, 310]. Moreover, inflammation, immune dysfunction and immune senescence in PLWH is further accentuated in relation to inflated immune responses against human cytomegalovirus (HCMV) [209]. The high worldwide prevalence of HCMV (40 – 100% seroprevalence) increases with aging and is especially high in PLWH (80 – 100% seroprevalence), with the immune deficit caused by HIV-1 infection allowing a higher frequency of HCMV reactivation in PLWH [206, 311-313].

Our conventional baseline NK cell receptor repertoire is distorted by HCMV infection and the subsequent immune response, generating a collection of NK cells with distinct phenotypic features [106]. Large fractions of the NK cell repertoire of PLWH coinfected with HCMV commonly express CD57 and NKG2C together with CD16 receptors that have lost the signaling adaptor subunit FcRγ [136, 158]. Given the association of HCMV with increased inflammation, skewed T cell responses, immune senescence and exaggerated NK cell adaptation, HCMV coinfection must be considered as a key factor modulating the immune compartment in HIV-1 infection [106, 136, 204, 314].

Therefore, we addressed whether alterations in NK cell TIGIT expression impact HIV-1-specific functions. We measured natural and antibody-dependent cytotoxicity of NK cells from PLWH against an *in vitro* HIV-1-infected $CD4^{pos}$ T cell line in the presence and absence of a blocking anti-TIGIT mAb and observed that

TIGIT blockade consistently potentiated HIV-1-specific NK cell responses. Although activated $CD4^{pos}$ T cells upregulate PVR expression, the extent of its modulation in active HIV-1 infection remains unresolved [249, 251-254, 302]. Here, we demonstrated that PVR is inducible and enriched on endogenous HIV-1 antigen-positive $CD4^{pos}$ T cells expanded from PLWH. When HIV-1 replication was activated in autologous $CD4^{pos}$ T cells *ex vivo*, NK cell effector responses against the infected cells increased with inclusion of anti-TIGIT blocking mAb. These data indicate that therapeutic antibodies preventing TIGIT:PVR interactions can benefit HIV-1 treatment strategies and increase cell-mediated effector responses against HIV-1-infected cells.

4.3 Materials and Methods

4.3.1 Study Subjects

This study was carried out in accordance with recommendations of the Canadian Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans. Protocols to obtain anonymized umbilical cord blood and peripheral blood from HIV-seronegative donors and from PLWH recruited through the Newfoundland and Labrador Provincial HIV Clinic were approved by the Health Research Ethics Authority of Newfoundland and Labrador, Canada. Peripheral blood was collected from study subjects after written informed consent in accordance with the Declaration of Helsinki.

4.3.2 Blood Sample Processing

Whole blood was collected by venipuncture in acid citrate dextrose vacutainers, after which plasma was collected following 10 min centrifugation at 500*g* and stored at -80°C. Cord blood mononuclear cells (CBMC) and peripheral blood mononuclear cells (PBMC) were isolated using the Canadian Autoimmunity Standardization Core consensus standard operating procedure (version: March 21, 2019). Freshly isolated PBMC were resuspended in freezing medium consisting of fetal calf serum (FCS) supplemented to 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis MO, USA) and cooled at 1°C per minute overnight to –80°C. Frozen PBMC were then maintained in liquid nitrogen until use.

4.3.3 PBMC Phenotyping

Isolated PBMC (10^6) were phenotyped using directly conjugated mAb (conjugate, clone in parentheses) against human CD16 (VioBlue, REA423), CD3 (VioGreen, BW264/56), CD4 (PE-Vio770, REA623), NKG2C (PE-Vio770, REA205), CD56 (APC-Vio770, REA196) from Miltenyi Biotec (San Diego CA, USA), CD57 (FITC, TB01), PVR (PE, 2H7CD155), TIGIT (AlexaFluor 647, MBSA43) from Thermo Fisher Scientific (San Diego CA, USA) and CD8 (PerCP, HIT8a) from Biolegend (San Diego, CA, USA) and fixed with 2% paraformaldehyde (Sigma-Aldrich) prior to data acquisition. Participants selected for functional assays were also phenotyped with anti-KIR2DL5 (PE, UP-R1) from Biolegend or polyclonal anti-Fc-receptor common gamma chain (FcRγ)-FITC from Millipore Sigma (Oakville ON, Canada) using Inside Stain Kit (Miltenyi Biotec) as per manufacturer's instructions. Data were acquired using the CytoFLEX flow cytometer and analyzed and illustrated using Kaluza software (both Beckman Coulter, Brea CA, USA) and GraphPad Prism Version 8.4.3.

4.3.4 Cell Culture

K562 (ATCC® CCL 243™), P815 (ATCC® TIB-64™), H9, CEM.NKR-CCR5 (NIH HIV Reagent Program, Division of AIDS, NIAID, NIH) and CEM.NKR-CCR5 PVR^{pos} (see below) cell lines were propagated in lymphocyte medium consisting of RPMI-1640 with 10% FCS, 200 IU/mL penicillin/streptomycin, 0.01 M HEPES, 1%

L-glutamine (all from Invitrogen, Carlsbad CA, USA) and 2.0 x 10^{-5} M 2-mercaptoethanol (Sigma-Aldrich) at 37°C, 5% CO_2. The CEM.NKR-CCR5 cell lines were maintained on a precise passage regimen as outlined [315-317]. Lenti-X™ 293T cells (TakaRa, Mountain View CA, USA) were propagated in DMEM (Sigma-Aldrich) with 10% tetracycline-free FCS (TakaRa) and 1 mM sodium pyruvate (Sigma-Aldrich) at 37°C, 5% CO_2.

4.3.5 PVR Gene Transfer and Expression in CEM.NKR-CCR5 cells

The recombinant Lenti-X™ pLVX-IRES lentiviral vector expression system (TakaRa) was used to introduce PVR into the CEM.NKR-CCR5 cell line. Briefly, the canonical PVR sequence was obtained from UniProtKB (P15151-1), synthesized by Invitrogen GeneArt (Thermo Fisher Scientific) and inserted (Rapid DNA Ligation Kit; Roche, Mannheim, Germany) into pLVX-IRES after SpeI and EcoRI restriction digestion (New England Biolabs, Ipswich, MA, USA) and agarose gel extraction/purification (QIAquick Gel Extraction; Qiagen, Toronto, ON, Canada). The pLVX-IRES/PVR expression vector was transformed into Stellar™ Competent Cells (TakaRa) from which midi-scale plasmid DNA was prepared (NucleoBond Xtra Midi, TakaRa), concentration determined using NanoDrop™ (Thermo Fisher Scientific) and positive and negative strands sequenced (TCAG, The Hospital for Sick Children, Toronto, ON, Canada) to ensure authenticity. Lenti-X™ 293 T cells were transfected with the pLVX-IRES/PVR expression vector using the Lenti-X™

Single Shot (TakaRa) packaging and transfection system. Lentiviral supernatants were collected 48 h after transfection, filtered through a 0.45 μm polyethersulfone filter to remove cellular debris, aliquoted and frozen at -80°C. A p24 ELISA [Leidos Biomedical Research, Inc., for the National Cancer Institute (NCI), Frederick, MD, USA], read at 450 nm on a Synergy HT BioTek microplate reader, was used to obtain viral titres and CEM.NKR-CCR5 cells were transduced with lentiviral supernatant and 4 μg/mL polybrene (Sigma-Aldrich) in 96-well round-bottom plates by 90 min 1200*g* spinoculation at 32°C. Transduction medium was replaced 24 h later with lymphocyte medium and cells were propagated, then sorted for PVR expression (2H7CD155 APC; Thermo Fisher Scientific) using a MoFlo Astrios EQ flow cytometer (Beckman Coulter).

4.3.6 HIV-1 Stock Generation

The HIV-1IIIB A17 variant (lot no. 9/03/92) was obtained through the NIH HIV Reagent Program [318]. The entire aliquot was used to infect 10^6 H9 cells. Cells were maintained in the minimal volume of lymphocyte medium required for growth and 7 days after infection supernatant was collected by 5 min 450*g* centrifugation followed by 0.45 μm polyethersulfone filtration. To generate high titre stock, 10^5 H9 cells were infected with 1 mL of infection supernatant, maintained in the minimal volume of lymphocyte medium required for growth and supernatant collected 14

days after infection as above. Stocks were split into single use vials and p24 quantity was determined by ELISA as above.

4.3.7 *In vitro* HIV-1 Infection

CEM.NKR-CCR5 and CEM.NKR-CCR5 PVR^{pos} cells (10^5) split 1:3 the day prior to infection were infected with 200 ng p24 HIV-1 IIIB A17 by spinoculation in a 15 mL tube at 32°C, 1200*g* for 90 min. Cells were cultured at 37°C 5% CO_2 for 1 h before adding 1 mL of lymphocyte medium. Experiments were performed 96 h after infection. HIV-1 infection was quantified by anti-p24 FITC [24-4, Santa Cruz (Dallas, TX, USA)] or gp120 expression [50 ng HIVIG (NIH HIV Reagent Program) per 10^5 cells followed by anti-human IgG Fc (PE, eBioscience)]. Anti-PVR-APC (2H7CD1555, Thermo Fisher) was used to assess PVR expression. Cells were fixed with 2% paraformaldehyde (Sigma-Aldrich) prior to data acquisition using a CytoFLEX (Beckman Coulter).

4.3.8 Chromium Release Assays

Cryopreserved PBMC were recovered overnight in lymphocyte medium at 37°C, 5% CO_2. Cells were recounted after recovery by trypan blue exclusion and used when > 75% viable. Target cells were labelled for 90 min with 100 µCi $Na_2{}^{51}CrO_4$ (PerkinElmer, Akron, OH, USA) at 37°C, 5% CO_2, washed four times in PBS containing 1% FCS before resting in 5 mL lymphocyte medium for 1 h at 37°C, 5% CO_2 to minimize spontaneous release and resuspended at 10^5 cells/mL. Where

indicated, PBMC and IgG_1 isotype control (11711, R&D Systems) or anti-TIGIT (MBSA43, Thermo Fisher Scientific) were preincubated for 30 min in a microtiter plate at a final mAb concentration of 5 μg/mL. NK cell CD16-mediated cytotoxicity was measured using ^{51}Cr labelled P815 cells and 30 ng per well IgG_1 isotype control (11711, R&D systems) or anti-CD16 (3G8, Biolegend). We used HIVIG pooled from inactivated human sera (NIH HIV Reagent Program) or control IgG from human sera (Sigma) at a final concentration of 10 μg/mL to measure ADCC. All ^{51}Cr release assays were conducted at E:T 30:1 (V_f = 300 μL) and cytotoxic activity was measured by ^{51}Cr release over 5 h. ^{51}Cr release was measured in 125 μL of supernatant on a Wallac 1480 Wizard gamma counter and percent specific lysis calculated by (experimental ^{51}Cr release – spontaneous ^{51}Cr release) / (maximum ^{51}Cr release – spontaneous ^{51}Cr release) x 100. Where indicated, percent increase was calculated by (% specific lysis anti-TIGIT-treated condition) – (% specific lysis IgG_1-treated condition) / (% specific lysis IgG_1-treated condition) x 100 and absolute increase in cytotoxicity was calculated by (% specific lysis anti-TIGIT-treated condition) – (% specific lysis IgG_1-treated condition).

4.3.9 Primary $CD4^{pos}$ T cell HIV-1 Reactivation

PBMC were depleted of $CD8^{pos}$ T cells (StemCell Technologies, Vancouver BC, Canada) and cultured in lymphocyte medium without phenol red supplemented to 50 IU/mL IL-2 (NCI) for up to 144 h. Aliquots were removed at 24 h intervals and

stained with anti-human CD4 (PE-Vio770), CD3 (VioGreen), from Miltenyi, CD8 (PerCP, Biolegend), PVR (APC, Invitrogen) and intracellular anti-HIV-1 p24 (FITC, Santa Cruz) using Inside Stain Kit (Miltenyi).

4.3.10 Primary $CD4^{pos}$ T cell HIV-1 Stimulation and Antibody-Dependent NK cell Activation Assay

PBMC from PLWH were resuspended at 2.5 x 10^6 cells/mL in lymphocyte medium without phenol red supplemented to 50 IU/mL IL-2. PBMC were left unstimulated or stimulated with 5 µg/mL phytohemagglutinin (PHA)-P (Sigma) and divided equally into three conditions: untreated or treated with 10 µg IgG/mL of purified Ab from plasma of HIV-seronegative individuals (CON Ab) or Ab from plasma of PLWH (HIV Ab). Briefly, Ab was purified by pooling heat inactivated (1 h at 56°C) plasma from HIV-seronegative donors or PLWH (matched for HCMV status). Pooled plasma was centrifuged at 10,000*g* for 20 min, diluted 1:1 in endotoxin-free binding buffer (0.2 M Na_3PO_4, Millipore Sigma) and purified using a 1 mL Cytiva HiTrap™ Protein G HP column (Millipore Sigma). Purified Ab was adsorbed on CEM.NKR-CCR5 and PHA-P-stimulated $CD8^{dep}$ PBMC from HIV-seronegative participants to reduce non-specific binding. Approximately 48 h after stimulation, PBMC from each condition were treated with IgG_1 (11711, R&D Systems) or anti-TIGIT (MBSA43, Thermo Fisher Scientific) at a final mAb concentration of 5 µg/mL and (i) incubated with V_f 500 µL IL-2-supplemented

lymphocyte medium for 24 h or (ii) labelled with 0.25 μg per 10^6 PBMC of anti-CD107a (H4A3; BioLegend) for 24 h (V_f 500 μL). Cells in condition (i) were stained using anti-human CD3 (VioGreen), CD4 (PE-Vio770) from Miltenyi, PVR (APC 2H7CD155, Thermo Fisher) and intracellular anti-HIV-1 p24 (FITC, Santa Cruz) using Inside Stain Kit (Miltenyi) and condition (ii) were assessed using CD3 (VioGreen), CD56 (APC-Vio770) from Miltenyi and CD8 (PerCP, Biolegend). Data were acquired using a CytoFLEX (Beckman Coulter).

4.3.11 Statistical Analysis

Statistical analyses were performed using GraphPad Prism Version 8.4.3 with two-sided *P*-values < 0.05 considered significant. Normality of data distributions were assessed using Shapiro-Wilk test. Significance in correlations were assessed using Spearman's rank correlation coefficient. Differences in means with standard deviation (SD) or medians with interquartile range (IQR, calculated as IQR = Q_3 – Q_1) between groups were compared by one-way ANOVA, Student's *t* or Mann-Whitney *U*-test as appropriate based on normality of data distribution. For paired analyses, Student's paired *t*-test was used when data were normally distributed, and non-parametric Wilcoxon signed rank test otherwise.

4.3.12 Figures

Adobe Illustrator 24.1.1 was used to construct figures and illustrations were created using BioRender.com.

4.4 Results

4.4.1 TIGIT Expression on Immune cells from Newborns, HIV-Seronegative Persons and PLWH

Widespread expression of TIGIT on immune cells makes it an attractive potential target to incorporate into HIV-1 treatment strategies. We applied an immune cell phenotyping panel to determine the extent TIGIT was expressed on immune cell subsets of 95 PLWH attending the Provincial HIV Clinic in St. John's, Newfoundland and Labrador. All participants were receiving cART and our selection was representative of the Provincial HIV Cohort of 188 individuals (2019). Their general demographics are outlined in Table 4.1 and our flow cytometry gating strategy is depicted in Figure 4.1 A. TIGIT was moderately expressed on $CD4^{pos}$ T cells [median (IQR) 22.0% (9.4%)] and to a greater extent on both $CD8^{pos}$ T cells [44.9% (21.3%)] and NK cells [73.6% (18.2%); Figure 4.1 B]. To address the significance of TIGIT expression on these immune cell subsets and evaluate whether levels relate to HIV-1, 'immune experience' or other chronic viral infections, we also analysed nascent immune cells (CBMC, n = 15) and PBMC from a cohort of HIV-seronegative individuals (n = 26). The latter group was matched to the PLWH cohort for age, sex and HCMV serostatus (Table 4.1).

Table 4.1| General Cohort Demographics

	PLWH	HIV-seronegative
n [undetectable HIV-1† (%)]	95 (84.2%)	26 (N/A)
age [years, mean (SD)]	51.6 (10.8)	51.1 (13.1)
male [n (%)]	72 (75.8%)	19 (73.1%)
years of HIV-1 infection [mean (SD)]	15.8 (10.3)	N/A
CD4 nadir‡ [cells/µl, median (IQR)]	211 (372.8)	N/A
HBV positive [n (%)]	4 (4.2%)	0
HCV positive [n (%)]	4 (4.2%)	0
CMV positive [n (%)]	77 (81.0%)	21 (80.8%)
NKG2C^{null} [n (%)]	4 (4.2%)	1 (3.8%)

† *< 40 copies HIV RNA per milliliter of plasma at time of testing.*

‡ *Lowest recorded CD4⁺ T cell count per microliter of peripheral blood.*

Comparison of TIGIT expression on CD4^{pos} T cells (Figure 4.1 C), CD8^{pos} T cells (Figure 4.1 D) and NK cells (Figure 4.1 E) between the three groups indicated significantly higher frequency TIGIT expression on CD4^{pos} T cells of PLWH than on either nascent CD4^{pos} T cells or those from HIV-seronegative participants (Figure 4.1 C). General aging/immune experience also elevates TIGIT levels on CD8^{pos} T cells from HIV-seronegative individuals in comparison to CBMC (Figure 4.1 D). However, there was no significant difference in TIGIT expression on CD8^{pos} T cells from cART-treated PLWH and the age-matched HIV-seronegative group (Figure 4.1 D). In contrast, TIGIT expression was elevated on NK cells from PLWH compared with the NK cells of both nascent controls and the matched HIV-seronegative participants (Figure 4.1 E). Although TIGIT expression on T cells from HIV-seronegative adults was elevated compared to cord blood T cells, there was no significant difference in TIGIT expression between cord blood NK cells and NK cells from HIV-seronegative adults (Figure 4.1 E).

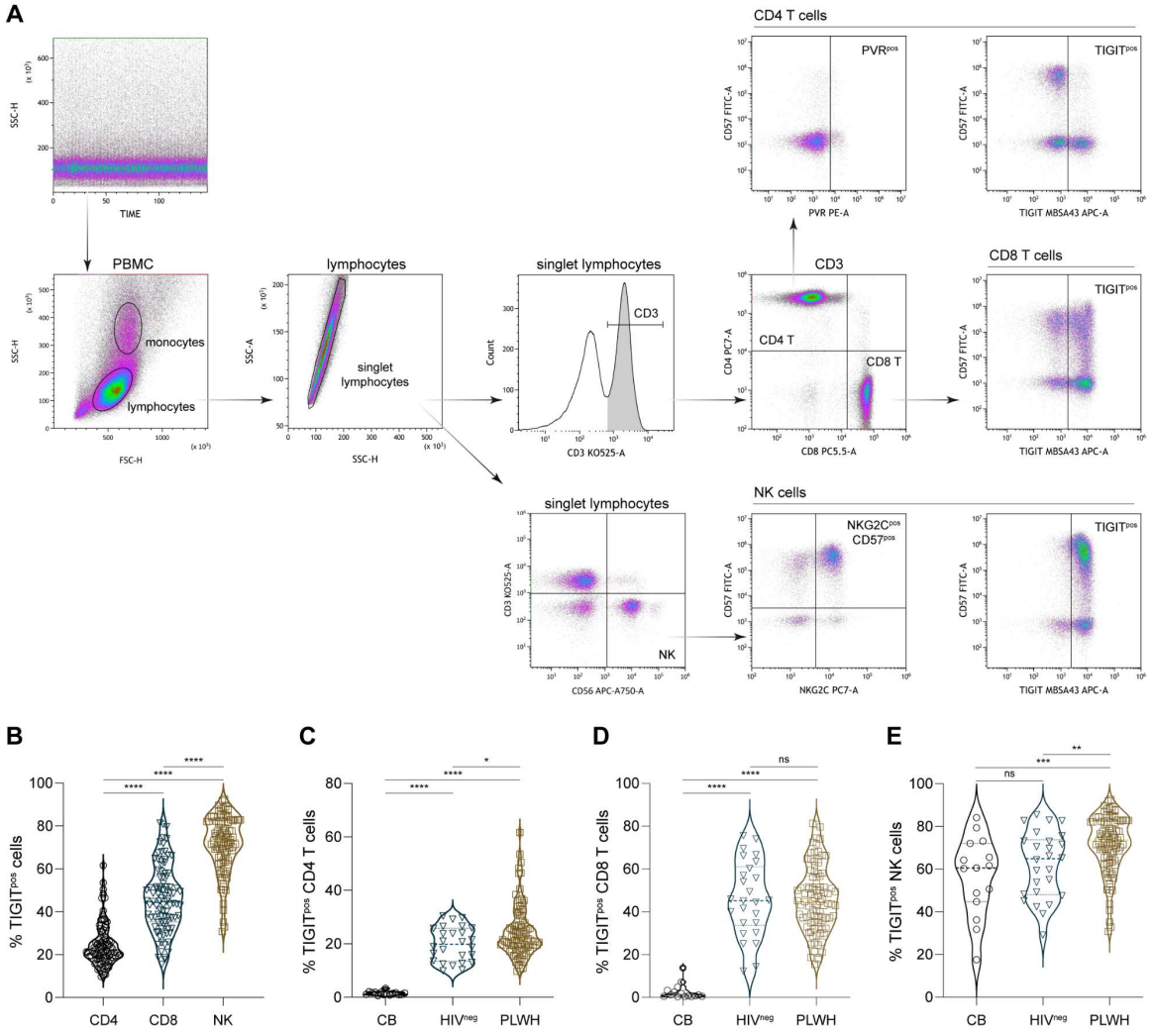

Figure 4.1 | TIGIT expression on immune cell subsets from cord blood and peripheral blood of PLWH and HIV-seronegative adults

(A) Quality of flow cytometry data acquisition was monitored by side scatter over time. Lymphocytes were identified by scatter characteristics and doublet exclusion. T cells were identified as $CD3^{pos}$ lymphocytes and distinguished by either CD4 or CD8 expression and NK cells were $CD3^{neg}CD56^{pos}$ lymphocytes. Subsets of $CD4^{pos}$ T cells, $CD8^{pos}$ T cells and NK cells were further demarcated by TIGIT and CD57 expression, $CD4^{pos}$ T cells were analyzed for PVR expression and NK cells for NKG2C and CD57 expression. (B) Compiled TIGIT expression levels on $CD4^{pos}$ T cells, $CD8^{pos}$ T cells and NK cells from PLWH (n = 95). Friedman Test $^{****}P < 0.0001$. Expression of TIGIT on (C) $CD4^{pos}$ T cells, (D) $CD8^{pos}$ T cells and (E) NK cells from nascent (CB, n = 15), HIV-seronegative (HIV^{neg}, n = 26) and PLWH (n = 95) was compared. Kruskal Wallis test $^{*}P = 0.0408$ $^{**}P = 0.0082$ $^{***}P = 0.0008$ $^{****}P < 0.0001$. Horizontal lines bisecting groups represent median with IQR.

4.4.2 Contribution of HIV-1 and HCMV to CD4^{pos} T cell, CD8^{pos} T cell and NK cell TIGIT Expression

To further determine how increased levels of TIGIT relate to HIV-1 infection, we assessed correlation between TIGIT expression on each immune cell subtype and CD4^{pos} T cell nadir, a marker for the historical extent of HIV-1 disease progression. Many participants with lower nadir CD4^{pos} T cell counts had higher levels of TIGIT expression on both CD4^{pos} T cells (Figure 4.2 A) and CD8^{pos} T cells (Figure 4.2 B). Although significant, these correlations were relatively weak, and in some instances, participants with high historical CD4^{pos} T cell counts had large fractions of CD4^{pos} T cells or CD8^{pos} T cells expressing TIGIT. In contrast, and despite TIGIT being elevated on NK cells from PLWH, there was no significant correlation between NK cell TIGIT expression and CD4^{pos} T cell nadir (Figure 4.2 C). Although chronic HIV-1 seems to contribute to increased levels of TIGIT on CD4^{pos} T cells and both CD4^{pos} T cell and CD8^{pos} T cell TIGIT expression correlates with HIV-1 disease progression, other co-factors appear to drive elevated NK cell TIGIT expression in PLWH.

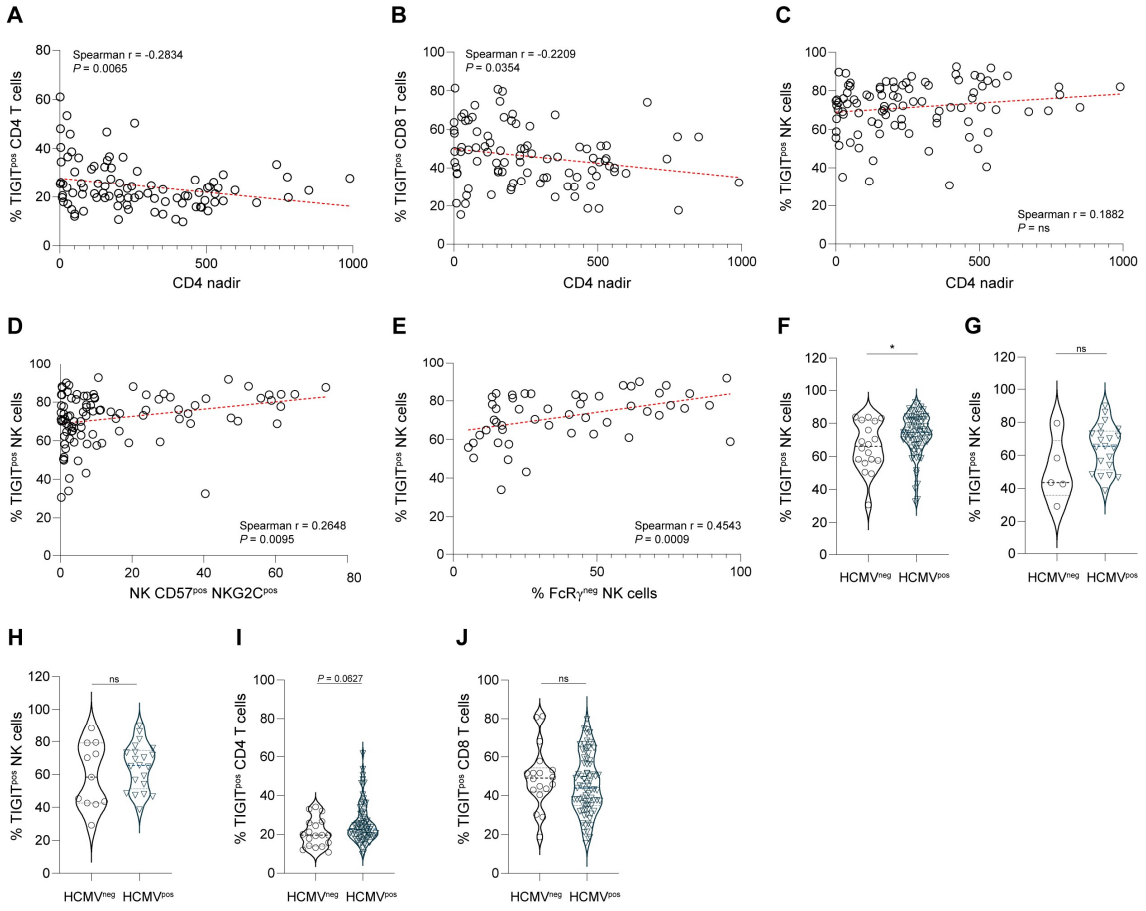

Figure 4.2 | TIGIT expression on immune cell subsets and impact of HIV-1 and HCMV infection

Correlation of TIGIT expression levels on (A) $CD4^{pos}$ T cells, (B) $CD8^{pos}$ T cells and (C) NK cells with $CD4^{pos}$ T cell nadir was evaluated for the PLWH cohort (n = 95) to gauge linkage with HIV-1 disease progression. The extent of TIGIT expression on NK cells was plotted against the percentage of NK cells that were (D) $CD57^{pos}NKG2C^{pos}$ (n = 95) or (E) $FcR\gamma^{neg}$ (n = 50) and correlation assessed. Individuals within the (F) PLWH or (G) HIV-seronegative cohort were grouped based on HCMV serostatus and NK cell TIGIT expression for those who were HCMV-seronegative (PLWH n = 18; HIV-seronegative n = 5) was compared to those who were HCMV-seropositive (PLWH n = 77; HIV-seronegative n = 21). Mann Whitney test $*P = 0.0256$. (H) Six additional HCMV-seronegative individuals were included for more robust comparison of TIGIT expression on NK cells from HCMV-seronegative (n = 11) and HCMV-seropositive (n = 21) HIV-seronegative individuals. HCMV-seronegative individuals were 64% male with mean age of 49.3 years, whereas HCMV-seropositive individuals were 73% male with mean age of 52.0 years. Student's *t*-test ns. The HCMV serostatus of PLWH was determined and (I) $CD4^{pos}$ T cell or (J) $CD8^{pos}$ T cell TIGIT expression for those who were HCMV-seronegative (n = 18) was compared to those who were HCMV-seropositive (n = 77). Mann Whitney test $P = 0.0627$; Student's *t*-test ns. Horizontal lines bisecting groups in (F – J) represent median with IQR.

Given the high worldwide prevalence of PLWH coinfected with HCMV and considering 81% of the PLWH in this study were HCMV-seropositive, we investigated whether known markers of NK cell adaptation to HCMV infection related to TIGIT expression. TIGIT was expressed at higher levels on $CD57^{pos}$ $NKG2C^{pos}$ NK cells (Figure 4.2 D) and on NK cells lacking FcRγ in PLWH (Figure 4.2 E) [244]. In addition, a higher percentage of NK cells expressed TIGIT in HCMV-seropositive compared to HCMV-seronegative PLWH (Figure 4.2 F). There was no significant difference in NK cell TIGIT expression between those who were HCMV-seronegative vs HCMV-seropositive within either the HIV-seronegative cohort (Figure 4.2 G) or a group of age- and sex-matched HIV-seronegative participants that included more HCMV-seronegative individuals (Figure 4.2 H). In contrast to NK cells, the HCMV serostatus of PLWH had no impact on $CD4^{pos}$ T cell (Figure 4.2 I) or $CD8^{pos}$ T cell (Figure 4.2 J) TIGIT expression. The proclivity of HCMV infection to accentuate inflammation and immune dysfunction in PLWH may be a co-factor driving increased levels of TIGIT expression on NK cells of PLWH.

4.4.3 PVR Expression on Circulating $CD4^{pos}$ T cells from PLWH

Interaction between TIGIT and its ligands inhibits immune cell activation. Since TIGIT expression is dysregulated in PLWH, we assessed expression of PVR, the predominant ligand for TIGIT, on $CD4^{pos}$ T cells within each study group and compared levels between groups. PVR was present at low levels on resting peripheral

blood $CD4^{pos}$ T cells, with higher expression on $CD4^{pos}$ T cells from PLWH compared to other groups (Figure 4.3 A) [241]. There was no significant difference in PVR expression on $CD4^{pos}$ T cells between the nascent and age-matched HIV-seronegative groups (Figure 4.3 A). PLWH who had a higher frequency of $CD4^{pos}$ T cells expressing PVR generally had lower historical $CD4^{pos}$ T cell counts (CD4 nadir), linking HIV-1 disease progression with frequency of circulating $CD4^{pos}$ T cells expressing PVR (Figure 4.3 B) [243]. Mirroring the increased TIGIT expression on NK cells from HCMV-seropositive PLWH noted in Figure 4.2 F, $CD4^{pos}$ T cells from HCMV-seropositive PLWH expressed higher levels of PVR than the $CD4^{pos}$ T cells of their HCMV-seronegative counterparts (Figure 4.3 C). Although $CD4^{pos}$ T cell PVR expression directly correlated with the extent to which TIGIT was expressed on $CD4^{pos}$ T cells (Figure 4.3 D), we did not see similar associations between elevated numbers of PVR^{pos} $CD4^{pos}$ T cells and either $TIGIT^{pos}$ $CD8^{pos}$ T cells (Figure 4.3 E) or $TIGIT^{pos}$ NK cells (Figure 4.3 F). These data shape a perspective that progression of HIV-1 infection contributes to TIGIT expression on $CD4^{pos}$ T cells, that elevated $CD8^{pos}$ T cell TIGIT can return to similar levels as in HIV-seronegative individuals with effective treatment and that other factors underlie increased TIGIT expression on NK cells from PLWH [241].

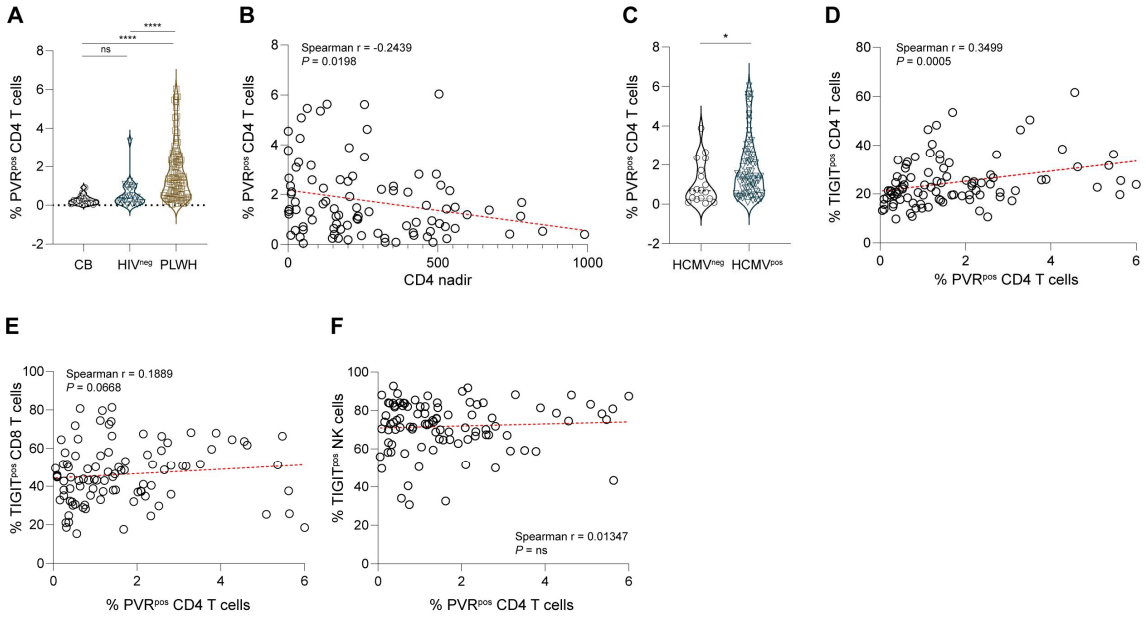

Figure 4.3| PVR expression on circulating CD4^{pos} T cells from PLWH and HIV-seronegative controls

(A) The extent of PVR expression on CD4^{pos} T cells from newborns (CB), HIV-seronegative (HIV^{neg}) adults or PLWH was measured and compared. Kruskal Wallis test ****P < 0.0001. (B) Correlation between PVR expression on CD4^{pos} T cells from PLWH (n = 95) and HIV-1 disease progression (CD4 nadir) was assessed and (C) expression of PVR on CD4^{pos} T cells from HCMV-seronegative (n = 18) or HCMV-seropositive (n = 77) PLWH contrasted. Mann Whitney test *P = 0.0290. Correlation between CD4^{pos} T cell PVR expression and percentage of (D) CD4^{pos} T cell TIGIT, (E) CD8^{pos} T cell TIGIT and (F) NK cell TIGIT expression is depicted. Horizontal lines bisecting groups in (A) and (C) represent median with IQR.

4.4.4 Effect of TIGIT Blockade on NK cell Cytotoxicity Against Conventional Targets

TIGIT is widely expressed on multiple immune cell subsets and has a role in regulating their function [70-75], yet limited information on its functional role in the context of HIV-1 infection exists and interpretation of that which is available differs [241, 243, 244]. To investigate cytotoxic activity of NK cells from PLWH in the context of TIGIT expression, we selected a subset (n = 26) of the PLWH cohort

(n = 95) for functional experiments. These representative individuals were selected based on age, sex, HCMV serostatus, years of HIV-1 infection and $CD4^{pos}$ T cell nadir (Table 4.2). All participants were on cART, however, samples from two individuals used for functional studies were collected when they had detectable viral loads (> 40 copies HIV-1 RNA/mL blood). We considered NK cell TIGIT expression in the selection to ensure the range of TIGIT expression within the entire cohort (Figure 4.1 B; range 43.8 – 89.7%) was represented within the functionally studied group (Figure 4.4 A, range 49.8 – 90.0%). PLWH with hepatitis B virus (HBV) or hepatitis C virus (HCV) coinfection, HCMV-seropositive participants with no functional NKG2C gene ($NKG2C^{null}$) and PLWH with < 10% baseline NK cell cytotoxicity against conventional K562 target cells were excluded from functional studies.

Table 4.2| Characteristics of PLWH Included in Functional Studies

n [undetectable HIV-1[†] (%)]	26 (92.3%)
age [years, mean (SD)]	55.4 (9.7)
male [n (%)]	21 (80.8%)
years of HIV-1 (years, mean (SD)]	17 (9.1)
CD4 nadir[‡] [cells/µl, median (IQR)]	188 (282)
CMV positive [n (%)]	16 (61.5%)

[†] *< 40 copies HIV RNA per milliliter of plasma at time of testing.*

[‡] *Lowest recorded $CD4^+$ T cell count per microliter of peripheral blood.*

To measure the effect of TIGIT engagement on NK cell cytotoxicity, we sensitized IgG Fc receptor (FcR)-expressing P815 cells for redirected NK cell lysis using monoclonal anti-CD16 antibody (Figure 4.4 B, right panel). In most cases,

engagement of TIGIT decreased NK cell activity against P815 cells, independent of donor HCMV infection status (Figure 4.4 B, left panel). Having established that engaging TIGIT inhibited NK cell CD16-mediated cytotoxicity for a large fraction of the subcohort, we tested whether preventing TIGIT:ligand interactions increased NK cell natural cytotoxicity (Figure 4.4 C, right panel). The K562 cell line is a conventional NK cell target that expresses high levels of PVR [319]. Natural cytotoxicity receptor-mediated killing was generally more robust than CD16-directed P815 lysis and preventing TIGIT:PVR interactions increased NK cell natural cytotoxicity by a mean ± SD of 15.6 ± 11.9% (Figure 4.4 C, left panel).

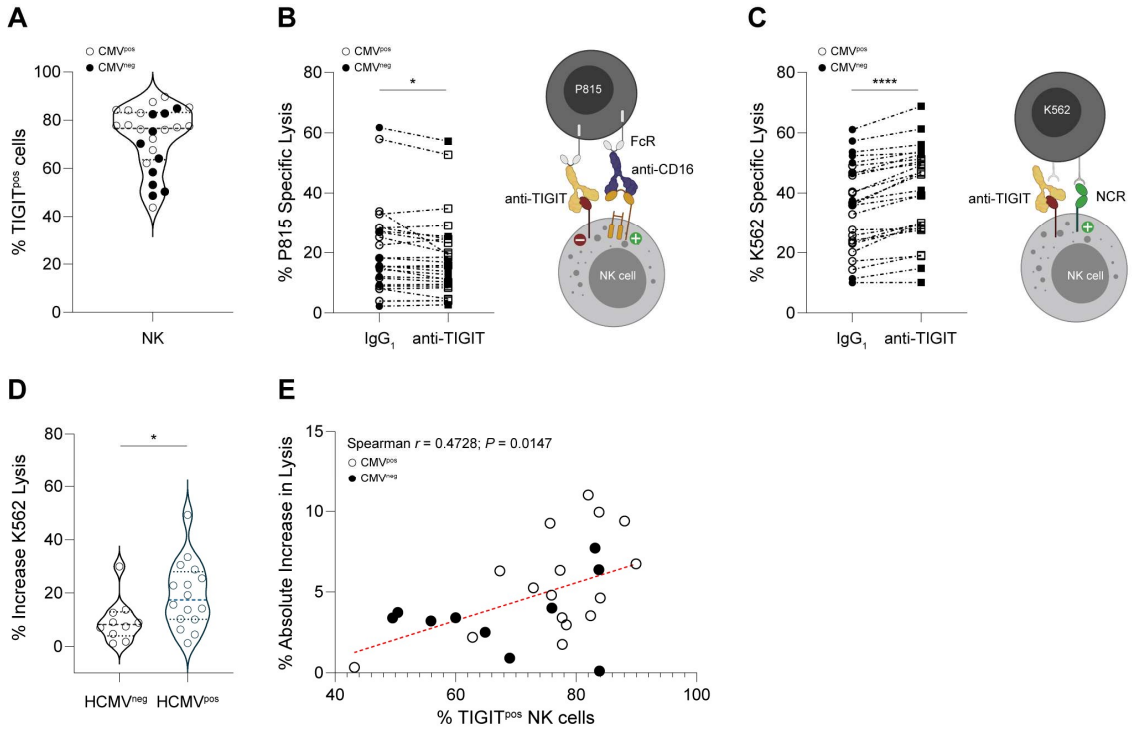

Figure 4.4 | Impact of TIGIT engagement on NK cell CD16-mediated and natural cytotoxicity

(A) The range of TIGIT expression on NK cells (49.8 – 90.0%) from PLWH selected for functional studies is depicted [median (IQR) 76.6% (19.6%)]. (B) NK cells were triggered to lyse P815 cells using anti-CD16 and the overall effect of NK cell TIGIT crosslinking was measured using IgG_1 isotype control (not depicted) or anti-TIGIT (represented by the yellow antibody). Percent specific lysis of P815 cells mediated through CD16 with either IgG_1 or anti-TIGIT was compared. Wilcoxon signed-rank test $^*P = 0.0351$. (C) NK cells were pretreated with anti-TIGIT to prevent interaction with PVR on K562 cells and lysis was measured and compared to lysis mediated by NK cells pretreated with IgG_1. Student's paired *t*-test $^{****}P < 0.0001$. (D) Individuals were grouped based on HCMV serostatus and percent increase in cytotoxicity of NK cells against K562 targets in the presence of anti-TIGIT vs IgG_1 was calculated from raw data in (C). Mann–Whitney *U*-test $^*P = 0.0309$. For all graphs, filled symbols represent HCMV-seronegative (n = 10) participants while open symbols depict HCMV-seropositive participants (n = 16). Horizontal lines bisecting groups in (A) and (D) represent median with IQR.

Although NK cells from both the HCMV-seronegative and seropositive groups responded to TIGIT blockade, the median (IQR) percent increase in natural cytotoxicity was greater for HCMV-seropositive [17.4% (17.9%)] than HCMV-seronegative [8.2% (9.0%)] individuals (Figure 4.4 D). This functional impact aligns

with the observation of higher NK cell TIGIT expression levels in HCMV-seropositive individuals within the greater PLWH cohort (Figure 4.2 F). Within this subset of PLWH, the absolute increase in natural cytotoxicity in response to TIGIT blockade directly related to the extent of TIGIT expression on NK cells (Figure 4.4 E).

4.4.5 HIV-1 Infection of PVR-Expressing CEM.NKR-CCR5 cells

After demonstrating that TIGIT engagement negatively impacted NK cell cytotoxicity through CD16 and that NK cell natural cytotoxicity is increased by blocking TIGIT:PVR interactions, we next determined the impact of TIGIT:PVR interaction on NK cell-mediated killing of HIV-1-infected cells. To do so, we transduced PVR into HIV-1-permissive CCR5-expressing CEM.NKR (CEM.NKR-CCR5 PVR^{pos}) cells (Figure 4.5 A), as they are resistant to NK cell-mediated lysis and constitutively express very low levels of CD112 [315, 317, 319]. Both the parental and PVR-transduced CEM.NKR-CCR5 cell lines were permissive for HIV-1 A17 infection and were infected at comparable levels based on intracellular p24 (Figure 4.5 B) and surface gp120 (Figure 4.5 C) detection 96 h post infection. During this time period, surface PVR mean fluorescence intensity (MFI) increased on CEM.NKR-CCR5 PVR^{pos} cells from 32,739 ± 3,318 on uninfected cells to 109,736 ± 36,409 for HIV-1 A17 infected cells, indicating that *in vitro* HIV-1 infection has a profound effect on PVR expression of this $CD4^{pos}$ T cell line (Figure 4.5 D) [254]. To determine

whether TIGIT has an impact on NK cell cytotoxicity in this setting, we used uninfected and HIV-1 A17-infected parental and PVR-transduced CEM.NKR-CCR5 cell lines in tandem as targets for HIV-1-specific cytotoxicity experiments.

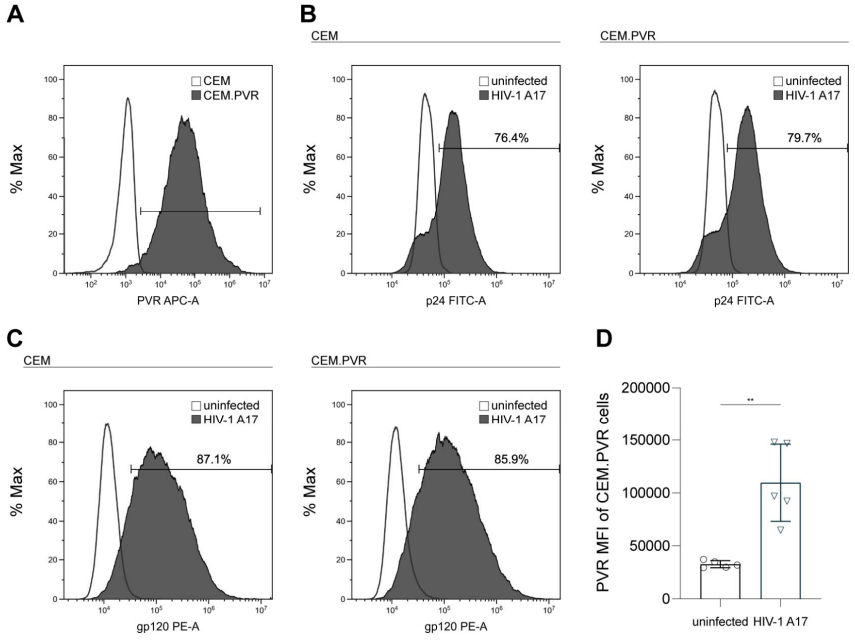

Figure 4.5 | PVR and HIV-1 antigen expression on CEM.NKR-CCR5 cells

Representative histograms from five independent experiments demonstrate (A) expression of PVR [median (IQR) 92.3% (5.1%)] on CEM.NKR-CCR5 PVR^{pos} (CEM.PVR) cells and relative levels of HIV-1 (B) p24 and (C) gp120 on CEM.NKR-CCR5 (CEM) and CEM.PVR cells 96 h after HIV-1 A17 infection. The impact of active HIV-1 infection on PVR expression was measured and (D) summarized based on MFI of PVR expression (n = 5) on uninfected CEM.PVR cells compared to CEM.PVR cells infected with HIV-1 A17 for 96 h. Student's *t*-test $^{**}P = 0.0015$. Error bars in (D) represent SD.

4.4.6 TIGIT Blockade Augments HIV-1-Specific NK cell Natural Cytotoxicity

Despite their natural resistance to NK cell-based killing, CEM.NKR-CCR5 cells infected with HIV-1 A17 became modestly susceptible to NK cell natural cytotoxicity (Figure 4.6 A, dataset one and two). Introducing PVR rendered uninfected CEM.NKR-CCR5 targets marginally susceptible to NK cell lysis as we noted slightly increased natural cytotoxicity against these cells compared to the parental line (Figure 4.6 A, dataset one and three). PVR expression also facilitated higher natural killing of HIV-1 A17-infected CEM.NKR-CCR5 PVR^{pos} targets than of the CEM.NKR-CCR5 cells not expressing PVR (Figure 4.6 A, dataset two and four) or the uninfected CEM.NKR-CCR5 PVR^{pos} targets (Figure 4.6 A, dataset three and four), likely through NK cell DNAM-1:PVR interactions [320]. As productive HIV-1 infection inflates cell surface PVR expression (Figure 4.5 D) [254], the increased killing noted for the PVR-transduced CEM.NKR-CCR5 cells infected with HIV-1 A17 in Figure 4.6 A may partly reflect an HIV-1-dependent increase in PVR levels. Blocking NK cell TIGIT receptors increased natural cytotoxicity against HIV-1 A17-infected CEM.NKR-CCR5 target cells expressing PVR (Figure 4.6 B), but had no effect on NK cell activity against infected parental CEM.NKR-CCR5, indicating that the ligand for TIGIT must be expressed for inhibition to occur and for anti-TIGIT mAb blockade to be effective. This supports the notion that PVR is the major inhibitory ligand for TIGIT expressed on these target cells and that its expression can modulate NK cell cytotoxicity against uninfected and HIV-1-infected targets.

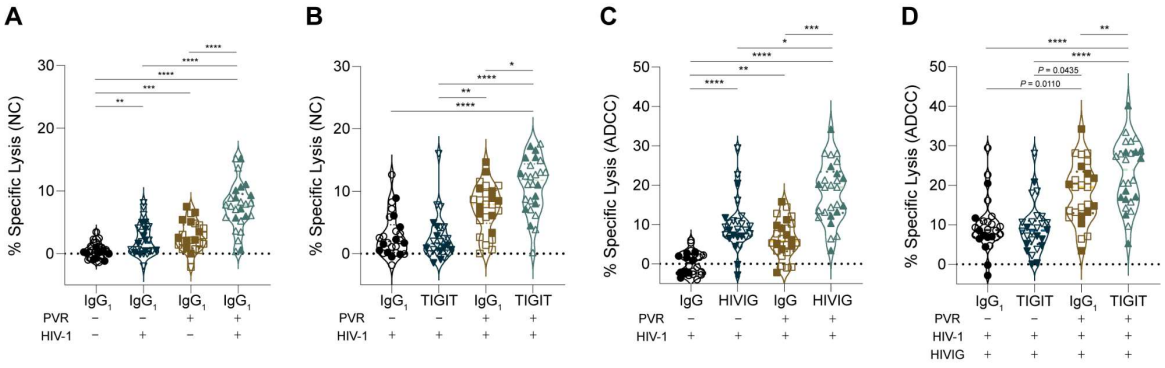

Figure 4.6 | Impact of TIGIT blockade on natural and antibody-dependent NK cell-mediated cytotoxicity

Cytotoxicity experiments were performed over 5 h with PBMC from PLWH and uninfected or HIV-1 A17-infected CEM.NKR-CCR5 or CEM.NKR-CCR5 PVR^{pos} target cells. (A) Natural cytotoxicity (NC) in the absence (-) or presence (+) of PVR was measured against uninfected (-) or HIV-1 infected (+) targets. One-way ANOVA with Tukey's multiple comparison $^{**}P = 0.0071$ $^{***}P = 0.0006$ $^{****}P < 0.0001$. (B) PBMC preincubated with control IgG_1 or anti-TIGIT mAb were tested for NK cell-mediated killing against HIV-1 A17-infected CEM.NKR-CCR5 or CEM.NKR-CCR5 PVR^{pos} targets. Non-parametric ANOVA with Dunn's multiple comparison $^{*}P = 0.0158$ $^{**}P = 0.0035$ $^{****}P < 0.0001$. (C) Control IgG or HIVIG was used to elicit ADCC against HIV-1 A17-infected targets with or without PVR and cytotoxicity was measured. Non-parametric ANOVA with Dunn's multiple comparison $^{*}P = 0.0158$ $^{**}P = 0.0016$ $^{***}P = 0.0002$ $^{****}P < 0.0001$. (D) PBMC were pretreated with control IgG_1 or anti-TIGIT mAb and the effect of TIGIT blockade on NK cell HIV-1-specific ADCC was measured against HIV-1 A17-infected CEM.NKR-CCR5 or CEM.NKR-CCR5 PVR^{pos} cells in the presence of HIVIG. Non-parametric ANOVA with Dunn's multiple comparison $^{**}P = 0.0076$ $^{****}P < 0.0001$. For all graphs, filled symbols represent HCMV-seronegative (n = 10) participants while open symbols depict HCMV-seropositive participants (n = 16). Horizontal lines bisecting groups represent median with IQR.

4.4.7 TIGIT Blockade Increases HIV-1-Specific NK cell ADCC

Data in Figure 4.4 B indicate that TIGIT engagement negatively impacted CD16 signaling. We also noted positive correlation between expression of NK cell TIGIT and markers of NK cell adaptation to HCMV (Figure 4.2 D, E). Despite the relationship between HCMV infection and loss of FcRγ subunits, the implications for NK cell cytotoxicity triggered through CD16 are unclear [158, 159, 161, 162,

271, 291], therefore, we examined the impact of TIGIT blockade on HIV-1-specific NK cell cytotoxicity triggered specifically through CD16 receptors. To accomplish this, we focussed NK cells onto HIV-1 A17-infected CEM.NKR-CCR5 or CEM.NKR-CCR5 PVR^{pos} cells using a standard pooled polyclonal anti-HIV Ig preparation from PLWH (HIVIG) or pooled Ig from HIV-seronegative individuals (IgG) as control. For both cell lines, HIVIG elicited NK cell-mediated ADCC against HIV-1 A17-infected targets compared to control IgG (Figure 4.6 C) and robust HIV-1-specific ADCC occurred independent of PVR expression (Figure 4.6 C, dataset two and four). TIGIT blockade increased HIV-1-specific ADCC responses against infected target cells expressing PVR, but not against target cells that did not express PVR (Figure 4.6 D). This again illustrates the selective benefit of TIGIT blockade when target cells, such as HIV-1-infected $CD4^{pos}$ T cells, express PVR.

Another inhibitory receptor expressed by NK cells, KIR2DL5, also selectively interacts with PVR [88, 321]. We measured the percentage of NK cells expressing KIR2DL5 to determine whether there was any association between NK cell KIR2DL5 and TIGIT expression or whether the percentage of NK cells expressing KIR2DL5 related to the impact of TIGIT blockade on individual functional responses. Approximately 25% (n = 7) of PLWH in the functionally studied cohort expressed KIR2DL5 on their NK cells with a wide range of frequency (2.7 – 23.0%; Figure 4.7 A, B). Within these experimental constraints and cohort studied, KIR2DL5 and

TIGIT expression were not linked (Figure 4.7 C) and KIR2DL5 expression had no bearing on NK cell cytotoxicity in response to TIGIT blockade (Figure 4.7 D).

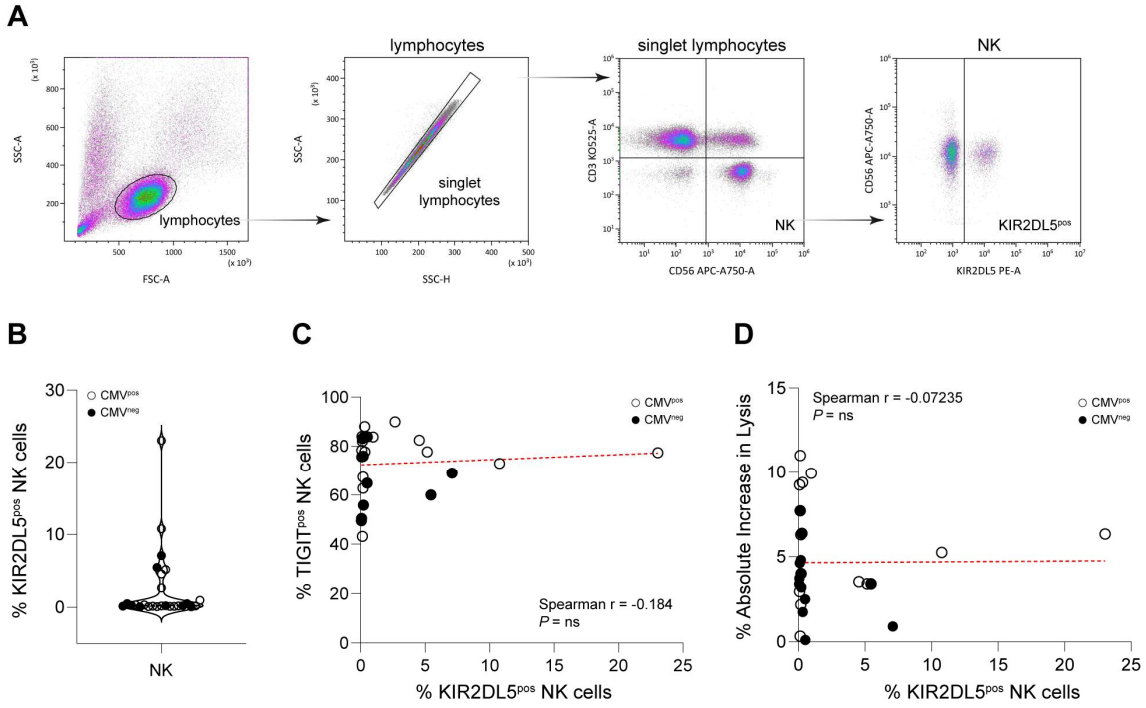

Figure 4.7| Expression of NK cell KIR2DL5 within the functionally studied PLWH cohort

(A) NK cells were defined as $CD3^{neg}CD56^{pos}$ singlet lymphocytes and $KIR2DL5^{pos}$ NK cells identified. (B) Summary data of NK cell KIR2DL5 expression for PLWH within the functionally studied cohort. Correlations between the percentage of NK cells expressing KIR2DL5 and (C) TIGIT or (D) absolute increase in % specific lysis (raw data from Figure 4.4 C) in the presence of anti-TIGIT mAb vs IgG_1 are depicted. For all graphs, filled symbols represent HCMV-seronegative (n = 10) participants while open symbols depict HCMV-seropositive participants (n = 16). Horizontal lines bisecting the group in (B) represent median with IQR.

4.4.8 TIGIT Blockade Increases HIV-1-Specific NK cell Cytotoxicity Against Autologous CD4^{pos} T cells

Whether *in vitro* disruption of NK cell TIGIT:PVR interactions can translate *in vivo* to strengthen NK cell activity against HIV-1 is the key issue for evaluating the immunotherapeutic potential of TIGIT blockade in PLWH. Our data demonstrate TIGIT blockade is effective when target cells express PVR, thus, for anti-TIGIT mAb to have a meaningful impact on NK cell cytotoxicity against HIV-1 *in vivo*, CD4^{pos} T cells harbouring HIV-1 must express PVR. In PLWH, circulating CD4^{pos} T cells express PVR (Figure 4.3 A)[243] and it is expressed to a greater extent on CD4^{pos} T cells in lymph nodes, the major compartment for HIV-1 reservoirs [241, 255, 256, 322]. Cell cycle activation induces PVR on primary CD4^{pos} T cells from both HIV-seronegative individuals and PLWH [242, 248]. To determine whether PVR was induced on *ex vivo* CD4^{pos} T cells supporting HIV-1 replication, we depleted CD8^{pos} T cells from PBMC of PLWH (CD8^{dep} PBMC) and, to control for exogenous factors modulating PVR expression, cultured otherwise unstimulated CD8^{dep} PBMC in interleukin (IL)-2 and measured intracellular CD4^{pos} T cell HIV-1 p24 (Figure 4.8 A) and surface PVR expression on HIV-1 p24^{pos} (Figure 4.8 B) and HIV-1 p24^{neg} cells (Figure 4.8 C). Data was included for analysis when p24 expression was detected on > 1,500 cells within the CD4^{pos} T cell gate. With this protocol, 9.8 ± 3.9% (mean ± SD) of CD4^{pos} T cells expressed p24 (Figure 4.8 D, left axis) with 74.8 ± 20.1% (mean ± SD) of this fraction being PVR^{pos} (Figure 4.8 D, right axis). In stark contrast, only

0.3 ± 0.4% (mean ± SD) of CD4^{pos} T cells not expressing p24 (Figure 4.8 C) were PVR^{pos} (Figure 4.8 D, right axis). Therefore, PVR expression is highly enriched on *ex vivo* CD4^{pos} T cells reactivated to express endogenous HIV-1 compared to uninfected CD4^{pos} T cells.

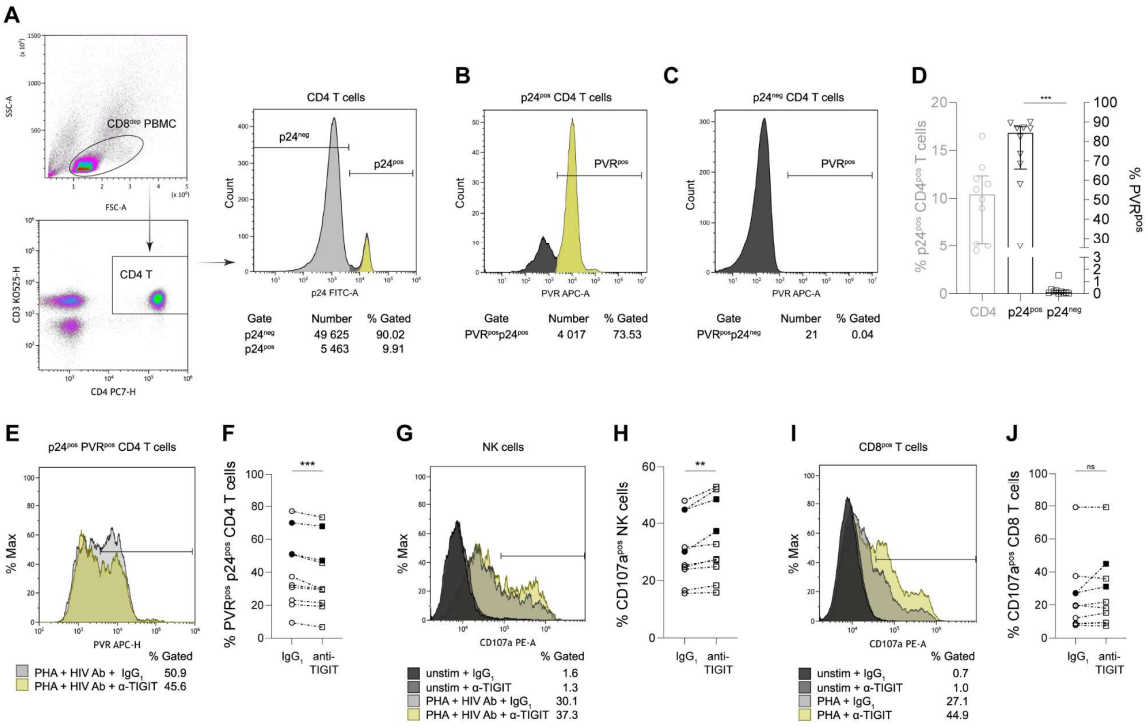

Figure 4.8| Effect of TIGIT blockade on HIV-1-specific NK cell and CD8^{pos} T cell activation and elimination of endogenously infected PVR-expressing CD4^{pos} T cells *ex vivo*

(A) PBMC from PLWH were depleted of CD8^{pos} T cells, treated with IL-2 and assessed for HIV-1 infection by p24 expression. Representative plots depict initial gating on CD3^{pos}CD4^{pos}CD8^{dep} lymphocytes and determination of the percentage of PVR expression for (B) p24^{pos} or (C) p24^{neg} cells. (D) Summary data as in (A – C) from 10 individuals. The left axis depicts percentage of CD4^{pos} T cells that were p24^{pos} 72 – 144 h after CD8^{pos} T cell depletion and the right axis represents percentage of PVR expressing cells in either the p24^{pos} or p24^{neg} CD4^{pos} T cell populations. ANOVA with Tukey's multiple comparison ***P = 0.0001. Error bars in (D) represent median with IQR. Following 72h PHA-P activation of PBMC, (E, F) loss of p24^{pos}PVR^{pos}CD4^{pos} T cells or (G, H) NK cell and (I, J) CD8^{pos} T cell degranulation was evaluated 24 h after treatment with either IgG$_1$ or anti-TIGIT mAb in the presence of anti-HIV-1 antibodies. Histogram plots for each analysis are depicted in (E, G, I) with data summarized for 10 participants in (F, H, J). Degranulation was assessed after gating on lymphocytes and enumerating CD107a expressing cells in either CD3^{neg}CD56^{pos} (NK cell) or CD3^{pos}CD8^{pos} (CD8^{pos} T cell) populations. *P*-value in (F, H, J) was calculated using Student's paired *t*-test **P = 0.0034 ***P = 0.0007.

Although allowing $CD8^{dep}$ PBMC from PLWH to 'spontaneously' express HIV-1 p24 permitted detection of selective PVR expression on $p24^{pos}$ $CD4^{pos}$ T cells, it did not result in sufficient expression of antigen to measure autologous HIV-1-specific killing. Thus, added stimulus with the mitogenic lectin PHA-P was required to reactivate endogenous HIV-1 within $CD4^{pos}$ T cells and allow spread to sufficient levels to measure autologous HIV-1-specific NK cell and $CD8^{pos}$ T cell responses from whole PBMC cultures. HIV-1 p24 was detected on 40.3 ± 21.8% (mean ± SD) of PVR^{pos} $CD4^{pos}$ T cell fractions (Figure 4.8 E and summarized in Figure 4.8 F). Addition of HIV-1-specific antibodies to induce ADCC with inclusion of anti-TIGIT mAb increased NK cell antibody dependent activation (Figure 4.8 G and summarized in Figure 4.8 H) and decreased the survival of $p24^{pos}$ PVR^{pos} $CD4^{pos}$ T cells compared to control conditions (Figure 4.8 E, F). Increased HIV-1-specific $CD8^{pos}$ T cell degranulation against autologous $CD4^{pos}$ T cells reactivated to express endogenous HIV-1 in the presence of anti-TIGIT mAb was noted for some individuals, however, overall $CD8^{pos}$ T cell degranulation was not significantly affected under these conditions (Figure 4.8 I and summarized in Figure 4.8 J). These data demonstrating *ex vivo* elimination of autologous p24-expressing targets corroborate *in vitro* data using cell lines overexpressing PVR and suggest that anti-TIGIT mAb treatment is a rational approach to augment NK cell lysis of HIV-1 infected targets *in vivo*.

4.5 Discussion

An important role for TIGIT in regulating immune responses was demonstrated in recent studies [240, 241, 243, 244]. The breadth of TIGIT expression on different lymphocyte subsets, together with increased expression of its ligand, PVR, on $CD4^{pos}$ T cells infected with HIV-1, combine to create a compelling target for checkpoint inhibition in PLWH. Several studies reported altered regulation of TIGIT expression levels in PLWH and showed that TIGIT modulates the function of immune effector cells, including HIV-1-specific $CD8^{pos}$ T cells [241, 243, 244, 323]. However, questions remain as to what factors underlie increased TIGIT expression on different immune cell subsets and what impact TIGIT expression has on effector cell functions in different contexts. We studied TIGIT expression on the immune cell subsets of over 90 PLWH and focused functional studies on NK cells from a representative subset. Our data indicate that upregulation of TIGIT on different immune cell subsets relates to distinct factors and that blockade of NK cell TIGIT interaction with PVR consistently increases natural cytotoxicity and ADCC against HIV-1-infected and other target cells.

In PLWH with chronic infection, TIGIT was expressed to the greatest extent on NK cells, followed by $CD8^{pos}$ T cells and $CD4^{pos}$ T cells. Compared to age-matched HIV-seronegative controls, TIGIT expression in PLWH was elevated on NK cells and $CD4^{pos}$ T cells, but not on $CD8^{pos}$ T cells. Expression of TIGIT on $CD4^{pos}$ T cells increases with age/immune experience and further increases with chronic HIV-1

infection, despite cART. Considering the mean duration of HIV-1 infection (15.8 ± 10.3 years) for the PLWH cohort, our data indicates that levels of TIGIT on $CD8^{pos}$ T cells of cART-treated PLWH return to similar levels as expressed by age-matched HIV-seronegative individuals. There was very little TIGIT expression on T cells in CBMC, however, TIGIT was expressed on a substantial proportion of cord blood NK cells, albeit at a lower frequency than on NK cells from PLWH. This indicates that TIGIT expression occurs as a natural aspect of NK cell ontogeny, while its expression on T cells reflects post-developmental aspects of T cell maturation. Levels of TIGIT on both $CD4^{pos}$ and $CD8^{pos}$ T cells of PLWH correlated with extent of HIV-1 disease progression as defined by $CD4^{pos}$ T cell nadir. Although several other studies associated elevated NK cell TIGIT expression with correlates of HIV-1 disease progression [240, 241, 243, 244], we found that the frequency of TIGIT expression on NK cells of PLWH correlated with the extent of NK cell adaptation to HCMV infection as indicated by loss of FcRγ or increased expression of NKG2C and CD57.

The influence of HCMV infection was also illustrated in functional modulation of NK cells by TIGIT engagement. Experiments using P815 and K562 NK cell targets verified the influence of TIGIT engagement on NK cell cytotoxicity. Engaging NK cell TIGIT compromised signaling through CD16 and blocking TIGIT:PVR interaction effectively augmented NK cell natural cytotoxicity. The NK cells from HCMV-seropositive PLWH with higher levels of TIGIT expression had larger increases in NK cell cytotoxicity in the presence of anti-TIGIT mAb compared

to NK cells from the HCMV-seronegative group. Considering the caustic association between HIV-1 and HCMV infection and the reciprocal role each virus has contributing to the others' reactivation, the higher prevalence of TIGIT^{pos} NK cells in PLWH compared to the HIV-seronegative group could reasonably be attributed to more frequent HCMV reactivation in PLWH. This possibility is consistent with the exaggerated extent of both HCMV-specific CD8^{pos} T cell memory inflation and NK cell adaptation to HCMV infection reported in PLWH [204, 324]. Just as an increase in the rate of NK cell adaptation to HCMV is accelerated by HIV-1 infection, so too may be the rate of NK cell TIGIT accumulation. These results underscore the need to consider HCMV coinfection in the context of NK cell studies with PLWH.

Most relevant to enhancing immune-mediated strategies to address the HIV-1 reservoir in PLWH, we demonstrated that TIGIT blockade increases NK cell cytotoxicity and degranulation in response to HIV-1-infected CD4^{pos} T cells *in vitro* and *ex vivo*. Transduction of an HIV-1-permissive CD4^{pos} T cell line with PVR showed that the increase in NK cell cytotoxicity induced by TIGIT blockade was dependent upon target cell PVR expression. We further showed that reactivation of HIV-1 in CD4^{pos} T cells from PLWH selectively increased PVR expression on infected cells, rendered infected cells susceptible to NK cell-mediated ADCC and that TIGIT blockade increased NK cell degranulation in response to HIV-1-bearing autologous target cells. Moreover, the reduction in HIV-1 antigen positive PVR^{pos} CD4^{pos} T cells in anti-TIGIT mAb conditions compared to control demonstrated accelerated

elimination of HIV-1^{pos} cells by NK cells. KIR2DL5 is another inhibitory receptor expressed by NK cells and CD8^{pos} T cells capable of binding PVR independently [88]. In preventing TIGIT:PVR interactions, we expected that PLWH with higher levels of KIR2DL5-expressing NK cells might not respond to anti-TIGIT mAb blockade as robustly as those not expressing KIR2DL5. Despite measuring KIR2DL5 expression on seven of the twenty-six participants within the functional cohort, we found KIR2DL5 expression to have no significant impact on NK cell natural or antibody-dependent functions in the presence of anti-TIGIT mAb. As the total number of participants identified with KIR2DL5^{pos} NK cells in the functional cohort was low, and only two individuals had > 10% KIR2DL5 expression on their NK cells, further investigation that includes a larger cohort of PLWH exhibiting higher levels of KIR2DL5 expression coupled with the consideration of differential NK cell education is required.

Conscripting our own immune system to purge HIV-1 reservoirs is an attractive approach not yet successfully applied. One major barrier to engaging NK cells in HIV-1 reservoir elimination is the limited expression of HIV-1-specific or HIV-1-related cell surface antigens during latency and early reactivation. Without a basis for specifically targeting HIV-1-infected cells, no amount of reinvigoration of NK cell activity will address the reservoir. The enriched presence of HIV-1 in CD4^{pos} T cells expressing PVR provides a means of targeting cells in which HIV-1 is reactivated and, under appropriate conditions, PVR expression promotes NK cell

activity. Since DNAM-1:PVR interactions favour effector cell-mediated killing and TIGIT:PVR engagement decreases or prevents killing, TIGIT blockade has a dual effect of reducing inhibitory signaling and enhancing activation [72, 325].

The use of anti-TIGIT mAb to prevent inhibitory TIGIT:PVR interactions increased NK cell activity against HIV-1-infected autologous $CD4^{pos}$ T cells. Circulating $CD4^{pos}$ T cells from PLWH express appreciable levels of TIGIT and $CD4^{pos}$ T cell TIGIT and PVR expression were positively correlated. In relation to HIV-seronegative controls, PVR expression on $CD4^{pos}$ T cells from PLWH was elevated, however, the symbiotic relationship between HIV-1 and HCMV infection that encourages sustained inflammation and immune activation is highlighted in our finding that levels of PVR were particularly enriched on circulating $CD4^{pos}$ T cells from HCMV-seropositive PLWH compared to HCMV-seronegative PLWH. Lymph node resident follicular helper $CD4^{pos}$ T cells or circulating $CD4^{pos}$ T cells in which the HIV-1 reservoir is concentrated are more likely to express TIGIT in combination with other inhibitory immune checkpoint receptors such as PD-1 and LAG-3 [71, 75, 326]. Therefore, TIGIT expression alone, or in combination with other inhibitory immune checkpoint receptors identifies $CD4^{pos}$ T cells enriched for HIV-1 infection. Introducing anti-TIGIT mAb could have even broader influence by lifting any inhibition of latent HIV-1 activation imposed through TIGIT:PVR interaction, thereby increasing HIV-1 antigen expression and the potential for immune recognition. Studies examining the influence of TIGIT blockade on lymph

node resident TIGIT-expressing $CD4^{pos}$ T cells harbouring HIV-1 are warranted. Humanized anti-TIGIT mAbs are in early trials for use in cancer therapy and anti-PD-1 treatment is being investigated in clinical studies targeting cancer and, to a limited extent, latent HIV-1 reservoirs expressing PD-1 [327, 328]. Treatment with anti-PD-1 was generally well-tolerated in PLWH with well controlled HIV-1 viral loads and future inclusion of PLWH immunotherapy trials will inform whether anti-TIGIT mAb is tolerated to the same extent as in HIV-seronegative participants [328].

In summary, we found that TIGIT expression is upregulated on T cells and NK cells of PLWH. The extent of expression relates to HIV-1 disease progression in the case of T cells and to HCMV coinfection in the case of NK cells. The NK cells of PLWH coinfected with HCMV exhibit greater increases in cytotoxicity with TIGIT blockade than NK cells from HCMV-seronegative PLWH and NK cells of PLWH respond to TIGIT blockade with increased activation against autologous $CD4^{pos}$ T cells when endogenous HIV-1 replication is stimulated. Thus, TIGIT blockade could have a synergistic effect as a component of immunotherapeutic strategies targeting the HIV-1 reservoir by releasing immune effector cells from inhibition and favouring HIV-1 replication in the $TIGIT^{pos}$ reservoirs being targeted. The impact of TIGIT blockade on recognition of HIV-1-infected cells supports broader application of NK cell-based therapies in other chronic conditions exploiting TIGIT:PVR interactions for immune regulation.

4.6 Conclusions

Immune dysfunction is a hallmark of HIV-1 infection and the expression of inhibitory receptors remain elevated on immune effector cells despite cART. TIGIT expression is dysregulated on NK cells from PLWH and directly relates to HCMV coinfection. Expression of PVR, the predominant ligand for TIGIT, is increased on resting *ex vivo* CD4^{pos} T cells and is further induced with HIV-1 infection. Preventing TIGIT from interacting with PVR augments NK cell and CD8^{pos} T cell activation against autologous HIV-1 infected CD4^{pos} T cells.

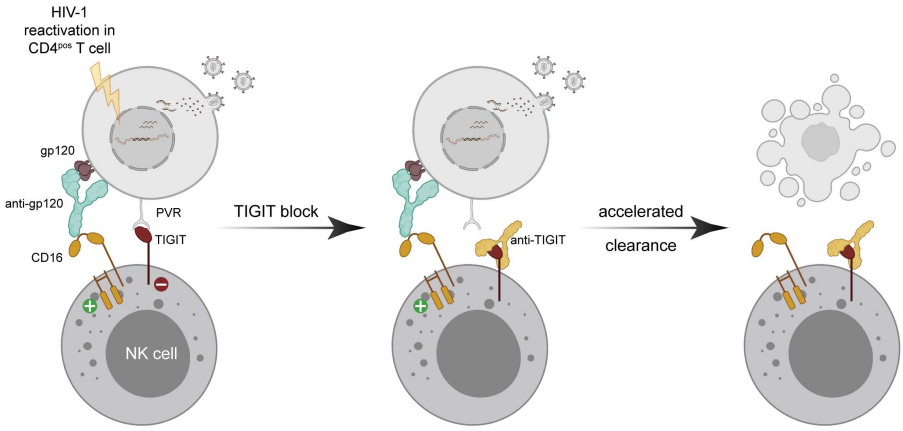

Figure 4.9| TIGIT blockade augments HIV-1-specific NK cell cytotoxicity

TIGIT expression is dysregulated on NK cells from PLWH and directly relates to HCMV coinfection. Expression of PVR, the predominant ligand for TIGIT, is increased on resting *ex vivo* CD4^{pos} T cells and is further induced with HIV-1 infection. Preventing TIGIT from interacting with PVR augments NK cell activation against autologous HIV-1 infected CD4^{pos} T cells.

5 Summary and Future Directions

HCMV has evolved many mechanisms to manipulate and evade both innate and adaptive immunity. It is a highly evolved, complex ancient virus with one of the largest genomes of all human viruses. The NK cell repertoire is particularly affected by HCMV infection in both phenotype and function, as demonstrated by the expansion of a subset of adapted NK cells identified by gain of NKG2C and CD57 markers combined with the loss of FcRγ and NKG2A and purportedly greater functional capacity for ADCC [17, 106, 136, 141, 159]. As our immune response is shaped and altered in the setting of chronic HCMV infection, questions arise as to (i) whether this process of NK cell adaptation improves upon their cytotoxic capabilities and, (ii) if HCMV infection does impact NK cell cytotoxicity, to what extent is the impact facilitated by NK cell adaptation itself or by inflammation in combination with the myriad other environmental factors closely associated with HCMV infection.

Both human- and virus-encoded cytokines produced during HCMV infection impact NK cell functions. The HCMV homologue of hIL-10, cmvIL-10, is expressed during active infection as a full-length variant and exhibits specificity for the hIL-10R despite overall low sequence homology with hIL-10 [181, 258, 263]. Although previous research identified that hIL-10, in combination with other stimulatory cytokines, positively impacted NK cell functions, the influence of hIL-10 or cmvIL-10 alone on NK cell function was previously unreported [273, 277-281, 329].

Investigating whether short-term exposure to cmvIL-10 affected NK cell functions or contributed to phenotypic changes associated with their adaptation revealed that while cmvIL-10 substantially enhanced NK cell natural cytotoxicity and ADCC, it did not rapidly introduce phenotypic changes reminiscent of NK adaptation to HCMV infection [329].

In general, IL-10 engages the hIL-10Rα subunit of hIL-10Rα/β heterodimers, but it can also interact with hIL-10β subunits, with expression of hIL-10β widely varying across immune cell types to fine tune responses and contribute to the functional pleiotropy of IL-10 [330] Dimerization of two hIL-10Rα/β heterodimers $(hIL\text{-}10R\alpha/\beta)_2$ results in (predominantly) STAT3 transcription factor phosphorylation, activation, and cytokine expression that differentially regulate immune responses [274]. STAT3 activation negatively regulates IFN-α/β and IFN-γ production by downregulating IFN-stimulated gene expression and opposing STAT1 signaling, a crucial and potent inducer of NK cell antiviral responses [112-114, 331]. These negative feedback mechanisms serve to prevent excessive proinflammatory and autoreactive responses. Extended STAT3 activation also impacts metalloproteases by increasing their enzymatic activity [332]. Matrix metalloprotease (MMP) and a disintegrin and metalloproteinases (ADAMs) are a class of enzymes that break down extracellular matrix proteins to enable cell migration and promote shedding of receptor-bound proteins, including cytokines [333]. MMPs and ADAM17 are expressed by NK cells and can act to cleave CD16

receptors from their cell surface [334-337]. With evidence that cmvIL-10 downregulates MMP-9 and its role in reducing cell surface receptor shedding, cmvIL-10 could have a dual benefit to NK cell function. First, by acting through hIL-10R to stimulate NK cell activity through pan activating receptors and second, by downmodulating MMP activity to prevent CD16 shedding, thereby increasing NK cell antiviral activity. However, there are also benefits to intact NK cell MMP activity. NK cells physically interacting with and attached to a target cell will more readily detach with MMP-mediated activating receptor (*e.g.* CD16) cleavage and subsequent immune synapse disassembly [338-340]. This means that with intact MMP activity, an NK cell will disengage from its target at a greater pace enabling subsequent engagement with and killing of the next aberrant or infected cell in serial succession. As only a small fraction of circulating NK cells expressed detectable amounts of the hIL-10R, increased activity observed by this subset of NK cells could be quickly disrupted if cmvIL-10 also dysregulates optimal MMP-mediated activating receptor cleavage to impair serial target killing. As decreased MMP activity can augment NK cell ADCC and antiviral activity by sustaining CD16 receptors, yet in other instances, intact MMP activity can promote NK cell detachment from target cells and potentiate serial killing, further studies to measure NK cell MMP levels and assess serial killing after short- and longer-term exposure to cmvIL-10 are warranted.

In response to short-term cmvIL-10 stimulation, increased NK cell cytotoxicity against target cells was rapid and dose-dependent, however, there were no significant differences in NK cell degranulation or IFN-γ production. This indicates that the means by which one chooses to measure NK cell function alone impacts how we perceive and interpret NK cell responses to stimulation. Conversely, complex regulation of NK cell responses may account for the seemingly discrepant findings that cmvIL-10 increases NK cell cytotoxicity but not markers of activation. IL-10R heterodimerization and subsequent STAT3 activation upon cmvIL-10 binding negatively regulates STAT1 and may explain in part why NK cell cytotoxic functions were augmented whilst there was no significant augmentation to IFN-γ stimulation during short-term exposure. However, extended (16 h) exposure to hIL-10 was recently shown to increase NK cell IFN-γ secretion and cytotoxicity by inducing metabolic changes that supported effector functions [341-344]. Since both short (5 h) and extended (16 h) exposure to hIL-10 augments NK cell effector functions and extended exposure induces metabolic changes in NK cells, it will be interesting to see whether cmvIL-10 causes the same metabolic alterations. Although not normally considered proinflammatory, hIL-10 is functionally pleiotropic, depending on the cell type with which it interacts. As hIL-10 influences NK cells to modify their antiviral responses and alter metabolic programming, the small subset of NK cells expressing hIL-10R and responding to cmvIL-10 could be

progenitors of the larger population of NK cells reshaped from prolonged exposure to HCMV.

The notion that a virus-encoded cytokine expressed during productive infection of permissive cells would influence NK cell function in favour of the host is counterintuitive. How HCMV can flourish despite robust IFN-α/β production, which typically inhibits virus replication and stimulates protective host factors and NK cell cytolytic activity, is also unclear. Nevertheless, dysregulated immune responses caused by cmvIL-10 can promote viral dissemination and long-term persistence in immune competent hosts [345, 346]. Despite short-term exposure to cmvIL-10 tilting NK cell function in favour of host defense, cmvIL-10 has immunosuppressive properties similar to that of hIL-10 [274]. During chronic infection, the immune response shifts away from a type I proinflammatory response to a predominantly immunosuppressive type II response, largely driven by hIL-10 (and presumably, cmvIL-10) to limit immune pathology and promote viral persistence [347-351]. It is apt that the master of immune evasion encodes a protein that mimics a master regulator of immune responses. Exposure to cmvIL-10 parallels the modulating effects of hIL-10: it inhibits monocyte proinflammatory cytokine production and HLA I and II expression, has a profound effect on DC maturation and disrupts NK cell / DC crosstalk [264, 265, 352].

DCs have a role in coordinating innate and adaptive immune responses, therefore, limiting their maturation and costimulatory receptor expression would

negatively impact antigen presentation, T cell stimulation and subsequent development of effective adaptive responses [267, 268]. Interplay between DCs and NK cells generally leads to NK cell activation and cytokine release, which, in turn promotes DC maturation or deletion (apoptosis) [108, 353-355]. Exposure to IFN-α/β during HCMV infection can assist DC maturation and subsequent production of proinflammatory soluble factors such as IL-12, IL-15 and IL-18, which enhance NK cell antiviral IFN-γ production [354, 356, 357]. In turn, NK cells provide a measure of quality control over immature DCs by delivering feedback signals to polarize DC maturation towards antiviral type I T cell responses and edit aberrant mature DCs out of circulation [358-361]. In the setting of HCMV infection, this interplay is dysregulated as adapted NK cells downregulate the transcription and expression of signaling receptor subunits for IL-12 and IL-18 and thereby suffer reduced responsiveness to these cytokines compared to canonical NK cells [143, 362]. Their diminished response to IL-12 and IL-18 reduces IFN-γ production, a key factor in DC polarization, thereby disrupting induction and maintenance of adaptive antiviral responses [363]. Furthermore, DCs express high levels of CD112 and PVR and their interaction with NK cell TIGIT reduces IL-12 expression and increases hIL-10 production, which feeds back to induce further NK cell TIGIT expression [71, 243]. Thus, for individuals with higher levels of $TIGIT^{pos}$ NK cells (*i.e.,* HCMV coinfected PLWH), the combined modulation of the NK cell compartment with higher levels

of TIGIT on a greater fraction of adapted NK cells could contribute to suppressed antiviral immunity.

Engaging TIGIT is implicated in control of immune pathology by shifting the balance towards IL-10-dominated type II immunosuppressive responses [71, 86, 364]. TIGIT-expressing memory B cells and T cells modify DC responses to suppress their maturation, reduce IL-6 and IL-12 secretion and promote IL-10 production [71, 323]. When exposed to hIL-10, immature DCs become resistant to NK cell editing and mature DCs become more susceptible to NK cell-mediated elimination, leading to progressive T cell dysfunction [365]. Both hIL-10 and cmvIL-10 severely modulate antigen presenting cells by inhibiting expression of costimulatory molecules, HLA, and the secretion of proinflammatory mediators such as IL-1β, IL-6, IL-12 and TNF-α to disrupt antiviral effector cell activation [264, 366]. Mature DCs exposed to cmvIL-10 are now susceptible targets for NK cell-mediated elimination, a process presumably augmented by the exposure of NK cells to cmvIL-10 themselves. Little is known about the impact of cmvIL-10 on NK cell-mediated cytolysis of DCs. If a similar trend follows with cmvIL-10, by encoding just one virokine, HCMV has the potential to disrupt downstream T cell responses and dysregulate the timely, productive generation of acquired immunity.

Host-derived IFN-α/β directly promotes NK cell activation through multiple activating receptors. Plasmacytoid DCs (pDCs), are natural IFN-producing cells that are the major source of IFN-α/β in response to viral infection [367, 368]. Although

poor antigen presenting cells, pDCs stimulate NK cells and promote $CD8^{pos}$ T cell cytotoxicity by polarizing antigen-experienced cells to type I based immune responses [369, 370]. High levels of IFN-α/β are produced by pDC up to 30 h after contact with HCMV-infected cells to induce robust NK cell IFN-γ production [371]. However, during productive HCMV infection, secretion of cmvIL-10 from HCMV-infected cells severely quells IFN-α/β production, presumably through STAT3-mediated inhibition of STAT1 signaling [372]. As we consider the multipotent immunomodulatory effect of cmvIL-10 on our immune system, HCMV-infected cells secreting cmvIL-10 can dampen pDC IFN-α/β production, preventing type I T cell polarization and NK cell activation / DC crosstalk. Although our experiments considered the effects of both cmvIL-10 and IFN-α/β on NK cell function independently, the HCMV strain used for our studies was impaired in cmvIL-10 expression. Thus, this virokine was not present in supernatants harvested during active infection of fibroblasts in Chapter 3. It would be prudent to examine infection kinetics and determine whether IFN-α/β expression is altered during active infection when intact *UL111a* is also present by using a more physiologically relevant strain of HCMV and infection model.

To study longer-term effects of HCMV infection on NK cells, we focused on a cohort of PLWH exhibiting varying degrees of NK cell adaptation to HCMV infection. Groups were distinguished by high or low expression of NKG2C and their functional responses evaluated. Despite PLWH exhibiting a clear hierarchy of

adaptation to HCMV in our study, we observed no difference between groups in NK cell cytotoxicity, degranulation or IFN-γ secretion through CD16 receptors in response to physical stimuli [271]. Studies using healthy controls suggested that HCMV infection imprints superior responses through CD16 receptors, often measured by IFN-γ release [124, 159]. This effect was attributed to HCMV-induced epigenetic changes in the promotor region and upstream CNS-I enhancer of the *IFNG* locus in NKG2C^{hi} or CD57^{pos} NK cells [161, 164]. Relaxation of DNA methylation allows access to the *IFNG* locus, thereby lowering the threshold for NK cell cytokine production following stimulation [143, 144]. However, varying degrees of NK cell IFN-γ production in response to different types of stimuli have been noted. For instance, IL-12 / IL-18 stimulation yields less IFN-γ production from FcRγ^{neg} NK cell populations compared to nonadapted canonical counterparts, however, combined phorbol 12-myristate 13-acetate / ionomycin results in their potent activation and IFN-γ secretion [143]. Degranulation responses to stimuli can also differ. Similar to our observations with Ab-opsonized target cells, Schlums *et al.* noted that adapted NK cells degranulated to the same extent as their nonadapted counterparts in response to physical CD16 stimulation [143, 271].

Although it is tempting to ascribe superior qualities to NK cells adapted to HCMV infection, it is the way in which adapted NK cells are stimulated that determines their variegated response. Not all input signals are created equal and convoluted signaling pathways yield differential responses. The environment to

which an adaptively-pliable NK cell is exposed during HCMV infection imprints a program that seems to favour ADCC in response to Ab-opsonized targets. Although this feature may benefit specific antiviral responses, this conditioning also reduces sensitivity to certain proinflammatory mediators and has the potential to prevent induction of appropriate innate and adaptive immunity to subsequent infectious challenges [362].

Considering that inhibitory immune checkpoint receptor expression is often dysregulated in settings of chronic infection, we examined the expression of select inhibitory receptors (*i.e.,* LAG3, Tim-3, PD-1, TIGIT) on NK cells from PLWH whilst considering the influence of HCMV coinfection. LAG3 and PD-1 were expressed at very low or undetectable levels on NK cells, therefore, we first considered the functional implications of Tim-3^{pos} NK cells in the setting of persistent immune activation during chronic HIV-1 and HCMV coinfection. Although others found Tim-3 to be expressed on a large fraction of circulating NK cells from healthy donors, we found that only ~5% of resting NK cells expressed Tim-3 with similar frequency between groups of HCMV^{neg} and HCMV^{pos} PLWH [271, 373-375]. Despite activation, levels of NK cell Tim-3 receptors remained unchanged in our cohort of PLWH. Tim-3 expression was unaffected by CD16 stimulation and Tim-3^{pos} NK cells from HCMV^{pos} PLWH produced significantly less IFN-γ than the total NK cell fraction [271]. PLWH have vastly lower levels of Tim-3^{pos} NK cells than HIV-1-seronegative counterparts and this loss of NK cell Tim-3 expression correlates with

elevated immune activation often seen in HCMV coinfected PLWH [301, 375-377]. Reduced Tim-3 expression can contribute to ongoing immune dysfunction in PLWH, thus, Tim-3 has the potential for a dichotomous role in regulating NK cell functions.

Our study that examined the role for Tim-3 in modulating NK cell functions in the context of PLWH contrasted with results from others who demonstrated that NK cell activation can rapidly augment Tim-3 expression [373, 374]. The immune environment and physical cues determine whether Tim-3 negatively regulates NK cell functions and marks hypofunctional NK cells or whether Tim-3 expression potentiates antiviral activity to identify a subset of activated and functionally competent NK cells [373, 374, 378]. Nuances in the source of NK cells, the techniques used in their isolation and selection, and their prolonged culture and/or inclusion of cytokines may also account for recorded discrepancies in Tim-3 expression and its functional implications between studies. The method of NK cell stimulation is also important. Both the stimulus and the manner by which Tim-3 is induced and regulated on NK cells determines how it influences NK cell function [378]. In our study, we stimulated NK cell CD16 receptors via the antigen binding fragment (Fab) of anti-CD16 antibodies rather than through the fragment crystallizable (Fc) region of an antibody attached to target cells. As Fc multimers evoke robust IFN-γ production from Tim-3^{pos} NK cells obtained from healthy controls, it would be prudent to contrast functional responses and phenotypic

changes of NK cells from PLWH using different mechanisms of activation (*e.g.* CD16 Fab versus target:Ab Fc) to determine whether IFN-γ responses from Tim-3^{pos} NK cells are similar or if our choice of stimulation inadvertently underestimated their potential for IFN-γ production.

Focusing on Tim-3 as an independent marker to identify dysregulated NK cells need be approached with caution. As such, we also examined the extent to which TIGIT was expressed on NK cells as expression of this marker is broadly dysregulated in HIV-1 infection [243, 244, 302]. We found that cART-treated PLWH had elevated TIGIT levels on CD4^{pos} T cells and NK cells compared to HIV-1-seronegative or newborn controls. Although higher levels of TIGIT on CD4^{pos} T cells correlated with HIV-1 disease progression, the extent to which NK cell TIGT was elevated was associated with NK cell adaptation to HCMV. Further, TIGIT is expressed to a greater extent on resting adapted NK cells compared with their conventional counterparts [244]. The increase in NK cells expressing TIGIT in PLWH may be attributed to the increased frequency and level of HCMV reactivation events encountered in the setting of chronic HIV-1 infection in addition to exacerbated immune inflammation, as compared to HIV-1 seronegative controls.

Cord blood NK cells expressed comparable levels of TIGIT to those derived from adult peripheral blood. TIGIT-specific NK cell education seemingly occurs during human ontogeny and its expression and function continues to be modified during post-natal development in response to environmental cues, particularly

during chronic viral infection. Ancillary to their primary education, NK cells receive inductive signals from environmental cues to fine-tune their functional responses [50, 55]. Although NK cells are predominantly educated through iKIR, expression of other inhibitory receptors (*e.g.* NKG2A or TIGIT) can contribute to nonclassical NK cell education [309]. Using murine models, He *et al*. demonstrated that TIGIT-expressing NK cells were highly functional, and acquisition of robust effector function is contingent upon host PVR expression during their functional maturation [309]. Inhibitory receptors are necessary to educate NK cells to sense missing self and render NK cells tolerant to cells expressing appropriate levels of cognate ligands. NK cells educated through TIGIT receptors have increased responses to targets lacking PVR, therefore, conditions supporting or increasing the expression of PVR *in vivo* will ligate TIGIT and impede NK cell-mediated functions [309]. NK cells are conditionally specific and reserve the ability to adjust their responses to ever-changing immune environments [379-381]. However, education through TIGIT:PVR interaction alone does not confer NK cell polyfunctionality (heightened degranulation and IFN-γ secretion), providing further illustration that NK cell education occurs along a continuum, with higher tuning from stronger inhibitory inputs allowing lower thresholds for NK cell activation and higher probability of both degranulation and IFN-γ secretion upon stimulation [50, 309].

In murine studies, TIGIT's paired receptor, DNAM-1, serves as an accessory signal for the differentiation and proliferation of adaptive LY49H^{pos} NK cells in

response to MCMV infection [133]. Expression of PVR and CD112 are upregulated *in vivo* on MCMV-infected DCs and macrophages providing critical interaction with and signaling through DNAM-1 (via Fyn and PKCη) to generate adapted NK cells [132, 133]. In HCMV infection, extracellular expression of both PVR and CD112 are downmodulated by HCMV-encoded UL141 to delay virus clearance and escape NK cell-mediated recognition [382, 383]. However, TIGIT-educated NK cells may facilitate a means by which to recognize HCMV-infected targets that have lost ligands for TIGIT, namely, PVR. By concomitantly preventing DNAM-1 and TIGIT engagement by sequestering their ligands within infected host cells, HCMV-infected cells can still be recognized by alternate NK cell stimulatory receptors, compensating for loss of signals through DNAM-1, and receive less inhibition through TIGIT with PVR missing from autologous cells. With HCMV having such an extensive set of genes that encode proteins to upset typical NK cell functions and interactions, NK cells may have evolutionarily compensated by shifting effector responses to predominantly function through CD16:Ab recognition of altered cells – the most notable feature of adapted NK cells. The apparent loss of NK cell plasticity and appropriate response to innate stimuli in combination with diminished capacity for helping DC maturation may have important implications for control of other chronic virus infection. However, simultaneous gain of specialization for Ab-dependent responses may allow for their exploitation in suitable environments and

raises the controversial question as to whether NK cells, and particularly ADCC, are realistic means by which to control HIV-1.

The main HIV-1 reservoir is not circulating and the principal obstacle to HIV-1 cure is the lingering and inaccessible virus sanctuary within T_{FH} cells residing in lymph node B cell compartments. This, combined with the lack of HIV-1 antigen expression on reservoir $CD4^{pos}$ T cells leaves no appropriate means to target them immunologically. Without specific cell surface markers or HIV-1 antigen expression/peptide presentation to identify infected cells, no level of competent effector cell function can eradicate HIV-1. Together with $CD8^{pos}$ T cells and NK cells, $CD4^{pos}$ T cells, including T_{FH} cells residing deep within lymph node tissues, express TIGIT [71, 75]. The $CD4^{pos}$ T cell fraction expressing TIGIT is enriched for integrated HIV-1 DNA and the frequency of $TIGIT^{pos}$ cells that also co-express PD-1 and LAG-3 correlates with the size of the HIV-1 reservoir [326]. Expression of TIGIT on $CD4^{pos}$ T cells, alone or in combination with other immune checkpoint receptors, identifies a subset of $CD4^{pos}$ T cells more likely to harbour latent HIV-1.

Although TIGIT expression can help identify HIV-1 reservoirs, these cells need to be activated or shocked into productive infection to express HIV-1 antigens or associated stress proteins enabling recognition by antiviral effector cells. Maintenance of stable HIV-1 reservoirs involves persistent inhibition through interactions between checkpoint inhibitors, such as PD-1 or TIGIT, and their ligands [235]. Consistent with the latency reversal noted with anti-PD-1 mAb, introducing

anti-TIGIT mAb to unleash negative regulation can help shock TIGIT-expressing $CD4^{pos}$ T cells into activation and shift latent HIV-1 into active production [235, 240, 326, 384-386]. Our study demonstrated that HIV-1 infected cells can be selectively targeted for NK cell ADCC using antibodies derived from plasma of PLWH and that TIGIT blockade further increases ADCC activity and autologous cytolytic $CD8^{pos}$ T cell functions. Targeting TIGIT as part of a cure strategy for HIV-1 could concurrently help force HIV-1 out of hiding, while augmenting $CD8^{pos}$ T cell and NK cell antiviral functions to bridge effector cell functions with recognition of HIV-1-infected cells – a multipronged 'shock and kill' approach.

The vast majority of PLWH rely on cART to maintain HIV-1 viremia below limits of current detection, yet a remarkable few (< 1%) 'elite controllers' possess the innate and spontaneous ability for prolonged control of HIV-1 below current limits of detection and maintain normal $CD4^{pos}$ T cell counts [387, 388]. Determining how this rare group of individuals control HIV-1 provides insight into current and future vaccine and cure strategies. Strong genetic overrepresentation of protective HLA B57 and HLA B27 alleles in elite controllers is associated with superior viremic control, and despite general immunological heterogeneity, strong Gag-specific polyfunctional $CD8^{pos}$ T cell responses and lower frequency of $TIGIT^{pos}$ $CD4^{pos}$ T cells are a common feature in aviremic PLWH [389-395]. Interestingly, elite controllers are less likely to develop neutralizing (n)Ab responses than viremic individuals and even rarer PLWH generate broadly (b)nAbs to HIV-1 that retain

protective qualities despite the ever-changing and immune-escaping HIV-1 envelope glycoproteins $(gp120/gp41)_3$ [396-401]. However, non-neutralizing antibodies are found at greater levels in HIV-1 controllers than viremic PLWH and these antibodies mediate protection by complexing infected cells to enable macrophage-dependent phagocytosis or NK cell-mediated ADCC [393, 402-405]. The rhesus macaque simian immunodeficiency virus (SIV) model of HIV-1 infection and vaccine trials involving human participants (RV144) demonstrate that despite the inability to generate bnAbs, eliciting potent non-neutralizing envelope-specific antibody responses can facilitate NK cell ADCC [396-401, 403, 406-409].

Although the RV144 vaccine trial involving Thai participants with HIV-1 CRF01_AE was partially protective (31.2%), a subsequent trial focussing on HIV-1 clade C participants in South Africa (HVTN 702) showed no efficacy, despite also inducing non-neutralizing antibodies [407, 410, 411]. The main correlate of protection elicited by the RV144 vaccine regimen was antibodies against a linear epitope containing lysine at position 169 (K169) in the variable loop 2 (V2) region of gp120 [407, 412-414]. Notably, vaccine efficacy against K169-bearing HIV-1 CFR01_AE was related to a genetic predisposition for FcR. This thesis has extensively considered the role for CD16 (FcγRIIIa) in mediating NK cell ADCC, however, the human family of low affinity IgG receptors also includes isoforms of FcγRII (CD32), with activating FcγRIIc (CD32c) being potentially relevant for ADCC [415]. Only ~20% of the population possess a functional form of the FcγRIIc receptor (a single

nucleotide polymorphism in *FCGR2C* produces either a functional or null gene), and NK cells co-expressing CD16 and FcγRIIc may demonstrate possible synergy for ADCC upon IgG_3- or IgG_1-mediated receptor crosslinking [415-418]. Further, FcγRIIc expression on B cells enhances humoral responses to vaccination and may contribute to enhanced antiviral activities of IgG_3-specific antibodies against gp120 V2, including NK cell ADCC in RV144 vaccinees [419, 420]. Single nucleotide polymorphisms within the *FCGR2C* gene (CT and TT) were associated with 91% vaccine protective efficacy against the CFR01_AE K169 HIV-1 strain and 64% against any HIV-1 strain, whereas vaccine protective efficacy for individuals with CC at this locus was only 15% against CFR01_AE K169 HIV-1 and 11% against any strain [421]. Expression of FcγRIIc is largely absent in Black Africans (South Africans, Luhya in Kenya and Yoruba in Nigeria) as the functional form of *FCGR2C* is rare or absent in this population [422]. As FcRs significantly contribute to acquired immunity, discrepant outcomes noted for vaccine-induced protective responses between RV144 and HVTN 702 could be partly attributed to genetic variations found within each group of individuals and the predominant circulating HIV-1 clade. However, these trial results hold promise for NK cells being a feasible means by which to control HIV-1 in an appropriate setting.

In our studies, we did not observe overt benefits for having a larger fraction of circulating NK cells possessing adaptive qualities and penchant for ADCC. With the high predisposition of PLWH for HCMV co-infection, it was necessary to

evaluate whether inflammation-associated inhibitory immune checkpoint receptors disrupted CD16-specific responses. NK cell TIGIT expression and engagement did affect responses triggered through CD16 receptors, however, blocking TIGIT was able to prevent this deficit and improve ADCC function over individual baseline activity. Phase I and II clinical trials of antibody therapy involving humaninzed anti-TIGIT mAb (*e.g.* Tiragolumab, Domvanalimab, Etigilimab) are ongoing in cancer and early stage studies indicate a favourable safety profile with effective TIGIT blockade. Experience in the cancer setting should help inform strategies for TIGIT blockade in PLWH, including whether better outcomes can be achieved when it is used in combination with other checkpoint inhibitors. For cure strategies that involve widespread reactivation of HIV-1 replication and purging of the exposed infected cells, it will be critical to determine which effector cells or functions can most rapidly be brought to bear against nascent HIV-1 replication [232, 240-243, 326].

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7 Appendix

7.1 Health Research Ethics Approval

Researcher Portal File #: 20180836

Dear Dr. Michael Grant:

This e-mail serves as notification that your ethics renewal for study HREB # 2017.220 – The influence of cytomegalovirus infection on maturation and aging of the immune system in HIV-infected individuals – has been **approved**. Please log in to the Researcher Portal to view the approved event.

Ethics approval for this project has been granted for a period of twelve months effective from November 23, 2020 to November 23, 2021.

Please note, it is the responsibility of the Principal Investigator (PI) to ensure that the Ethics Renewal form is submitted prior to the renewal date each year. Though the Research Ethics Office makes every effort to remind the PI of this responsibility, the PI may not receive a reminder. The Ethics Renewal form can be found on the Researcher Portal as an "Event".

The ethics renewal will be reported to the Health Research Ethics Board at their meeting dated November 19, 2020.

Thank you,

Research Ethics Office

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