Adaptive NK Cell Frequency and Function in Cytomegalovirus-Infected People Living with HIV

Khlood Alsulami

Department of Medicine

Division of Experimental Medicine

McGill University, Montreal

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LIST OF ABBREVIATIONS

Ab	Antibody
AdapNK	Adaptive NK
ADCC	Antibody dependent cellular cytotoxicity
ADNKA	Antibody-dependent NK activation
AIDS	Acquired immunodeficiency syndrome
aKIR	Activating killer immunoglobulin-like receptor
APOBEC3	Apolipoprotein B mRNA editing complex 3
ART	Antiretroviral therapy
AS	Atherosclerosis
ATP	Adenosine triphosphate
BM	Bone marrow
bNAb	Broadly neutralizing antibody
BP	Blood pressure
CaAS	Carotid atherosclerosis
CAD	Coronary artery disease
CCR5	C-C chemokine receptor 5
CD	Cluster of differentiation
CD3ζ	CD3 zeta
CD4	$CD4^+$ T cells
CD8	$CD8^+$ T cells
CDC	Centers for Disease Control and Prevention
CHD	Coronary heart disease
CI	Confidence interval
CIMT	Carotid artery intima- media thickness
CK-MB	Creatine kinase-MB
CLP	Common lymphoid progenitors
CLR	C-type lectin receptor
CMV	Cytomegalovirus
cNK	Conventional NK cells
CoAS	Coronary atherosclerosis
CRP	C-reactive protein
CS	Cigarette smoke
CSW	Commercial sex worker
CT	Computed tomography
CTL	Cytotoxic T lymphocytes
CTLD	C-type lectin-like domains
CVD	Cardiovascular disease
CXCR4	C-X-C chemokine receptor 4
DAD	Data collection on adverse effects of anti-HIV drugs
DAP10	DNAX- activating protein 10
DAP12	DNAX- activating protein 12
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
EC	Elite controller
EIA	Enzyme immunoassay
Env	Envelope

Fc	Fragment crystallizable
FcR	Fc receptor
FcRγ	Fc receptor gamma
FcγRIII	Fc gamma receptor III
FDA	Food and Drug Administration
GAG	Group antigen
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GP120	Glycoprotein 120
GPCR	G-protein coupled receptor
GRID	Gay-related immune deficiency
hCMV	Human CMV infection
HDL	High density lipoprotein
HESN	HIV exposed seronegative
HIV	Human immunodeficiency virus
HS	Heparan sulfate
Hs-CRP	High sensitivity C-reactive protein
HSC	Hematopoietic stem cells
HTLV	Human T-cell leukemia virus
ICAM-1	Intracellular adhesion molecule-1
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IHD	Ischemic heart disease
iKIR	Inhibitory killer immunoglobulin-like receptor
IL-6	Interleukin-6
IN	Integrase
iNK	immature NK cells
Kb	Kilobase
KIR	Killer immunoglobulin-like receptor
KS	Kaposi sarcoma
LDL	Low density lipoproteins
Lin	lineage
LN	Lymph nodes
LPS	Lipopolysaccharide
LT	Lymphoid tissue
LTNP	Long term nonprogressor
mAb	monoclonal Ab
mCMV	Murine CMV infection
MFI	Mean fluorescence intensity
MHC-l	Major histocompatibility complex-l
MI	Myocardial infraction
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
mNK	mature NK cells
MPER	Membrane proximal external region
MSM	Men who have sex with men
MT	Microbial translocation

NCR	Natural Cytotoxicity Receptors
NK	Natural killer
NKP or pre-NKP	pre-NK progenitor
OD	Outer domain
Ois	Opportunistic infections
PBMC	Peripheral blood mononuclear
PCR	Polymerase chain reaction
PEP	Post exposure prophylaxis
PHAC	Public Health Agency of Canada
PHI	Primary HIV infection
PLWH	People living with HIV
POL	DNA polymerase
PR	Protease
PrEP	Pre exposure prophylaxis
PVD	Peripheral vascular disease
PYs	Person-years
RNA	Ribonucleic acid
rNKP	refined-NKP
ROS	Reactive oxygen species
RR	Relative risk
RT	Reverse transcriptase
sCD14	Soluble CD14
sCD163	Soluble CD163
SH2	Homology 2 domain
SHP-1	Homology 2 domain phosphatase
SIV	Simian immunodeficiency virus
SLT	Secondary lymphoid tissues
SM	Sooty mangabey
SMC	Smooth muscle cells
ssRNA	Single stranded ribonucleic acid
Syk	Spleen tyrosine kinase
TC	Total cholesterol
TCRF	Traditional Cardiovascular risk factors
TG	Triglyceride
TNF-α	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TPV	Total plaque volume
ULBP	UL16-binding protein
UNAIDS	Joint United AIDS Program on HIV/AIDS
V1-V5	Variable loop 1-5
VC	Viremic controller
VL	Viral load
Zap-70	Zeta-chain associated protein kinase 70

ABSTRACT

The majority of people living with HIV (PLWH) are cytomegalovirus (CMV) co-infected. Infection with both of these viruses is associated with immune activation and a higher risk of health complications such as cardiovascular disease (CVD).

CMV infection expands a population of natural killer (NK) cells having adaptive properties. Adaptive NK (adapNK) cells are positive for NKG2C, CD57 and CD16. Most AdapNK cells have potent antibody dependent (AD) responses such as AD cellular cytotoxicity (ADCC) and AD NK cell activation (ADNKA).

I hypothesized that adapNK cells play a role in HIV pathogenesis in the context of CMV co-infection. To test this hypothesis, I addressed the following research questions: 1) Does *NKG2C* genotype influence protection from HIV infection and/or HIV control? 2) Is the frequency of adapNK cells a determinant of total plaque volume (TPV) in CMV^{+}PLWH and $CMV^{+}HIV^{-}$ subjects? 3) Does age or time on antiretroviral treatment (ART) influence the frequency and functionality of adapNK cells in CMV^{+}PLWH? 4) Does HIV infection compromise adapNK cell functionality?

In chapter II, I genotyped subjects from an HIV primary infection (PI, i.e. HIV susceptible) cohort and HIV exposed seronegative (HESN i.e. HIV resistant) persons for three possible *NKG2C* genotypes where the gene product of the $NKG2C^{-}$ allele is not cell surface expressed. When I compared the distribution of the three possible *NKG2C* genotypes in PI and HESNs, I found no $NKG2C^{-/-}$ carriers in HESNs. Thus, the $NKG2C^{-/-}$ genotype was associated with HIV susceptibility in HIV exposed persons. I next compared the viral load (VL) setpoint in carriers of the three *NKG2C* genotypes finding no significant between-genotype differences in the VL set point in PLWH. I also showed that there was no correlation between the frequency of $NKG2C^{+}$ adapNK cells and mean fluorescent intensity (MFI) of $NKG2C^{+}$ expression on adapNK cells and VL set point. Thus, these genotypes played no role in HIV control.

In chapter III, I investigated the frequency of adapNK cells in four groups enrolled in the Canadian HIV and Aging Cohort Study (CHACS): CMV^+PLWH, CMV^+HIV^-, CMV^-PLWH, and CMV^-HIV^- persons. All CHACS participants were >40 yrs of age; all PLWH were on ART. The frequency of adapNK cells was significantly higher in CMV^+ than in CMV^- persons, while there was no significant difference in the frequency of these cells in CMV^+PLWH compared to CMV^+HIV^- persons. High frequencies of adapNK cells were associated with a reduced prevalence of coronary atherosclerotic plaque in CMV^+PLWH and CMV^+HIV^- persons, suggesting a protective role in this pre-clinical CVD setting.

In chapter IV, I showed that the frequency of adapNK cells was higher in CMV^+PLWH than in CMV^+HIV^- persons aged <40 yrs but did not differ significantly in these two groups when from persons aged >40 yrs. However, in the PLWH there was a significant positive associated between age and time on ART. Experiments dissociating age and ART duration revealed that time on ART was the parameter that negatively influenced not only the frequency of adapNK cells but also the frequency of adapNK cells secreting IFN-γ and TNF-α upon AD stimulation in an ADNKA assay. A higher frequency of adapNK than cNK cells from CMV^+PLWH and CMV^+HIV^- responded to AD stimulation by secreting IFN-γ and TNF-α indicating that treated HIV infection did not compromise the function of adapNK cells.

The results presented in this thesis show that $NKG2C^+$ adapNK play a role at the level of susceptibility to HIV infection in those exposed to HIV but plays no role in HIV control in those infected. AdapNK cells play a role in reducing the risk of pre-clinical atherosclerosis in CMV^+ persons. The AD functionality of adapNK and cNK cells is maintained in in the setting of treated HIV infection. The duration of ART impacts the frequency and likely also the functionality of adapNK in CMV^+PLWH.

RÉSUMÉ

La majorité des individus vivant avec le VIH (PLWH) sont co-infectés par le cytomegalovirus (CMV). Le VIH comme le CMV activent le système immunitaire et augmentent les risques de complications liées aux maladies cardio-vasculaires (CVD).

L'infection CMV amplifie une population de cellules tueuses naturelles (NK) aux propriétés adaptatives. Les cellules NK adaptatives (adapNK) expriment NKG2C, CD57 et CD16, et répondent fortement à des stimulations anticorps-dépendantes (AD) en augmentant leur état d'activation (ADNKA) et en lysant les cellules cibles opsonisées (ADCC).

Mon projet de thèse repose sur l'hypothèse que les cellules adapNK jouent un rôle dans la pathogénicité du VIH chez les individus co-infectés par le CMV. Différentes questions ont alors été soulevées pour répondre à cette hypothèse : 1) est-ce que le génotype *NKG2C* protège et/ou aide à contrôler l'infection VIH ? 2) est-ce que la fréquence (%) de cellules adapNK est lié au volume total de plaques d'athérosclérose (TPV) chez les individus CMV^{+}PLWH et les $CMV^{+}HIV^{-}$? 3) est-ce que l'âge ou la durée du traitement antirétroviral (ART) affecte le % et la fonction des cellules adapNK chez les individus CMV^{+}PLWH ? et 4) est-ce que l'infection VIH affecte la fonction des cellules adapNK ?

Dans le chapitre II, j'ai réalisé le génotype d'individus engagés dans les cohortes « HIV primary infection » (PI, i.e. susceptibles au VIH) et « HIV exposed seronegative » (HESN, i.e résistants au VIH) pour le locus *NKG2C* ($NKG2C^{+/+}$, $NKG2C^{-/-}$, $NKG2C^{+/-}$). La comparaison des deux cohortes montre qu'il n'y a aucun individu $NKG2C^{-/-}$ chez les HESN, suggérant une association entre ce génotype et la susceptibilité à l'infection VIH. Je n'ai pas observé d'association significative entre l'un des génotypes *NKG2C* et la charge virale au plateau (VL) chez les PLWH. De même, je n'ai pas observé de corrélation entre le % de cellules adapNK ni le niveau d'expression de NKG2C sur ces cellules et la VL, démontrant ainsi l'absence d'association entre NKG2C et le contrôle du VIH.

Dans le chapitre III, j'ai évalué le % de cellules adapNK chez des individus CMV^+PLWH, CMV^+HIV^-, CMV^-PLWH et CMV^-HIV^- engagés dans la cohorte « Canadian HIV and Aging Cohort Study » (CHACS). Tous ces individus sont > 40 ans ; ceux infectés par le VIH sont sous traitement ART. Le % de cellules adapNK s'est avéré significativement plus élevé chez les individus CMV^+ que CMV^-, mais équivalent chez les individus CMV^+PLWH et CMV^+HIV^-. Le % de cellules adapNK était significativement associé à une réduction du TPV et donc du risque d'athérosclérose chez les individus CMV^+PLWH et les CMV^+HIV^-, suggérant un rôle protecteur de ces cellules dans les maladies CVD.

Dans le chapitre III, j'ai montré que les individus CMV^+PLWH <40 ans ont un % significativement plus élevé de cellules adapNK que les CMV^+HIV^- <40 ans, mais que cette différence ne persistait pas >40 ans. La durée de traitement ART étant positivement corrélé à l'âge des PLWH, des expériences permettant de dissocier l'effet de l'âge de celui du traitement ont révélées que la durée du traitement ART, plus que l'âge, impacte sur le % de cellules adapNK ainsi que leur capacité à s'activer en produisant de l'IFN-γ et du TNF-α dans un test ADNKA. Nos résultats montre également que les cellules adapNK d'individus CMV^+PLWH et CMV^+HIV^- produisant davantage d'IFN-γ et de TNF-α que les cellules NK conventionnelles (cNK), suggérant le maintien de leur fonctionnalité chez les PLWH traités.

Mes résultats montrent que les cellules $NKG2C^+$ adapNK jouent un rôle dans la susceptibilité à l'infection VIH et non dans le contrôle du virus chez les individus infectés. Ces cellules jouent également un rôle significatif dans la réduction du risque d'athérosclérose préclinique chez les individus CMV^+. Si les fonctions AD des cellules adapNK et cNK semblent conservées chez les PLHW traités, la durée du ART impacte sur le % et la fonctionnalité des cellules adapNK chez les individus CMV^+PLWH.

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AUTHOR CONTRIBUTION

This thesis is based on the following articles, which are referred to in the text by Roman numbers:

Chapter I: Introduction and Literature Review

- **Author contribution**: Ms. Khlood Alsulami wrote this chapter. Nicole F. Bernard, Ph.D. edited and approved the chapter.

Chapter II: Influence of NKG2C Genotypes on HIV Susceptibility and Viral Load Set Point

- **Khlood Alsulami,** Naomi Bolastig, Franck P. Dupuy, Tsoarello Mabanga, Louise Gilbert, Zahra Kiani, Jean-Pierre Routy Julie Bruneau, Réjean Thomas, Cécile Tremblay, Christos M. Tsoukas, Jason Szabo, Pierre Côté, Benoit Trottier, Roger LeBlanc, Danielle Rouleau
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- **Author contribution**: I designed the study, performed the experiments, analyzed the results, prepared all the figures and contributed to writing the manuscript. Ms. Naomi Bolastig performed some of the experiments, analyzed the results and wrote a first draft of the manuscript. Franck P. Dupuy, Ph.D. participated in the data analysis for figure 1 and figure 3 and edited the manuscript. Ms. Tsoarello Mabanga and Ms. Louise Gilbert managed the database of subject samples and results and performed some of the experiments. Zahra Kiani, Ph.D. and Ms. Tsoarello Mabanga contributed to me and Ms. Bolastig in the technical aspects of the experiments performed in this manuscript. Dr. Jean-Pierre Routy, Dr. Julie Bruneau, Dr. Réjean Thomas, Dr. Cécile Tremblay, Dr. Christos M. Tsoukas, Dr. Jason Szabo, Dr. Pierre Coté, Dr. Benoit Trottier, Dr. Roger LeBlanc and Dr. Danielle Rouleau recruited and clinically followed subjects included in this study and contributed to editing the manuscript. Nicole F. Bernard, Ph.D. designed the study, provided supervision and administrative support, obtained funding for the study and contributed to editing drafts of the manuscript.

Chapter III: High Frequencies of Adaptive NK Cells are Associated with Absence of Coronary Plaque in Cytomegalovirus Infected People Living with HIV

- **Khlood Alsulami,** MSc, Manel Sadouni, MD, Daniel Tremblay-Sher MD, Jean-Guy Baril, MD, Benoit Trottier, MD, Franck P. Dupuy, PhD, Carl Chartrand-Lefebvre, MD, Cécile Tremblay, MD, Madeleine Durand, MD, Nicole F. Bernard, PhD
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- **Author contribution**: I designed the study, performed the experiments and data analysis, prepared all the figures, and wrote the first draft of the manuscript. Dr. Manel Sadouni provided training and statistical support for the analyses performed in this manuscript, participated in the data analysis for the information presented in tables 1, 2 and 3 and participated in generating the first draft of the manuscript. Dr. Daniel Tremblay-Sher contributed to database management of the subjects enrolled in the Canadian HIV and Aging Cohort Study (CHACS) and contributed to editing the manuscript. Dr. Jean-Guy Baril, Dr. Benoit Trottier, Dr. Cécile Tremblay and Dr. Madeleine Durand recruited and followed study subjects enrolled in the CHACS, provided clinical samples for the experimental component of the study and contributed to manuscript preparation. Dr. Madeleine Durand oversaw the CHACS, contributed to study design and conception and obtained funding for the study. Dr. Carl Chartrand-Lefebvre oversaw the imaging component of the study and contributed to manuscript preparation. Dr. Franck P. Dupuy supervised the experimental component of the study, contributed to experimental design and data analysis and contributed to manuscript preparation. Dr. Nicole F. Bernard conceived the study, supervised the experimental and data analysis components of the study, provided administrative support and contributed to manuscript preparation.

Chapter IV: The Frequency and Function of NKG2C^{+}CD57^{+} Adaptive NK cells in Cytomegalovirus Co-infected People Living with HIV decline with Duration of Antiretroviral Therapy

- **Khlood Alsulami,** Franck P. Dupuy, Louise Gilbert, Marc Messier-Peet, Madeleine Durand, Cécile Tremblay, Jean-Pierre Routy, Julie Bruneau, Jean-Guy Baril, Benoit Trottier and Nicole F. Bernard
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Chapter V: Discussion

- **Author contribution**: Ms. Khlood Alsulami wrote the chapter Nicole F. Bernard, Ph.D. edited and approved the chapter.

Contribution to other articles:

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  Nicole F. Bernard, **Khlood Alsulami**, Erik Pavey and Franck P. Dupuy

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- **Contribution to the manuscript:** I performed a literature review, and contributed to writing, and revising the manuscript for publication.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

- In chapter II, I investigated the link between *NKG2C* genotypes and 1) HIV susceptibility/protection and 2) levels of untreated VL set point in PLWH. I showed that the absence of the *NKG2C*$^{-/-}$ genotype among HIV exposed seronegative (HESN) subjects suggests that carriage of this genotype was associated with decreased HIV susceptibility. Contribution to original knowledge in this chapter include: 1) *NKG2C* genotyping NKG2C (*NKG2C*$^{+/+}$, *NKG2C*$^{+/-}$ *and NKG2C*$^{-/-}$) of a large, well characterized group of HIV susceptible PLWH and HESN participants. 2) Access to a large dataset permitting calculation of HIV VL set point, a better marker for rate of HIV disease progression than a single VL measure. 3) Ability to deconvolute study subjects by route of exposure. 4) Assessment of the correlation between VL set point and frequency of NKG2C^{+} NK cells and mean fluorescence intensity (MFI) of NKG2C expression on adapNK cells in CMV^{+}PLWH who were carriers of the three possible *NKG2C* genotypes (*NKG2C*$^{+/+}$, *NKG2C*$^{+/-}$ *and NKG2C*$^{-/-}$).

- In chapter III, I investigated the expression of NKG2C^{+}CD57^{+}adapNK cells in CMV^{+}PLWH, CMV^{+}HIV^{-}, CMV^{-}PLWH and CMV^{-}HIV^{-} participants who were enrolled in Canadian HIV and Aging Cohort Study (CHACS). I showed for the first time that the association between the high frequency of NKG2C^{+}CD57^{+}adapNK and the absence of coronary plaque in CMV^{+}PLWH participants. Contributions to original knowledge in this chapter include: 1) Access to data generated by computed tomography imaging measuring pre-clinical markers of atherosclerosis such as total plaque volume (TPV). 2) The use of a fluorochrome-conjugated antibody panel and flow cytometry gating strategy to identify the frequency of adapNK cells versus conventional cNK cells. 3) The ability to gate on and examine the association of adapNK cells frequency and coronary plaque using Poisson regression analysis after adjustment for cardiovascular risk factors. 4) Demonstration that the frequency of adapNK cells was correlated negatively with total plaque volume (TPV) in all CMV^{+} and CMV^{+}PLWH subjects.

- In chapter IV, I showed for the first time the influence of age and duration of time on antiretroviral therapy (ART) on NKG2C^{+}CD57^{+} adapNK cells frequency and functionality in CMV^{+}PLWH versus CMV^{+}HIV^{-} individuals who were stratified as >40 and <40 yrs of age. A particularly novel aspect of this work was investigating the impact of HIV infection on adapNK cells and cNK cells in CMV^{+}PLWH versus CMV^{+}HIV^{-} individuals. Contribution to original knowledge in this chapter include: 1) The investigation of adapNK cell frequency in study subjects who were above 40 yrs age among CHACS participants, and <40 yrs of age among participants of the HIV primary infection (PI) cohort and CMV infected or CMV uninfected HIV uninfected persons who

were < 40 yrs of age. 2) The inclusion of a fluorochrome-conjugated antibody panel and flow cytometry that allowed the measurement of the secretion of the IFN-γ, TNF-α and CCL4 and CD107a externalization as functional markers of adapNK cell activity. 3) A demonstration that HIV infection compromised neither adapNK cell nor cNK cell function.

CHAPTER I

1. INTRODUCTION AND LITERATURE REVIEW

1.1.HISTORY/DISCOVERY OF HIV

In 2008, the Nobel Prize in Physiology and Medicine was awarded to Dr. Françoise Barré-Sinoussi from the Institute Pasteur and Dr. Luc Montagnier from the World Foundation for AIDS Research and Prevention, both in Paris, France for their discovery of the human immunodeficiency virus (HIV) in the early 1980s (1). This virus was found to be the causative agent of the acquired immunodeficiency syndrome (AIDS), which is characterized by a progressive depletion of $CD4^+$ T cells causing impairment of cellular immunity and increasing susceptibility to opportunistic infections (Ois) and malignancies (2). AIDS was initially recognized in the United States of America in 1981. The Centers for Disease Control and Prevention (CDC) reported five cases of *Pneumocystis carinii* (now *Pneumocystis jiroveci*) pneumonia (PCP) in previously healthy homosexual men in Los Angeles, CA (3). Shortly after, additional cases of life threatening Ois and a malignancy, Kaposi's sarcoma, were reported in this population (4). The virus was successfully isolated from the lymph nodes (LN) of AIDS patients and cultured (5). It was first thought that the virus belonged to human T-cell leukemia virus (HTLV) family. Although HIV and HTLV virus share common entry routes to the host via parenteral exposure (blood transfusion and drug injection), and sexual exposure and both viruses lead to a chronic infection, the new virus was eventually found to not belong to the HTLV family. The disease caused by HIV was originally termed Gay-Related Immune Deficiency (GRID) as it was thought to only affect gay men. When it became clear that heterosexual men and women could also be HIV-infected, the disease caused by HIV infection was renamed AIDS.

Since its initial recognition, HIV infection has spread rapidly throughout the world. According to the Joint United Nations Program on HIV/AIDS (UNAIDS) report released in 2021, 38.4 million people world-wide are HIV seropositive and 1.5 million were newly infected in this year. Of these, 28.2 million are on antiretroviral therapy (ART). The highest prevalence is in sub-Saharan Africa, where 60% of people living with HIV (PLWH) are not receiving treatment (6). In Canada, in 2018, there were 2,561 PLWH, a number that increased by 8.2% from the previous year. The Public

Health Agency of Canada (PHAC) tracks HIV cases in the Canadian provinces. Ontario was the highest number with 39.2% of cases in Canada. Quebec had the second highest prevalence rate of 29.9% of cases followed by Alberta with 11.7% and British Columbia with 7.8%.

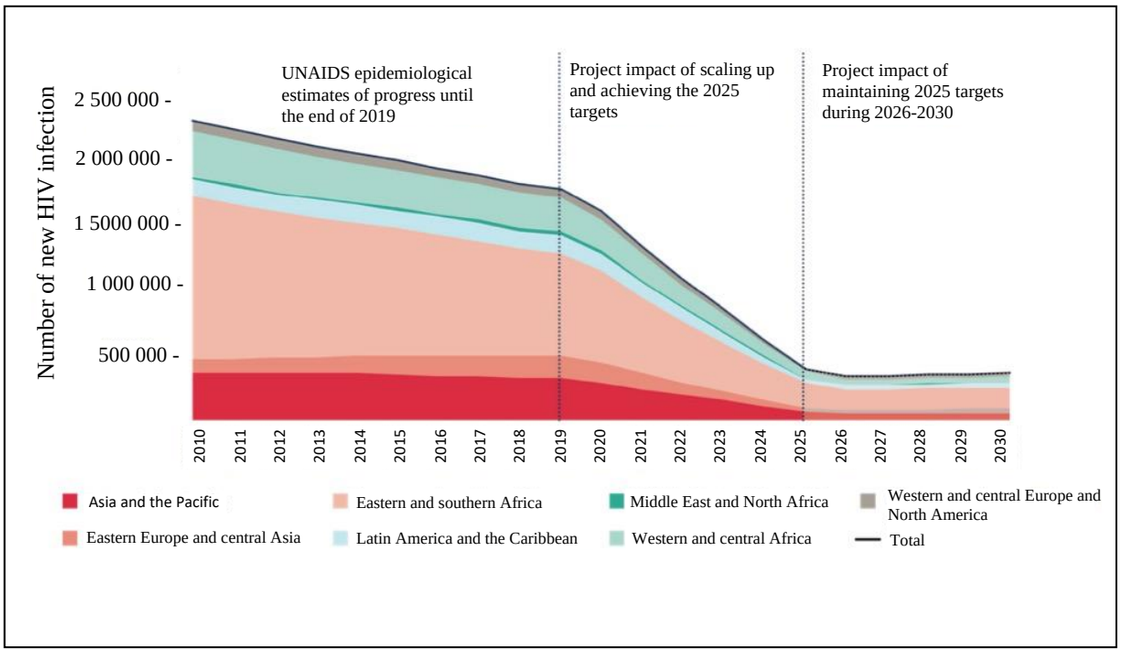

Figure 1.1. Epidemic modeling shows the worldwide prevalence of HIV among adults and the reduction in annual infection rates by 2030

Figure adapted from UNAIDS. (https://www.unaids.org/en/resources/documents/2020/prevailing-against-pandemics).

1.2. ORIGIN OF HIV

There are two main types of HIV: type 1 (HIV-1) and type 2 (HIV-2). HIV-1 originated from chimpanzees (cpz) infected with the simian immunodeficiency virus SIVcpz and while HIV-2 is most closely related to SIVsm that infects sooty mangabeys (SM) (7, 8). With a few exceptions, each primate species had its own type of SIV. HIV and SIV belong to the genus *Lentivirus* of the *Retroviridae* family. Investigators have screened for the presence of SIV in 70 non-human primate species from sub-Saharan Africa. More than half of these (n=40) harbored SIV-related lentiviruses, which were within a single lentivirus lineage that was distinct from lentiviruses in other mammalian species (9, 10). Furthermore, species of African non-human primates that are natural hosts for SIV do not develop AIDS as is the case for SIVsm infection in SMs. In contrast, this same SIVsm causes severe pathogenicity and an AIDS-like syndrome in rhesus macaques (RM). It is commonly accepted that the HIV-1 pandemic originated from the lentivirus SIVcpz that infects the sub-species *Pan troglodytes' troglodytes*, from Central West Africa (11, 12).

1.3. HIV GENETIC DIVERSITY AND CLASSIFICATION

Although HIV-1 and HIV-2 have a high level of genetic diversity, these viruses share similarities including intracellular mechanisms of replication and route of transmission. Despite the similarities between HIV-1 and HIV-2, the diseases caused by these two viruses differ in their epidemiology, diagnosis, and pathogenesis. For example, HIV-2 is restricted to West Africa and is less pathogenic than HIV-1, which has spread world-wide and causes a more aggressive disease course. Infection with HIV-2 is characterized by lower plasma HIV RNA levels, slower decline in $CD4^+$ T cell counts, lower rates of sexual transmission, and slower progression to AIDS than HIV-1 (13, 14).

For the remainder of this thesis, I will use HIV to refer to HIV-1, unless otherwise specified. HIV can be categorized into four groups: Main (M), Outlier (O), Non-M (N) and Putative (P). Each of these groups originated from distinct transmission events from chimpanzees to humans. Group M HIV causes more than 90% of HIV/AIDS cases. Based on the genomic sequence, group M HIV can be divided into ten subtypes (A, B, C, D, F, G, H, I, J and K) and some of these subtypes can be further divided into sub-subtypes. For example, Figure 2.1 shows that subtype A includes six sub-subtypes (A1, A2, A3, A4, A6 and A7), subtype B includes sub-subtype B1, subtype D includes sub-subtypes D1, D2 and D3 and subtype F includes sub-subtypes F1 and F2 (15). Forty-eight to 60% of PLWH worldwide are infected with HIV subtype (or clade) C, whose prevalence is rapidly increasing in Eastern and Southern Africa, India as well as in China, Brazil, Uruguay, and neighboring countries (16-19). HIV subtype B only accounts for 11% of worldwide infections and is most prevalent in Western countries. The group O, N and P HIV are very rare and have not been seen outside of Central West Africa in small populations in Cameroon. Group P virus originated in gorillas. Group P HIV is the last HIV group identified and only two cases have been reported to date (20). Most studies have focused on HIV subtype B, due to its prominence in North America and Europe. The most significant difference between HIV subtypes is variation in the *ENV* gene, which encodes the envelope (Env) surface glycoprotein 120 (GP120) and transmembrane glycoprotein 41 (GP41) (21). I will elaborate on ENV structure in a later section of this thesis.

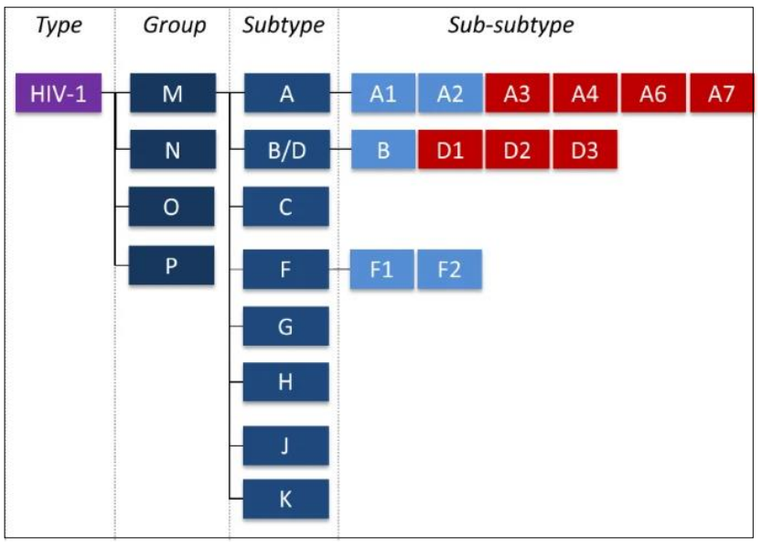

Figure 2.1. HIV-1 virus classification based on HIV-1 genetic diversity

Figure adapted from https://retrovirology.biomedcentral.com/track/pdf/10.1186/s12977-018-0461-y.pdf,(15)

1.4. HIV VIRAL GENOME AND STRUCTURE

HIV is a positive sense single stranded (ssRNA) retrovirus. The genome of viruses with a positive sense RNA can act as a messenger RNA and can be translated into viral proteins by the host cell's ribosomes. HIV uses its reverse transcriptase (RT) enzyme to transform its ssRNA into double stranded deoxyribonucleic acid (dsDNA), which is then integrated into the human genome (22). The characteristic cone-shaped core exists within the virion. The HIV virion has a spherical shape, with a diameter of 100 to 120 nm. Two copies of ssRNA are packaged in the capsid. The three viral enzymes: protease (PR), RT and integrase (IN), and the structural proteins; capsid (CA), matrix (MA) and nucleocapsid (NC) are inside the particle (23). More details on viral enzymes and the function of HIV encoded proteins will be explained below. See figure 3.1.

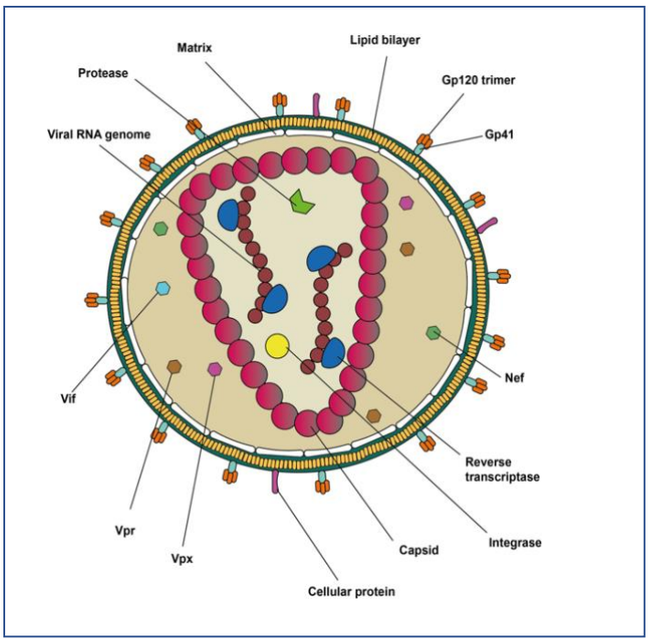

Figure 3.1. Schematic structure of the HIV virion

The virus is composed of a capsid core that contains the genetic material. The capsid is surrounded by a phospholipid bilayer envelope acquired from the host cell plasma membrane as it buds from infected host cells. The viral membrane has numerous Env spikes made up of glycoproteins GP120 and GP41. The HIV genome contains two helices of RNA molecules. The RT enzyme is responsible for the conversion of the ssRNA into dsDNA. The IN enzyme incorporates the viral genome into the host cell's genome. (Made by K Alsulami using BioRender.com).

The size of the HIV genome is around 9.7 kilobases (kb). It contains 9 genes that encode 15 viral proteins. Of the three major genes, *GAG*, for group antigen, codes for structural proteins CA, MA, NC and P6, *POL*, for DNA polymerase, codes for the enzymes PR, RT and IN, *ENV* codes for GP120 and GP41. Four genes code for the accessory proteins: negative regulator factor (NEF), virus infectivity protein (VIF), virus protein r (VPR) and viral protein unique (VPU). Two genes encode regulatory proteins, i.e. transactivator of transcription (TAT) and regulator of expression of the virion proteins (REV) (24). TAT and REV proteins are required for viral replication while NEF, VIF, VPR and VPU are known as auxiliary proteins that are dispensable for viral growth (see figure 4.1).

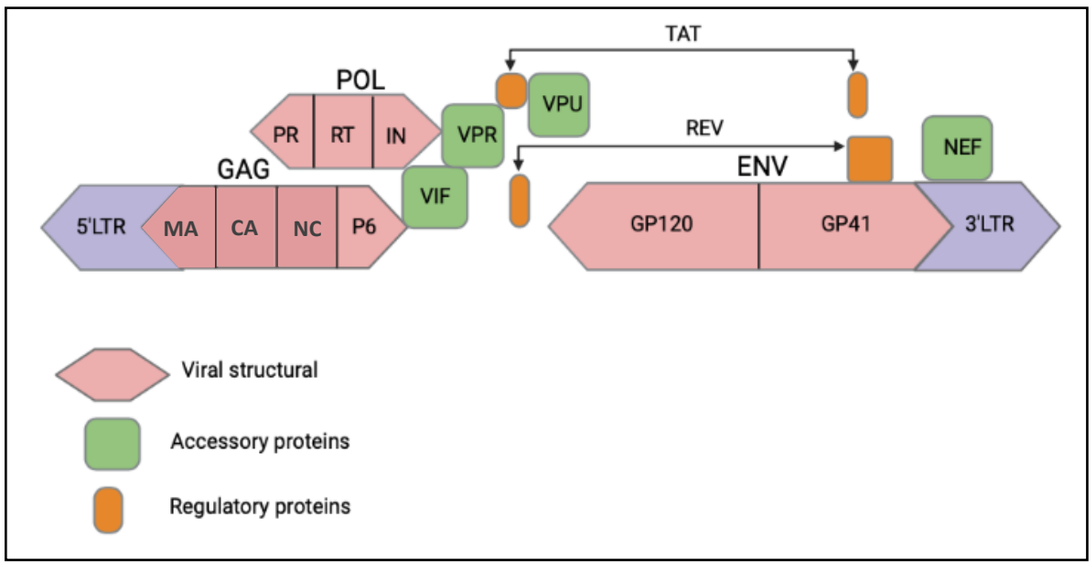

Figure 4.1. Schematic of HIV genome

The genome is composed of 3 genes (*GAG, POL* and *ENV* in light pink, 4 accessory genes in light green (*VIF, VPR, VPU* and *NEF*) and 2 regulatory genes in orange (*TAT* and *REV*). (Made by K Alsulami using BioRender.com).
(25).

GAG protein comprises CA, MA, NC and the P6 RNA binding protein. CA assembles into a conical core that consists of two identical ssRNA molecules attached to the NC proteins. MA encloses the capsid that itself is surrounded by a phospholipid bilayer (23) as shown in figure 3.1. GAG proteins play a role in viral assembly, interactions with several host proteins, regulation of viral gene expression and virus intracellular trafficking and budding. POL is made up of the viral enzymes PR, RT and IN, which are essential for viral replication. PR hydrolyzes peptide bonds on GAG and GAG-POL polyproteins, processing the resulting subunits into mature functional proteins within the virion, the infectious form of the virus outside the host cell (26, 27). This step is necessary to generate infectious virions (28, 29). RT has three biochemical activities: RNA dependent DNA polymerase activity, ribonuclease H (RnaseH) activity and DNA-dependent DNA polymerase activity, which together convert viral RNA to complementary dsDNA (30). IN plays a role in the integrations of HIV dsDNA into the host genome (31). ENV is a surface glycoprotein heterodimer composed of GP120 and GP41 that form homotrimeric "viral spikes" required for binding to, and entry of, the HIV virion into host $CD4^+$ T cells (32). VIF, VPR, VPU and NEF were originally named "accessory" proteins because their function was not essential for HIV replication *in vitro* (33, 34). However, there is now evidence that these proteins have an important role in viral replication, pathogenesis, and disease progression.

VIF and VPR are involved in the late stage of HIV viral replication. VIF increases the viral infectivity at the time of virus production suggesting this protein plays a role during virus assembly or maturation (35). Specifically, VIF targets a family of host immune factors known as apolipoprotein B mRNA editing complex 3 (APOBEC3) proteins, which are intrinsic restriction factors inhibiting HIV replication by inducing mutations in the viral genome during synthesis of cDNA from viral RNA, thus impeding the viral life cycle (36-38). VPR is critical for efficient viral replication in $CD4^+$ T cells and macrophages (39). It has been postulated that in macrophages, VPR facilitates the degradation of an as yet undefined host anti-viral factor, which inhibits the incorporation of ENV into virions (40, 41). NEF and VPU proteins downmodulate CD4 receptors from the surface of HIV-infected cells. NEF targets pre-existing CD4 at the plasma membrane while VPU targets the newly synthesized CD4 molecules in the endoplasmic reticulum (ER) (42). NEF protein is expressed at high levels after early infection, and it is critical for HIV viral replication and pathogenesis *in vivo* by enabling immune escape of HIV-infected cells. PLWH who are infected with HIV lacking NEF do not progress to AIDS, or they do so very slowly (43, 44). Likewise, RMs infected with SIV that does not express NEF had low viral load (VL) levels and do not progress to simian AIDS (45). NEF has the ability to downregulate major histocompatibility complex-class I (MHC-I) antigens from the cell surface (46) and enhances HIV replication (47). The mechanism behind how NEF downregulates CD4 is not known. However, the direct interaction between CD4, which is a transmembrane glycoprotein, and NEF, which is a myristoylated protein that targets the cytoplasmic domain of CD4 might a play a role in CD4 downregulation (48-50). Table 1.1 shows the contribution of HIV gene products to disease progression.

Table 1.1 HIV gene product function

Protein	Major Functions
ENV	• A glycoprotein composed of GP120 and GP41. GP120 is expressed on the surface of HIV virions and infected cells and interacts with CD4 on target cells.
GAG	• A polyprotein composed of MA (P17), CA (P24), NC (P7) and RNA binding protein (P6) (51). GAG is important for virus maturation, assembly and establishment of a productive infection (51).
POL	• A polyprotein composed of the viral enzymes PR, RT and IN. PR processes the GAG and GAG-POL polyproteins during HIV maturation. RT converts HIV RNA into viral DNA. IN inserts viral DNA into the DNA of CD4 cells. •
VIF	• Disrupts the anti-viral activity of the human enzyme APOBEC3G by targeting it for ubiquitination and degradation.
VPR	• Stimulates G2 cell-cycle arrest. Involved in HIV infection of macrophages
VPU	• Elicits CD4 degradation and enhances virion release
NEF	• Mediates downregulation of CD4 (52) that enhance release of infectious virions expressing HIV ENV to promote viral infection and replication (53, 54) • Downregulates MHC-I and MHC-II (55, 56) expression that protect HIV infected cells from host cytotoxic T lymphocyte (CTL) responses
REV	• Regulates viral gene expression. Mediates nuclear export to the cytoplasm of incompletely spliced viral RNA molecules
TAT	• Acts as a transcriptional activator, increases RNA POL II elongation on the viral DNA template

1.5. VIRAL LIFE CYCLE

Since its first isolation from cell culture, there have been thousands of publications on the function of HIV viral proteins and the HIV replication cycle. The HIV replication cycle consists of three stages: HIV entry into host cells, replication within host cells and budding of new virions from host cells (figure 5.1).

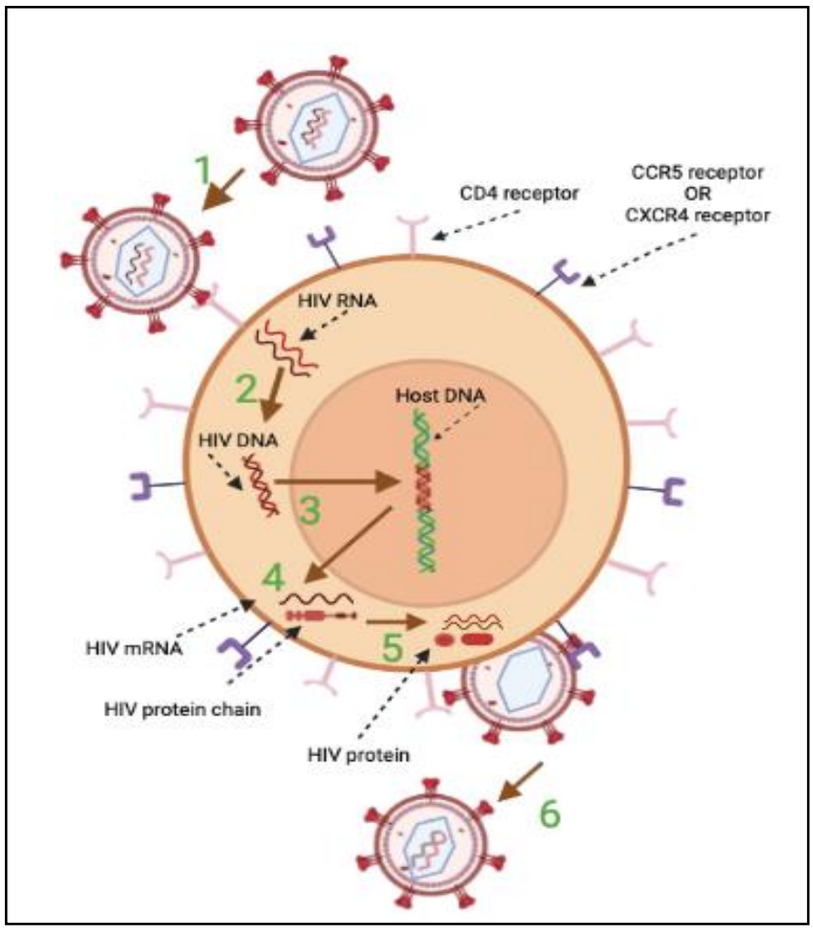

Figure 5.1. HIV replication cycle

https://www.thewellproject.org/hiv-information/hiv-drugs-and-hiv-lifecycle. (Made by K Alsulami using BioRender.com).

1.5.1. Viral entry

Viral entry starts when HIV ENV GP120 binds to the CD4 receptor expressed on the surface of host cells such as $CD4^+$ T cells, monocytes, macrophages, Langerhans cells, brain microglia and dendritic cells (57-63). The interaction between GP120 and CD4 results in a conformational change in GP120 structure that facilitates exposure of structures that allow the binding of co-receptors such as the C-C chemokine receptor 5 (CCR5) or the C-X-C chemokine receptor 4 (CXCR4). These co-receptors belong to the 7-transmembrance G-protein coupled receptor (GPCR) superfamily. CCR5 is expressed most abundantly macrophages, dendritic cells and T cells (64-67) while CXCR4 is expressed on T cells, mast cells, hematopoietic and endothelial cells (68, 69). Consequently, the interaction of GP120 with CCR5 or CXCR4 co-receptors triggers the GP41 subunit to undergo a complex series of conformational changes that promote

fusion between the virus and host cell membranes allowing GP41 to penetrate the cell membrane and form a six-helix bundle. (See figure 6.1).

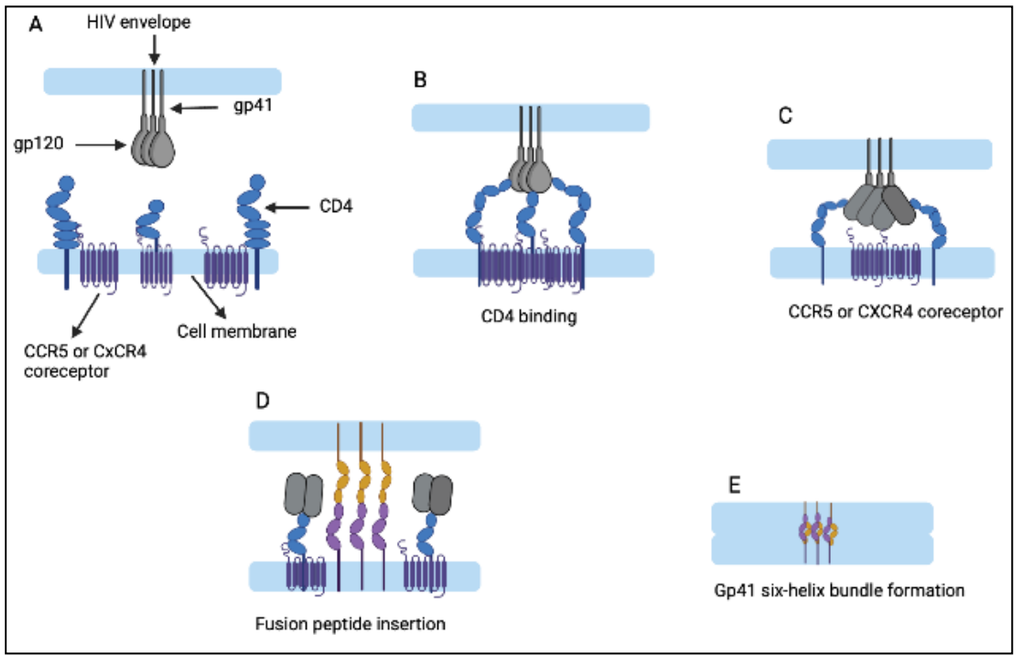

Figure 6.1. HIV entry into target cells

A) The major players involved in HIV entry. B) Binding of CD4 on target cells to GP120 on virions or HIV-infected cells. This results in a conformational change that exposes a hidden coreceptor binding site on GP120. C) Binding of GP120 to the CCR5 or CXCR4 co-receptors results in another conformational change that brings the HIV ENV into proximity with the cell surface. D) GP41 is triggered by CCR5 or CXCR4 co-receptor binding to undergo a conformational change, which promotes the insertion of fusion peptides into the cell membrane, E) allowing GP41 to penetrate the cell membrane and form a six-helix bundle. (Made by K Alsulami using BioRender.com) (70).

1.5.2. Viral replication (early phase) and viral integration

After virus fusion and entry, the virus is uncoated and releases its genetic material into the cytoplasm of the host cell. The RNA dependent DNA polymerase activity of RT uses HIV's ssRNA as a template for reverse transcription to generate an RNA-DNA duplex. The RT's RnaseH activity degrades the positive strand RNA of the RNA-DNA duplex while the DNA-dependent DNA polymerase activity generates a DNA strand complementary to the remaining DNA strand to generate dsDNA that contains all of the virus's genetic material. Once this has happened the viral DNA can be integrated into the host cells (30). RT lacks a proofreading domain. The consequence of this is the introduction on mutations at high frequencies leading to

the generation of quasispecies that include variants having the potential to escape the antiviral activity of drugs and host immune responses (71-73). Integration is an essential step in the HIV life cycle (74). In this step, the newly formed of HIV DNA enters the host cell's nucleus where HIV integrase inserts the HIV DNA into the host cell's DNA. This integrated HIV DNA is called a provirus that can either remain inactive for years or complete the HIV replication cycle by producing new infectious copies of itself (74).

1.5.3. Viral replication (late phase)

When the HIV provirus lies in transcriptionally active cells it uses the host's RNA polymerase enzyme to create copies of the HIV genomic material and shorter strands of RNA called messenger RNA (mRNA). This mRNA is used as a template for generating long chains of HIV precursor proteins that are cut by PR into smaller active proteins and assembled into mature virions (75, 76). Last, the newly assembled virus pushes out from the host cells. During the budding phase the new virus takes with it part of the host cell's outer envelope. This envelope is studded with protein/sugar combination called HIV glycoproteins. These glycoproteins are important for the virus to bind to CD4 and co-receptors present on new HIV susceptible target cells. The new copies of HIV can now move on to infect other cells as shown in figure 5.1.

1.6. ANTI-RETROVIRAL THERAPY

As of April 2022, there are 31 antiretroviral drugs approved for controlling HIV infection (https://clinicalinfo.hiv.gov/en/guidelines/pediatric-arv/drug-abbreviations). According to WHO 2022, ART is recommended to treat PLWH and should be started as soon as HIV infection is diagnosed. Early initiation of effective treatment that lowers VL to below the limit of detection using standard assays allows PLWH to achieve a normal life span and reduces the chance of HIV transmission to others. Without treatment, HIV eventually depletes $CD4^+$ T cells, which are helper cells needed to generate $CD8^+$ T cell and B cell responses. When $CD4^+$ T cell counts fall below 200 cells/mm^3, the host becomes susceptible to AIDS defining OI that leads to death.

Combination ART consists of a minimum of two drugs from two different drugs classes, which block different stages of HIV's life cycle.

Combination ART reduces HIV VL, which in turn reduces morbidity and mortality in PLWH. Antiretroviral drugs are classified according to the steps of the viral life cycle they inhibit. There are six classes of drugs categorized based on their molecular mechanism. Typical ART drug classes include nucleoside reverse-transcriptase inhibitors (NRTI), non-nucleoside reverse-transcriptase inhibitors (NNRTI), PR inhibitors (PI), integrase strand transfer inhibitors (INSTI), fusion inhibitors (Fis) and post attachment inhibitors also known as entry inhibitors (77). NRTIs were the first class of drugs to be approved by the Food and Drug Administration (FDA). NRTIs and NNRTIs block the conversion of HIV RNA to dsDNA. PIs and INSTIs block HIV PR and IN enzymes, blocking HIV replication at the maturation and integration steps, respectively. Fis and post-attachment inhibitors block HIV ENV from attaching to the CCR5 co-receptor and entering HIV-susceptible cells.

ART can increase $CD4^+$ T cell counts within a few weeks of treatment initiation. New, long-acting injectable cabotegravir (CAB-LA) was the first injectable ART regimen approved by the FDA. CAB-LA is an INSTI, which should be taken every two months as an injection into the gluteus muscle. Although, most of the individuals who received CAB-LA had injection site reactions, the treatment was shown to be safe and to have potent anti-HIV activity (78).

1.7. HIV ENV AND ITS CONFORMATIONS

The viral ENV protein is a trimer assembled of heterodimers made up of GP120 and GP41 glycoproteins. HIV ENV is the only HIV gene product expressed on the surface of virions and infected cells (79). As such, it plays a critical role in HIV entry into the host cells and is an important target for antibody (Ab) dependent (AD) functions. GP120 forms the outer part of the trimer, GP41 is buried at the trimer interface and anchors ENV on the plasma membrane (80-82). GP120 can be divided into three distinct parts: the inner domain, the outer domain (OD), and the bridging sheets. The OD of HIV ENV GP120 plays an important role in vaccine design due to

the presence of the CD4 receptor binding sites and other conserved epitopes for broadly neutralizing Abs (bNAbs) (see figure 7.1). There are six viral ENV regions targeted by bNAbs: the CD4 binding site (CD4bs) (83-89), the GP120 V1/V2 glycan region (90-92), the V3 glycan region (93-95), the interface between GP120 and GP41 ENV glycoproteins (96), the GP41 fusion domain (97) and the membrane proximal external region (MPER) (98, 99). Attempts to design an HIV vaccine that induces bNAbs able to protect against infection have failed. This is likely due to the high level of affinity maturation that Abs go through to become bNAbs, the high mutation rate of HIV leading to HIV quasispecies that differ extensively in the ENV region and extensive glycan coverage of the ENV surface that shields ENV from being recognized by Abs and bNAbs (100).

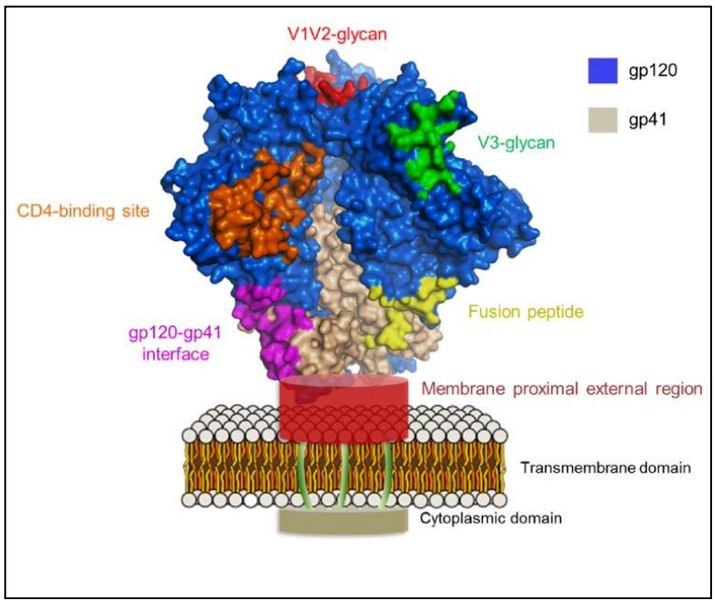

Figure 7.1. HIV Env glycoprotein

The structure of HIV trimer is displayed with GP120 and GP41 colored in blue and beige respectively. The six viral ENV regions targeted by bNAbs are the V1 and V2 loops (red) and V3 loop (green), CD4 binding site (orange), the interface between GP120 and GP41 (purple), the fusion peptide region (yellow) and the membrane proximal external region (brown) (101).

GP160 is a heavily glycosylated protein. The GP160 core protein is 90 kDa with the remaining molecular mass contributed by N-linked, and to a lesser extent, O-linked glycosylation (102-104). The sum of the GP120 attached glycan sugars is referred to as the “glycan shield”. This shield plays a role in protection of the GP120 protein and can be targeted by neutralizing Abs that help

HIV evade host immune responses. GP120 contains approximately 25 N-linked glycosylation sites of which almost 4 are in the inner domain, 7-8 in the V1, V2 and V3 variable loops, and the rest are in the OD of GP120. In the physical protein structure, the conserved regions (C1-C5) of the GP120 glycoprotein are hidden in its interior, while the five variable regions (V1-V5) have major sequence heterogeneity. The V3 loop is located under the V1/V2 region at the apical center of the ENV trimer, while the V4 and V5 loops have less movement upon binding the ENV receptor during the membrane fusion process (105).

GP120 mediates the initial attachment of HIV to the CD4 receptor and a second chemokine coreceptor on the surface HIV susceptible cells (106). The interaction of GP120 with the CD4 receptor triggers major conformational changes in the ENV protein such as movement of the V1/V2 and V3 loops. The changed ENV conformation exposes structures that allow binding to the CCR5 and CXCR4 coreceptors (107). The interaction of GP120 with the co-receptors promotes additional conformational changes in GP41. Normally, unliganded ENV is present on the surface of virions and infected cells in a “closed” conformation (108, 109) . This is because HIV NEF and VPU downmodulate CD4 from the surface, preventing it from interacting with GP120, which drives ENV from a “closed” to an “open” conformation (110, 111). However, GP120 can be shed from the surface of infected cells to bind CD4 on uninfected bystander cells leading to ENV assuming an open conformation on these bystander cells (107, 112, 113).

1.8. HIV TRANSMISSION

HIV transmission between humans can occur through sexual intercourse, contact with HIV infected blood and body fluids, broken skin or mucosa such as can occur through the sharing of contaminated needles, injection drug use, blood transfusion or organ transplantation, and mother to child transmission, *in utero*, perinatally or through breastfeeding (5, 114).

The risk of transmission is higher for unprotected receptive than insertive anal intercourse because the rectal canal has only one layer of epithelial cells (115). Extensive breaks in epithelial integrity at this site allows HIV direct access to target cells and allows the establishment of infection within this mucosal site. Interestingly, it has been demonstrated that circumcision reduces the chance of

HIV virus transmission from females to males by about 50– 60%. The reason for this is that in the foreskin of the uncircumcised penis contains a high percentage HIV susceptible cell including $CD4^+$ T cells, dendritic cells, and Langerhans cells. This makes the foreskin a portal of HIV virus entry during sexual transmission. Oral sex has a low probability of HIV transmission compared to vaginal and anal intercourse, while there is no evidence that HIV can be transmitted through contact with the sweat, tears, urine, and feces of PLWH (116).

There are several ways to prevent HIV transmission, including condom use, avoiding needle sharing, and using pre-exposure prophylaxis (PrEP) and post-exposure prophylaxis (PEP) with ART. PEP consists of a combination of three HIV medications: tenofovir (either TAF or TDF), emtricitabine (FTC) and raltegravir (RAL) taken by HIV uninfected persons exposed to HIV for four weeks to reduce their risk of HIV seroconversion (www.webmd.com/hiv-aids/post-exposure-prophylaxis). PEP should be started as soon as possible, within 72 hours of exposure to HIV (117). On the other hand, PrEP is prescribed for those at risk of HIV infection. It involves taking Truvada, a single pill that contains the NRTIs tenofovir disoproxil fumarate (TDC) and FTC or Descovy, a single pill that contains tenofovir alafenamide (TAF) and FTC either daily or before and after a planned a high-risk exposure to HIV (118-120). When PEP or PrEP are taken, the HIV drugs get into the bloodstream, genital and rectal tissues in a manner that blocks HIV replication preventing the establishment of infection.

1.9. STAGES OF INFECTION

PLWH who do not receive ART, typically progress through three major stages of infection characterized by biological markers and clinical manifestations. The first stage is “acute infection”, “recent infection” “early infection” or” primary HIV infection (PHI)”, terms that are often used inter-changeably in the literature. A recent review on this topic described acute infection as the time between the transmission event that led to the establishment of an productive HIV infection and when the infected person is viremic and has detectable GAG p24 antigen in the plasma but no diagnostic anti-p24 Abs in the plasma (121, 122). This period is also referred to as PHI. Recent infection typically refers to the first 6 mo of infection. Early infection

can be used to refer to acute or recent infection after which begins the chronic phase of infection (122).

Early HIV infection is separated into several phases. The eclipse phase of HIV infection is defined as the time from HIV acquisition to the time HIV RNA is first detected in plasma. It lasts approximately 10 days and during this phase, no diagnostic tests can detect HIV infection (123, 124). Following the eclipse phase, six Fiebig stages of early HIV infection have been described based on the emergence of immunologic and virologic markers (125). These stages are illustrated in figure 8.1. Fiebig stage I, which typically spans 5 days, refers to the period when HIV first appears in the blood as detected by an HIV RNA polymerase chain reaction (PCR) assay in a person negative for HIV GAG CA (or p24) antigen, Abs to p24 detected by enzyme immunoassays (EIA) and by Western blot (75, 126). Fiebig stage II is characterized by positive HIV RNA and p24 antigen tests, which appear in the blood once HIV RNA levels rise above 10,000 copies/ml of plasma (75). Fiebig stage III lasts 14 days and is characterized by the presence of a detectable plasma HIV RNA by PCR, p24 antigen and anti-HIV p24 IgM Abs using a sensitive (3^{rd} generation) EIA and a negative Western blot. HIV second 2^{nd} and 3^{rd} generation tests were developed in 1987 and 1991, respectively. The specificity and sensitivity of HIV 2^{nd} and 3^{rd} generation assay was improved to detect IgG and IgM Abs to p24 and to reduce the time interval between infection and a positive test using EIA assays from 4-6 weeks to 2-3 weeks, respectively (127). Fiebig stage IV, which lasts 19 days, is characterized by a positive HIV RNA PCR test, a waning p24 antigen test, a positive 3^{rd} generation HIV p24 Ab EIA and an indeterminate confirmatory HIV Western blot where not all bands detecting HIV gene products are present. In Fiebig stage V, which lasts 88 days, HIV RNA VL has declined from its peak, though the HIV VL PCR test remains positive. In this stage, the p24 antigen test may be positive or negative, the anti-HIV p24 Ab test is positive using 2^{nd} and 3^{rd} generation EIA tests and the confirmatory Western blot detects all bands except for the one for p31. The final Fiebig stage VI is known as “early chronic phase infection” and its duration is open ended. It is characterized by the same test results as Fiebig stage V with the exception that the confirmatory Western blot assay detects a full complement of bands (125).

$CD4^+$ T cells decline precipitously within a few weeks of infection and recover partially thereafter (128, 129). The most dramatic decline occurs in the gastrointestinal (GI) tract since most of the $CD4^+$ T cells at this site co-express CCR5 (128, 129). Within 2 to 4 weeks post infection, 50-90% of patients experience a symptomatic acute retroviral syndrome characterized by non-specific flu-like symptoms such as fever, rash, and sore throat (130, 131).

The second stage of HIV infection is the "chronic or latent phase". In this stage, an HIV VL set point is established, that remains stable throughout this phase. HIV actively replicates throughout this phase and $CD4^+$ T cells rapidly turn over (132). The VL set point determines how fast HIV will progress. The higher the VL set point the faster $CD4^+$ T cells in a treatment naïve infected person will decline and the faster the progression to AIDS and death (133, 134). The third stage of HIV infection is AIDS. AIDS is diagnosed when the $CD4^+$ T cell count drops below 200 cells/mm^3 or when an AIDS defining OI is diagnosed, whichever comes first. Figure 8.1 shows a schematic of the changes in HIV viremia and $CD4^+$ T cells counts over the course of the three stages of HIV infection.

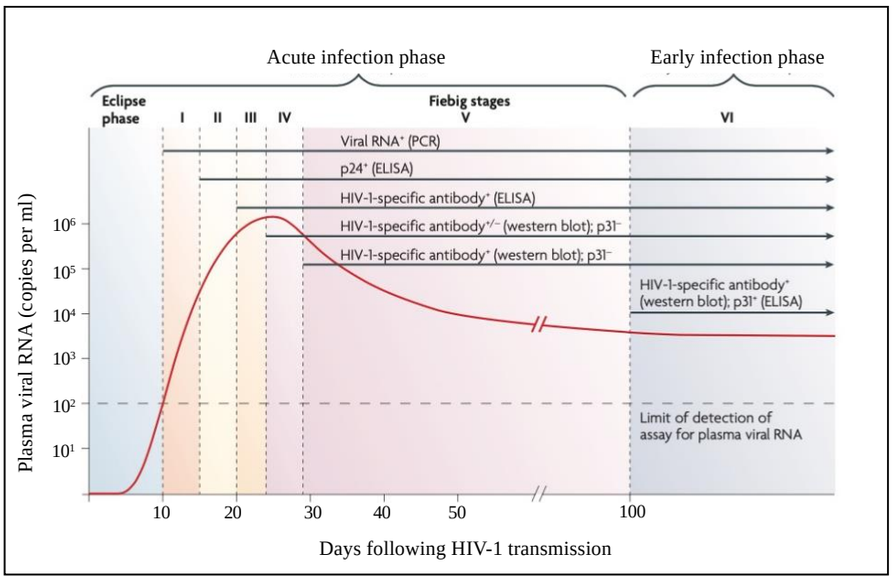

Figure 8.1. Schematic representation of the period of acute and early chronic HIV infection

According to the CDC, the sooner people start ART after diagnosis, the more they will benefit from treatment. HIV tests to diagnose HIV infection are very accurate. However, there is no test available that can detect the virus immediately after the infection. Table 2.1 provides a list of tests that can be used to diagnose acute HIV infection. The results of these tests together with

knowledge of the date of the start of symptoms consistent with a symptomatic seroconversion can be used to date the HIV exposure that resulted in the establishment of a new infection (122).

Table 2.1. Test used to diagnose and date HIV infection

Test	Purpose	Time from infection to detection	Advantages
HIV RNA (VL)	Measures the level of HIV RNA copies/ml of plasma using quantitative PCR, nucleic acid amplification assays (135).	11 – 12 days	• High specificity
p24 antigen	Measures the level of HIV p24 core protein which can be present in the plasma of newly infected people. This test is useful for diagnosing a recent HIV infection only when the p24 Ab EIA is negative (135).	14 – 15 days	• HIV p24 can be detected prior to seroconversion. • High specificity
p24 Ab EIA	Detects the presence of IgG and IgM anti-HIV p24 Abs (136). The 2nd generation HIV EIA assay was improved by adding the recombinant antigen for HIV p24 that reduces the duration of the Ab negative window. The 3rd, 4th and 5th generation EIA assays have the ability to detect anti-HIV-1 and HIV-2 IgG, IgM Abs and p24 Ag within 2 weeks post-infection (127).	3 – 6 weeks	• Most common test • High specificity and sensitivity
Western blot (WB)	This is a confirmatory test performed when the p24 Ab EIA test is positive. It separates the blood proteins via electrophoresis and detects the specific HIV proteins by Western blot. This test can detect bands specific for GP160, GP120, P24 and P31 (137, 138) When an indeterminate result is obtained (i.e. when bands to only a subset of HIV gene products are detected on the same day that a person is positive for the VL PCR test or the p24 antigen test or has experienced a symptomatic HIV infection, this date can be used to time when HIV infection occurred by subtracting 35 days.	4– 6 weeks 35 days for an indeterminant WB	• High specificity and sensitivity • Used to confirm a positive P24 antibody EIA test
Rapid HIV test	Detects anti-HIV IgG and IgM in oral fluid, whole blood and plasma or serum samples (136, 139).	4 – 6 weeks	• Results available faster than the above tests (30 min or less for result) • Low cost (an advantage in low-income countries). • High specificity and sensitivity (136).

1.10. MECHANISMS OF SPONTANEOUS HIV CONTROL IN PEOPLE LIVING WITH HIV (PLWH)

Several yrs after HIV was discovered and PLWH were followed for long enough, researchers identified a small subset of PLWH who spontaneously maintained high CD4 counts for 7 or more yrs. When HIV VL assays became available in the mid-1990s some of these were found to have an undetectable VL without receiving ART. Long term non progressor (LTNP) was the term used to identify PLWH who maintained CD4 counts >500 cells/mm^3 for 7 or more yrs (140, 141). Some LTNPs had detectable VLs. LTNPs who maintained undetectable VLs without ART were called Elite Controllers (Ecs) (142, 143). The LTPNs who maintained VLs of <2000 or <3000 copies/ml plasma (depending on the cohorts) were called Viral Controllers (VCs) (142). The EC population represents less than 1% of all PLWH (142-145). LTNPs make up approximately 5% of HIV infected individuals depending on the duration of infection used to define this population (77). Together these three groups are sometimes referred to as controllers or slow progressors (146). Several explanations have been postulated to account for the ability of ECs to control their VL to undetectable levels with high and stable CD4 counts (142). Certain HLA types, such as HLA-B*14, HLA-B*27 and HLA-B*57 is more common in controllers than in non-controllers (147-151). This implicates $CD8^+$ T cell responses in HIV control (152). Controllers are more likely to have HIV-specific polyfunctional $CD8^+$ T cell responses than non-controllers. HIV specific $CD8^+$ T cells from ECs had a greater proliferative capacity, higher cytotoxic activity and secreted more tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), chemokine CC motif ligand 4 (CCL4), and interleukin 2 (IL-2) compared to those from patients with progressive disease (153-155). $CD8^+$ T cell function was inversely correlated with VL (156). Recently, a study examined HIV proviral integration sites in a group of highly virus suppressed ECs and long term successfully treated PLWH (157). They found that ECs were more likely to have proviral HIV reservoirs integrated into genetic areas that were transcriptionally silent than HIV proviral sequences from successfully treated HIV-infected individuals. The implication of this finding was that proviruses in this group of ECs were unlikely to reactivate. Since these ECs also maintained HIV-specific T cells responses, if their provirus did re-activate their T cells responses could rapidly control HIV limiting the reseeding of the HIV reservoirs (157).

1.11. CORRELATES OF HIV PROTECTION IN HIV-EXPOSED SERONEGATIVE (HESN) INDIVIDUALS

Some individuals remain HIV uninfected despite multiple high-risk exposures to the virus. Such individuals are known as HIV-exposed seronegative (HESN) persons (158). Studying HESN individuals has the potential to provide clues on correlates of immune protection against HIV infection. Several HESN cohorts have been studied. These include HESN subjects with high-risk sexual behavior such as female commercial sex workers (CSWs) in Kenya, HIV-discordant couples, men who have sex with men (MSM), parenterally exposed HESN such as intravenous drug users and children born to HIV infected mothers (159-163). A better understanding of the mechanisms underlying their apparent resistance to HIV infection is required to develop strategies to protect against HIV infection.

Epidemiological and functional studies have shown evidence for the role of HIV-specific T cells, natural killer cells (NK) cells and Ab responses in protection from HIV infection. However, these responses were not described in all HESN cohorts (164, 165). Some have found anti-HIV specific T cell responses in HESNs (166-168). However, these T cell responses are likely to be markers of exposure to HIV rather than a mechanism for protection (164, 165). Following HIV exposure, it takes 10 to 14 days to develop an effector T cell response to HIV, which is too late for such a response to prevent the establishment of a new infection during the eclipse phase when HIV is susceptible to being extinguished (169). On the other hand, NK cells are primed to respond to virally infected cells such as HIV infected cells that downmodulate cell surface HLA expression (170-174).

In a cohort of CSW in Nairobi, Kenya, immune quiescence was proposed to be a mechanism of protection from infection in the subset who met the criteria for classification as HESNs. As HIV replicates preferentially in activated $CD4^+$ cells, quiescent CD4 cells represent a barrier to infection (175).

There exists a variant of the gene encoding the CCR5 co-receptor for HIV entry having a 32 base pair deletion. The allele encoding this *CCR5Δ32* variant has a frequency of approximately 10% in Caucasians with a higher frequency in Northern European populations that declines in populations

living further south (176). Homozygosity for *CCR5Δ32* occurs in approximately 1% of Caucasians and confers potent resistance to HIV infection (177). While homozygosity for *CCR5Δ32* confers potent protection from HIV infection, only a subset of Caucasians and no Asian or Black HESNs, are resistant to HIV infection due to carriage of this genotype. The *CCR5Δ32* mutation encodes a receptor that is not expressed on the cell surface thereby preventing HIV from entering $CD4^+$ T cells. Several HIV infected individuals needing treatment for leukemias have received bone marrow stem cell transplants from individuals who were *CCR5Δ32* homozygotes (178, 179). Some of these transplant recipients reconstituted their immune system with target cells unable to express CCR5 and maintained undetectable VLs for yrs without ART (179) . While it is not feasible to apply such therapies widely to treat HIV infection, these examples do illustrate the proof of concept that lack of CCR5 expression can lead to HIV remission. Strategies aimed at knocking out CCR5 expression are being explored as therapies for HIV. The use of zinc finger nucleases (ZFN) and clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) are one of the strategies being explored to knock out CCR5 expression. ZFNs are engineered proteins with zinc finger domains that can bind to target regions of DNA and conduct gene editing via dsDNA breaks. This strategy was safely used to modify CCR5 in autologous $CD4^+$ T cells to treat PLWH (180). CRISPR-Cas9 was successfully tested in human cells and able to modify CCR5 genes (181).

1.12. IMMUNE RESPONSES TO HIV

1.12.1. HIV-specific Cytotoxic T lymphocyte (CTL) responses

$CD8^+$ T cells, also known as CTLs play an important role in immune responses to intracellular pathogens such as HIV. CTL T cell receptors (TCR) recognize complexes of 8-10-mer peptides derived from the sequence of HIV gene products and autologous MHC-I antigens. CTL activation requires engagement of the TCR with cognate MHC-I/peptide complexes as a first signal, co-stimulation through engagement of CD28 on T cells with CD80 or CD86 on antigen presenting cells (APCs, 2^{nd} signal) and cytokine mediated differentiation and expansion such as IL-2 binding to high affinity IL-2 receptors on T cells (3^{rd} signal) (182). CTL activation leads to 1) the secretion of cytokines such as TNF-α and IFN-γ, which have anti-viral activity, 2) the externalization of cytotoxic granules containing perforin and granzyme-B, which kill HIV infected target cells, 3) the destruction of infected cells via Fas/FasL interaction when $CD8^+$ T cells express FasL receptors

and bind Fas on the target cells. And 4) the secretion of chemokines (CCL3, CCL4, CCL5) that bind the CCR5 co-receptor for HIV entry in a manner that blocks HIV entry into new $CD4^+$ T cell targets (183).

The importance of CTL in HIV control is illustrated by work done in SIV infected RMs. Depletion of $CD8^+$ T cells reverses SIV control leading to an increase in VL when depletion occurs in the chronic phase of infection or the inability to establish a set point VL when depletion occurs in primary SIV infection, until CD8 cells numbers recover (184). HIV-specific CTLs exert immune pressure on HIV infected cells. This is exemplified by the advantage conferred to HIV infected cells by HIV mutations that escape recognition by CTLs (185).

1.13. HIV-specific Ab responses

While the induction of neutralizing Abs by vaccination is a strong correlate of protection against many infections, induction of neutralizing anti-HIV Abs by vaccination has largely failed. PLWH develop hypergammaglobulinemia characterized by high levels of Abs to many specificities, including to HIV gene products (186). Most of the HIV-specific Abs in PLWH are non-neutralizing. While a subset of PLWH develop neutralizing Abs and in some cases bNAbs, these Abs typically do not recognize autologous contemporaneous HIV viral isolates and thus are ineffective at controlling HIV due to the rapid rate of evolution of HIV that leads to escape from any immune pressure exerted by these Abs (187, 188). Only Abs binding to the envelope surface spike can directly neutralize virions (97, 189). In PLWH, the production of anti-HIV ENV Abs occurs is in a sequential order. For instance, anti-GP41 Abs appear first a median of 13 days following detectable plasma viral RNA, while anti-GP120 Abs appear at median of 28 days after plasma RNA is first detected, anti-HIV p24- and p17-specific IgG Abs appear a median of 18 and 33 days from this time point, respectively and anti-p31 Abs detecting integrase appear a median of 53 days following detectable plasma RNA (186).

Previous studies have reported that bNAbs are not routinely made in HIV infection. The natural development of bNAbs can take several yrs to develop in PLWH (190). A study by Doria-Rose et al. and Sather et al. reported that ~25% of PLWH who were not receiving ART produced bNAbs after one year of infection (190, 191). Furthermore, a subset of PLWH, known as “elite neutralizers”, produce extremely potent bNAbs. Certain bNAbs from children have a broad range of neutralization specificity during early stages of infection within the first 2 yrs of life. These bNAbs are broader and more potent than bNAbs in adults (192, 193). Therapy using single and combinations of bNAbs is being explored. Infusion of bNAbs has been successful in suppressing of VL in PLWH and may be useful as an alternative therapy for HIV (194). A study done by Caskey et al., showed infusion of the potent bNAb, 10-1074, which targets the V3 glycan on HIV ENV, was safe and was able to temporarily suppress VL (195). Success of this type of monoclonal Ab (mAb) therapy for PLWH depends on having their having viral isolates that are sensitive to the bNAb (195, 196). Several strategies are being explored to improve the use of bNAbs therapeutically, such as infusions of bNAbs with more than one specificity, modifications of the Fc region of these Abs to increase their persistence and screening recipients for the presence of viral isolates resistant to the bNAbs in the Ab cocktails. The possibility of also using these strategies to prevent HIV infection in high-risk individuals is also being explored.

The RV144 HIV vaccine trial was the first to show significant, through modest, efficacy in protecting against HIV infection (197, 198). This trial was started in 2003 in Thailand and enrolled over 16,000 participants. Administration of this vaccine reduced the rate of infection by 31.2% (197). The results of the RV144 trial showed that the vaccine elicited the production of non-neutralizing IgG Abs (nNAbs) to the V1/V2 regions of ENV (198). Secondary analyses showed that anti-HIV ENV V1/V2 loop-specific Abs induced by this vaccine supported ADCC activity that correlated with a reduced risk of HIV infection (198-200).

1.14. NATURAL KILLER (NK) CELL

1.14.1 NK cell development

Until 1970, lymphocytes were classified as either T cells or B cells, which both had the ability to recognize pathogens specifically. In 1975, Rolf Keissling and colleagues in Sweden described the presence of cells in the spleen of adult mice that were neither T nor B cells. This was the first observation of a new subset of lymphocytes. Keissling named these cells "natural" killer cells since they were activated and responded to target cells without prior sensitization (201). A few years later, Klas Kärre demonstrated that NK cells were able to kill tumor cells with downregulated cell surface levels of MHC-I. Kärre proposed that NK cells killed through mechanisms involving recognition of "missing-self" as NK cells do not kill healthy self-cells (202).

NK cells make up 5-15% of peripheral blood mononuclear cells (PBMCs). These cells belong to innate immune system. NK cells detect and destroy virally infected cells, tumor cells, as well as stressed cells (203, 204). NK cells are found in the blood, as well as in lymphoid and non-lymphoid tissues such as bone marrow (BM), thymus, LNs, tonsils, liver, skin, gut and lungs (205).

NK cells develop and mature from hematopoietic stem cells (HSCs) in the BM and secondary lymphoid tissues (SLTs) such as tonsils, spleen and LNs. During NK cell development, a subset of HSCs become common lymphoid progenitors (CLPs). CLPs give rise to pre-NK progenitor (pre-NKP) cells, which are the earliest lineage restricted NK cells. Pre-NKP cells express CD122 and lack expression of lineage (Lin) markers. After the pre-NKP stage, in the presence of IL-5, they differentiate into immature NK cells (iNK) cells, which then develop into mature NK cells (mNK) cells within the BM. Finally, NK cells egress to the periphery. The detailed steps of NKP cell development into iNK cells and mNK cells is not fully characterized. A study done by Chen et al. demonstrated that a subpopulation of $CD34^{+}Lin^{-}CD45RA^{+}$ cells, which express CD10 could give rise to T cells, B cells, NK cells and DCs (206). This finding was confirmed by Miller et al. who reported that a subset of $CD34^{+}Lin^{-}CD45RA^{+}$ cells in the BM gives rise to NK cells, B cells and myeloid cells under similar culture conditions (207). Others showed that $CD3^{-}CD56^{+}$ NK cells can be generated *in vitro* from $CD34^{+}$ cells isolated from cord blood, BM, fetal liver, thymus and SLTs when stimulated with IL-2 or IL-5 (208-211).

There are two major subsets of circulating NK cells that differ from each other based on the surface expression of CD56 and CD16 (FcγRIIIa). $CD56^{dim}CD16^{bright}$ NK cells predominate in peripheral blood making up 90% of circulating NK cells. $CD56^{bright}$ $CD16^{neg/dim}$ NK cells represent 10% of circulating NK cells (212). The $CD56^{dim}CD16^{bright}$ NK cells were originally reported to be more naturally cytotoxic than the $CD56^{bright}CD16^{neg/dim}$ NK cells, which produce high levels of cytokines such as IFN-γ, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) (212). However, more recent information challenges this functional dichotomy showing that $CD56^{dim}CD16^{bright}$ can also secrete cytokines/chemokines upon stimulation (213).

NK cell development is separated into six stages based on the cell surface markers expressed at each stage. Expression of CD244 is specific to stage 1, which is the earliest lineage restricted NK cells stage also referred to as pre-NKP cells. Expression of CD7 and CD127 defines stage 2a and CD122 expression identifies stage 2b NK cells, which are also referred to as "refined-NKP" (rNKP) cells. In stage 3 and 4 NK cells are identified as iNK. Stage 3, iNK cells express NKG2D, while the hallmark of iNK cells in stage 4a is high expression levels of CD56, also known as $CD56^{bright}$. Stage 4b iNK cells express NKG2A, CD337 (NKp30), CD335 (NKp46), and NKp80. Stage 5 NK cells express lower levels of CD56 as seen on $CD56^{dim}$ NK cells and CD16 (FcγRIIIa). Finally, stage 6 NK cells are mNK cells that express Killer Immunoglobulin-like Receptors (KIRs) and the maturation marker CD57 (214). Figure 9.1 shows the NK cell development stages in BM and SLTs and what markers they express.

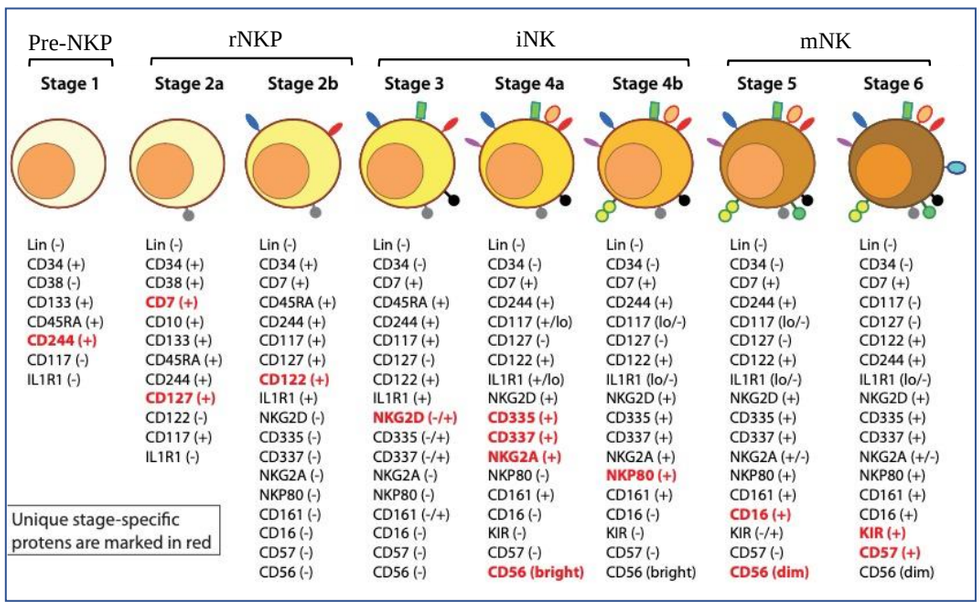

Figure 9.1. Schematic of different stages of human NK cell development and maturation in BM and LNs

Six different stages of NK cell development are depicted with their phenotypic markers in black. Markers highlighted in red are ones considered to be the hallmark of each stage (214).

1.14.2 NK cell education

NK cell education is an essential developmental process required to make NK cells functionally competent. The functional competency of NK cells is defined by their ability to elicit a potent and quick response that can include cytolysis and cytokine/chemokine secretion. The principles of NK cells education have been an area of active research over the past decade due to the importance of the education process for NK cell function. In this process, inhibitory NK cells receptors such as inhibitory KIRs, NKG2A and LILRBs contribute to shaping NK cell responsiveness by interacting with self MHC class I ligands (215-219). NK cells bearing inhibitory receptors to self MHC are resting under normal circumstances but become hyperresponsive when they encounter target cells in the periphery lacking self MHC molecules. This phenomenon, termed “missing self” recognition allows NK cells to eliminate such aberrant cells (202, 220) . The interaction of inhibitory NK cell receptors and their MHC-I ligands promote NK cell responsiveness through a process called “education” or “licensing”, which are critical for NK cells to sense virally infected cells, tumor cells or stressed cells leading to their clearance (217, 218). See figure 10.1 shows the model of uneducated and educated NK cells.

The ligands for activating NK cell receptors are less well characterized (221). Many inhibitory NK cell receptors have activating counterparts with similar extracellular domains (222). Despite this sequence similarity the interaction between activating NK cell receptors and their ligands has been difficult to demonstrate. One exception to this is KIR2DS1 which has been shown to bind HLA-C group 2 antigens (223, 224). KIR2DS1 contributes to NK cell education by tuning down NK cell responses in donors homozygous for HLA-C2 (225). It should be noted that in the absence of inhibitory NK cell receptors ligation of activating NK receptors can activate NK cells (226). For instance, KIR3DS1, which is an activating NK cell receptor, interacts with its ligand, HLA-F, expressed on CD4 T cells that are either HIV infected or not. This interaction activates KIR3DS1^{+} NK cells to produce CCL4, IFN-γ and CD107a (227).

As NK cell receptors are stochastically expressed on the surface of NK cells, approximately 10% of NK cells express no receptors to self HLA (228) . These uneducated NK cells remain hyporesponsive (217, 219, 229). This is important in maintaining tolerance and avoids NK cell mediated autoimmune responses.

NK cell education is a tunable process, that varies according to the number of NK cell receptor/ligand pairs contributing to NK cell education, the surface density of inhibitory NK cell receptors, the expression level of self MCH-l, and the binding avidity of receptor/ligand interactions, which is due to allele-encoded variations in both receptors and ligands involved in NK cell education (172).

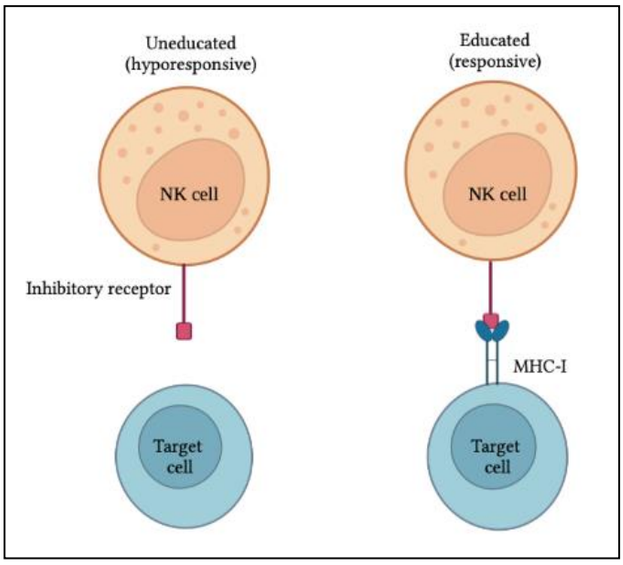

Figure 10.1. NK cells education model

The education model states that NK cell education requires positive engagement of inhibitory receptors with MHC-l on target cells. The lack of this interaction results in cellular hyporesponsiveness. Figure adapted from Hoglund and Brodin, 2010 (230). (Made by K Alsulami using BioRender.com).

NK cell education has been well-studied in the context of HIV infection. HIV downmodulates the expression MHC-I molecules on the surface of infected cells to evade CTL responses (170, 171, 231). However, this downmodulation makes infected cells more susceptible to NK cell recognition and killing through “missing-self” mechanisms (172). The potency of NK cell responses to HIV-infected cells is directly linked to the potency of NK cell education. This

observation has been confirmed by epidemiological studies in which infected individuals carrying the *KIR3DL1/Bw4*801* genetic combination showed slower time to AIDS than carriers of other *KIR/HLA* genotypes (232). Altogether, NK cell education is a tunable and resettable process determined by engagement of both inhibitory and activating NK cell receptors that orchestrate the balance between generating responsive and self-tolerant NK cells.

There are multiple proposed mechanisms that might explain NK cell education such as the arming model, the disarming model and the tuning model (233). In the arming model, which is known as the "positive model", NK cell precursors require interactions with MHC-l molecules. If NK cells do not encounter MHC-l molecules, they will remain unarmed and therefore hypo-responsive. In this model, NK cells are initially hypo-responsive and require the interaction between KIRs and their MHC-l ligands to acquire functional competency (218). In this model of NK cell education, the ligation of KIR with MHC-l induce inhibitory signaling and causes the phosphorylation of ITIMs leading to recruitment and activation of the SHP-2 domain and protein tyrosine phosphatase-1 (SHP-1). This inhibitory pathway leads to heightened cytolytic response in the licensed NK cells against target cells that lack MHC-l. Moreover, evidence for this model is supported by murine studies that have shown that deletion of ITIMs or SHP-1 in NK cells leads to hypo-responsive and uneducated NK cells (218, 234, 235).

In contrast, the disarming model, NK cells are always activated, and it is the expression of inhibitory receptors that prevent the cells from becoming hyporesponsive. The lack of expression of inhibitory receptors for self-MHC and prolonged activation therefore results in hyporesponsive NK cells (219, 236). The tuning model proposes that NK cell education is fine-tuned by total inhibitory input received where cells having more inhibitory input during education achieve greater functional capacity, which can be tuned down if activating signals are received from activating NK cell receptors interacting with their ligands (237, 238).

1.14.3. NK cell receptors

NK cell receptors fall into two broad categories: activating and inhibitory receptors. The integration of signals received from these receptors governs NK cell activation status and function. NK cell receptors can also be classified into those with C lectin-like domains and immunoglobulin (Ig)-like domains. Inhibitory NK cell receptors usually recognize MHC-1 antigens as ligands. Abnormal cells that have downregulated MHC-I molecules abrogate NK cell inhibitory signals, if these abnormal cells also express ligands for activating NK cell receptors, NK cells are activated to secrete cytokines, chemokines and elicit cytolysis that can eliminate aberrant MHC-I deficient target cells. This phenomenon is known as “missing self-recognition”. NK cell inhibitory receptors possess a signaling motif in their long cytoplasmic tails allowing the delivery of negative signals. This signaling motif is called an immunoreceptor tyrosine-based inhibitory motif (ITIM) and is defined by the consensus sequence: Ile/Val/Leu/Ser-x-Tyr-x-x-Leu/Val where “x” can be any amino acid. The ITIM’s tyrosine residue becomes phosphorylated and serves as a docking site for the recruitment of tyrosine-specific phosphatases such as Src (Sarcoma) homology 2 domain phosphatase 1 (SHP-1), Src homology phosphatase 2 (SHP-2) and Src homology 2 (SH2)-domain containing inositol polyphosphate-5-phosphatase (SHIP). These phosphatases can dephosphorylate signaling molecules such as CD3ζ and DAP12, ultimately inhibiting NK cell activities such as cytolysis and cytokine/chemokine secretion (239, 240). Unlike inhibitory receptors, most activating NK receptors lack a signaling motif in their short cytoplasmic tails and need to associate with adaptor molecules to transmit their signals. These adaptor molecules have immunoreceptor tyrosine-based activation motifs (ITAMs), which carry the amino acid sequence: Asp/Glu-x-x-Tyr-x-x-Leu/Ile-x_{6-8}-Tyr-x-x-Leu/Ile (239). NK cells use three ITAM-containing adaptor molecules: DAP12, FcεRIγ and CD3ζ. Upon activating NK cell receptor ligand binding, the tyrosine residues in the ITAM become phosphorylated and recruit spleen tyrosine kinase (Syk) and Zeta-chain-associated protein kinase 70 (Zap-70) that initiate a signaling cascade leading to the activation of NK cell functions (239).

NK cell activation status is determined by the integration of inhibitory and activating signals. If activating signals are predominate, NK cells will become activated and will release their cytotoxic granule contents to initiate cytolysis and produce cytokines/chemokines. On the other

hand, if inhibitory signals predominate, the NK cell will remain in a resting state (239). When activating and inhibitory receptors are present in the same immunological synapse, the phosphatases, such as SHP-1, recruited by inhibitory receptor ITIMs prevent phosphorylation of activating NK cell receptors resulting in the predominance of inhibitory signals under these conditions (240, 241).

1.14.3.1CD16 (FcγRIII)

CD16 is an activating receptor, which is expressed on the surface of NK cells, monocytes, and macrophages. CD16 has two Ig-like extracellular domains and a short cytoplasmic tail. CD16 uses ITAM containing adaptor proteins such as CD3ζ and FcεRIγ to transmit activating signals (242).

There are two structurally and functionally distinct types of CD16, CD16a (FcγRIIIA) and CD16b (FcγRIIIB). CD16a and CD16b are encoded by the *FCGR3A* and *FCGR3B* genes, respectively (243). $CD56^{dim}$ NK cells, monocytes, macrophages, and dendritic cells express CD16a. Few $CD56^{bright}$ NK cells express this receptor (212). CD16a and CD16b have 96% sequence similarity in their extracellular Ig binding domains (244). CD16b is expressed on neutrophils and is the only Fc receptor (FcR) anchored to the cell membrane by a glycosyl-phosphatidylinositol (GPI) linker (244). From now on, CD16a will be referred as CD16 as this is the FcR expressed on NK cells.

The main function of CD16 on NK cells is to mediate antibody-dependent cellular cytotoxicity (ADCC) leading to killing of target cells and antibody-dependent NK activation (ADNKA), which activates NK cells to produce cytokines/chemokines and release the contents of cytotoxic granules that lead to target cell cytolysis (213, 245) (See figure 11.1). The involvement of NK cells in ADCC as a result CD16 ligation by target cell-bound Abs highlights the importance of these responses in viral infections such as HIV (246).

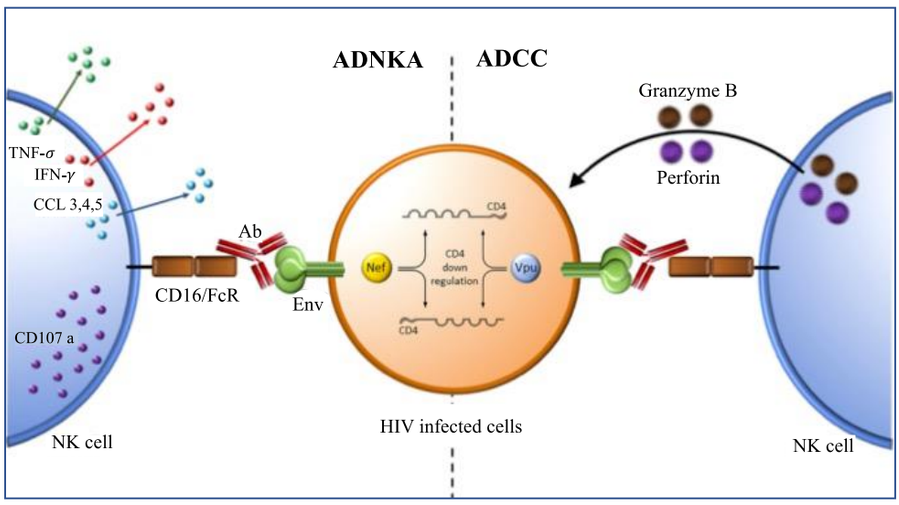

Figure 11.1. ADNKA and ADCC activity in HIV infected cells

HIV NEF and VPU downregulate CD4 expression on the surface of the infected cells, preventing CD4 from interacting with HIV ENV. The antigen combining site of bNAbs (shown in red) bind the ENV conformation while the Fc region of the bNAbs bind the activating receptor CD16 on NK cells, thus activating the ADCC of NK cells. ADNKA assesses the secretion of TNF-α (green) and IFN-γ (red), and expression of the degranulation marker CD107a (purple) from NK effector cells (left) whereas ADCC assesses the cytopathic effects of cytolytic granules containing perforin and granzyme B released by NK cells on antibody opsonized target cells (right) From (146).

1.14.3.2. Killer cell Immunoglobulin-like receptors (KIRs)

KIRs are a large family of Ig-like receptors, which are expressed stochastically on NK cells and other lymphocytes subsets such as CD4 and CD8 T cells (247). To date, The *KIR* gene family includes 14 loci (2 of which are pseudogenes) encoding 9 genes located within a 100 to 200 kilobase region of the leukocyte receptor complex located on chromosome 19 (19q13.4) (248-250). KIRs include both activating and inhibitory receptors. *KIR* genes are organized into two broad haplotypes, A and B, which are made up of centromeric and telomeric regions separated by a recombination hotspot. The genes within the telomeric and centromeric regions are in linkage disequilibrium with each other (251). *KIR* haplotype A, is non-variable and includes genes that encode mostly inhibitory KIRs (251). Group B haplotypes are more variable in terms of the number of genes present and include variable number of genes encoding activating KIRs. The KIR loci exhibit variable levels of allelic polymorphism. *KIR* haplotypes are still in the process of being fully characterized as this requires full length sequencing in multiple populations (250).

The first 12 *KIR* haplotypes sequenced were found to be made up of combinations of 8 centromeric and 6 telomeric *KIR* gene motifs (250).

KIR nomenclature is based on their protein structure. The number following "KIR" refers to the number of extracellular Ig-like domains they have, while "D" stands for domain. The next letter in the proteins' name is either "L" or "S" for a long or short cytoplasmic tail, respectively. As described above in the NK cell receptor section, those with a long cytoplasmic tail are inhibitory while those with a short cytoplasmic tail are activating receptors. The next number indicates an individual KIR protein, for instance, KIRs with a long intracellular chain (i.e 3DL1-3, 2DL1-3, 2DL5) are inhibitory KIRs (iKIRs) while those with short intracellular domains (3DS1, 2DS1-5) are activating KIRs (aKIRs) (252).

KIRs possess 2 or 3 extracellular Ig-like domains, which can interact with subsets of MHC-l ligands. The KIR2D proteins are subdivided into two groups depending on the origin of the membrane distal Ig-like domains present. Type I KIR2D proteins are (KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4 and KIR2DS5) have a membrane distal Ig-like domain similar in origin to the KIR3D D1 Ig-like domain but lack at a D0 domain. While type II KIR2D proteins, KIR2DL4 and KIR2DL5 have a membrane-distal Ig-like domain of similar sequence to the D0 domain present in KIR3D proteins but lack D1 domains. See Figure 12.1. Long cytoplasmic tails contain two ITIMs which transduce inhibitory signals to NK cells, but short cytoplasmic tails have one ITIM and a positively charged amino acid residue in their transmembrane region that allows them associated with the DAP12 signaling molecule to generate an activation signal (253).

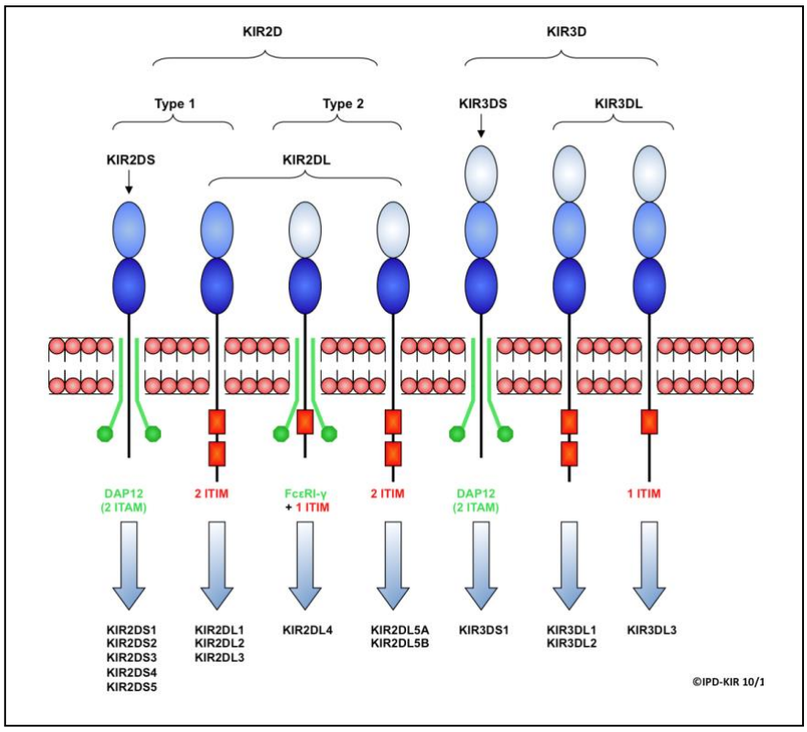

Figure 12.1. Schematic representation of KIR protein structures

The figure shows the characteristics of two and three Ig-like domain KIR proteins. The association of activating KIR to adaptor molecules is shown in green, whereas the ITIMs of inhibitory KIR are shown as red boxes. Figure adapted from IPD-KIR. https://www.ebi.ac.uk/ipd/kir/about/

The major ligands of iKIRs are subsets of MHC-1 molecules, which are expressed on the surface of healthy cells. The ligands for KIR2DL2 and KIR2DL3 are HLA-C antigens belonging to the C1 group, the ligands for KIR2DL1 bind the HLA-C2 group antigens (254). The interaction of KIR2DL1 with HLA-C2 appears stronger than the interaction of KIR2DL3 with HLA-C1 (254). The ligands for KIR3DL1 are a subset of HLA-B allotypes known as HLA-Bw4. HLA-Bw4 differs from the remaining HLA-Bw6 antigens by sequences at amino acid 77 to 83 of the HLA heavy chain (255). HLA-Bw4 allotypes with an isoleucine at position 80 (Bw4*80I) have a higher avidity for KIR3DL1 than those with a thymidine (Bw4*80T) at this position (256) . KIR2DS1, KIR2DS2 and KIR3DS1 are the activating counterparts of KIR2DL1, KIR2DL2 and KIR3DL1, respectively. The extracellular domains of the activating receptors a have a high degree of sequence similarity with their inhibitory counterparts but have a much lower affinity or none for the same ligands (257, 258). The non-classical MHC-I antigen, HLA-F, is ligands of KIR3DS1 (259). As mentioned earlier, KIRs are stochastically expressed on NK cells, so that there exists a variety of NK cell clones expressing different combinations of KIRs, each with its

own ligand specificity. This ensures that some NK cells will be able to rapidly recognize transformed cells or virally infected cells with downmodulated levels of HLA-I ligands. Although many of MHC-I ligands have been identified for various KIRs, several have remained more elusive, particularly those for activating KIRs.

Several studies have been shown that combinations of KIR and HLA-I variants are associated with outcomes that can affect human health. In the context of HIV infection, co-expression of HLA-A and B ligands containing Bw4 motif with interacting KIRs is associated with protection from infection and slower time to AIDS (260). Boulet et al. showed that carriage of the *KIR3DS1* homozygous genotype is associated with reduced risk of HIV infection (261). HLA-F is expressed on the surface of uninfected $CD4^+$ cells and at a lower level on HIV-infected cells (226, 227). The mechanism underlying the protective role of *KIR3DS1*, may be the ability of $KIR3DS1^+$ NK cells to interact with HLA-F on HIV-infected cells, which stimulates these $KIR3DS1^+$ NK cells to secrete anti-viral factors such as IFN-γ, TNF-α and secrete CCL3, CCL4, and CCL5, which block HIV entry into new target cells (227, 262-264). In an earlier study, the carriage of *KIR3DS1* with HLA-Bw4*80I alleles in PLWH was associated with a slower progression to AIDS (265) . However, this finding has been difficult to explain since KIR3DS1 does not bind HLA-Bw4*80I (265).

In summary, co-carriage of genes encoding certain KIRs and their MHC-I ligands is associated with a variety of human disease outcomes. This suggests a role for direct NK cell activity obtained though NK cell education in the control of certain viral infections. Identifying the unknown ligands for KIRs and understanding how their receptor ligand interactions affect NK cell function may provide crucial information required to develop more efficient vaccines and therapies from human health complications.

1.14.3.3.Natural cytotoxicity receptors (NCRs)

NCRs were identified in the late 1990s as activating receptors. NCRs are expressed on the surface of NK cells and some T cell subsets. The NCR family includes NKp46 or CD335, NKp44 or CD336 and NKp30 or CD337 (266). They are encoded by *NCR1, NCR2 and NCR3* genes, respectively.

NKp46 has two extracellular C2-type Ig-like domains with a very short cytoplasmic tail. NKp44 and NKp30 have one extracellular Ig-like domains. The NKp46 and NKp30 cytoplasmic regions lack ITAMs but contain an arginine residue that associates with FcRγ and CD3ζ adaptors molecules that can initiate activating signals (267). NKp30 and NKp46 are expressed on mature resting and activated NK cells. Both NKp46 and NKp30 have reduced surface expression on adaptive NK cells due to the downregulated expression of the FcRγ signaling chain required for the surface expression of these receptors (268, 269).

NCR ligand binding stimulates NK cell cytolysis and secretion of IFN-γ and TNF-α. The expression level of NCRs on NK cells and the presence of their ligands on tumor cells can affect the ability of these effector cells to kill tumour cells. NCRs have been implicated in the clearance of virally infected cells. For example, it has been demonstrated that melanoma cells express ligands for NKp46, which activate NK cells to eliminate these tumour cells. The interaction of NKp46 with its ligands plays an important role in killing HIV, influenza and CMV infected cells (270, 271).

NCRs have been implicated in some autoimmune diseases, such as type 1 diabetes. The ligand for NKp46 is upregulated on pancreatic β cells leading to the migration of $NKp46^{+}$ NK cells into pancreatic islets. Upon binding the NKp46 ligands in islets, $NKp46^{+}$ NK cells degranulate and kill the islets cells, which are a source of the insulin hormone needed to regulate blood glucose levels (272).

Heparan sulfate (HS) glycosaminoglycans are expressed on the surface of cells and play a key role in cell-cell communication and disease. HS glycosaminoglycans is a member of the glycosaminoglycan family, which plays a vital role in tumor progression allowing cancer cells to proliferate (273). All three NCRs have been reported to bind to HS glycosaminoglycans. NCRs possess a distinct HS glycosaminoglycans binding specificity. The affinity of NKp30 and NKp44 binding to HS glycosaminoglycans is higher than that of NKp46. Thus, NK cells may use NCRs to sense changes in HS glycosaminoglycans in the tumor microenvironment that lead to NK cell cytolytic activity that eliminates target cells (274). In conclusion, the identification of NCR ligands has raised their profile as potential targets for clinical applications.

1.14.3.4.NKG2 receptors

NKG2 receptors are members of the C-type lectin receptor (CLR) family. These receptors are expressed on the surface of the human and mouse NK cells and a subset of $CD8^+$ T cells. CLRs are a large group of proteins that are characterized by the presence of one or more C-type lectin-like domains (CTLD) (275), which are a subfamily of lectins that require Ca^{2+} to bind sugar moieties (276). These molecules were originally named for their ability to bind to carbohydrates in a Ca^{2+} dependent manner via conserved residues within the CTLD. CLRs can recognize endogenous and exogenous ligands and contribute to numerous physiological functions. For instance, CLRs can use diverse intracellular signalling pathway to modulate immunological responses.

One of the members of this family is NKG2A. It is expressed as type II transmembrane glycoprotein, which forms heterodimers with CD94 through disulfide bonds (277). See figure 13.1, which shows the structure of NKG2A/CD94 and NKG2C/CD94 receptors and their interaction with HLA-E molecules (278). NKG2A is an inhibitory receptor, which contains two ITIMs in its cytoplasmic tail while NKG2C and NKG2D are activating receptors, which transmit activating signals via adaptor molecules such as DNAX activating proteins DAP10 and DAP12 (279).

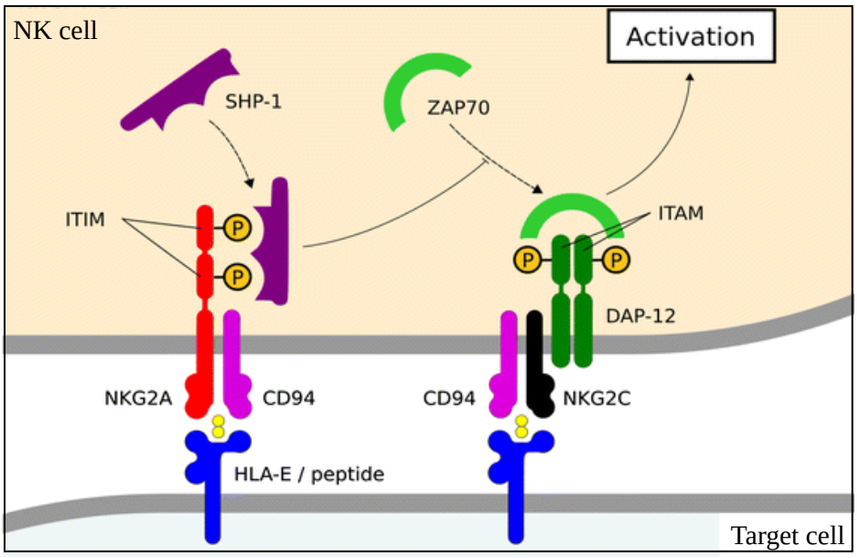

Figure 13.1. NKG2A inhibitory and NKG2C activity signal transduction and regulation in NK cells

NKG2A/CD94, is an inhibitory receptor shown to the left in red and purple. NKG2C/CD94 is an activating receptor shown on the right in purple and black. Both these receptors interact with HLA-E molecules shown in blue complexed with peptides derived from the leader sequence of HLA-A, -B, -C and -G or pathogen derived peptides. The interaction of NKG2C/CD94 with HLA-E/peptide leads to binding of the adaptor molecule DAP12 (dark green) containing ITAMs in its cytoplasmic tail. This triggers recruitment of ZAP-70 (light green) and initiation of the signalling cascade resulting NK cell activation. The interaction of NKG2A/CD94 with HLA-E/peptide promotes the phosphorylation of NKG2A ITIMs, recruitment of SHP-1 proteins (dark purple) that dephosphorylate of ZAP-70 proteins, preventing its binding to ITAM domains and inhibiting the activation pathway (280).

The HLA-E heavy chain forms a complex with beta-2 microglobulin and 9-mer peptides derived from the leader sequence of many HLA-A, -B, -C and -G allotypes (281). Although HLA-I (A, B and C) molecules are downmodulated in some viral infections including HIV, in some cases such as in CMV infection, HLA-E expression is maintained on the surface of infected cells due to binding peptides derived from CMV encoded UL40 in order to avoid NK cell recognition through NKG2A; this favors NK cell cytolysis (282).

CMV infection expands a population of NKG2C^{+} NK cells, that are negative for the inhibitory NKG2A/CD94 receptor (283). This NK cell subset has memory-like properties and are called adaptive NK (adapNK) cells. The adapNK cells can be activated via the interaction of NKG2C with HLA-E complexed with peptides derived from the CMV UL40 gene product (284). AdapNK cells will be discussed more thoroughly in a later section of this thesis.

Natural killer group 2 member D (NKG2D) is an activating receptor that is expressed on the surface of NK cells. NKG2D uses certain UL16 binding proteins (ULBP) and MHC class I polypeptide-related sequence A (MICA) and MICB, which are not expressed on healthy cells, but are upregulated on virus- and bacteria- infected cells as ligands (285, 286). Engagement of NKG2D by their ligands on target cells can directly activate NK cells inducing cytokines and perforin/granzyme-mediated cytotoxicity leading to killing of virus infected cells (221).

1.15. Adaptive NK (AdapNK) cells

NK cells are innate immune lymphocytes, which provide cytokines critical for early host defenses against pathogens. However, recent studies showed that some NK cells can have adaptive immune features such as long-term persistence, virus induced clonal expansion and epigenetic modifications (287).

1.15.1. NK cell adaption to murine (mCMV)

During an infection, naïve T cells respond to pathogens by becoming activated and by proliferating to form a larger sized clone of antigen specific effector cells (288). Subsequently, the effector population contracts as activated T cells undergo apoptosis and cell numbers drop precipitously (289). The third phase of a response to infection is the memory maintenance phase in which long-lived memory cells patrol for the previously encountered antigen (290). The fourth phase occurs when memory cells reencounter their cognate antigen and undergo a recall response by rapidly and robustly re-expanding and developing into effector cells (291). NK cells share many features with CD8^{+} T cells (221, 268, 292). The mouse model of mCMV infection in

C57Bl/6 mice was used to illustrate that NK cells can protect against mCMV infection by undergoing these four phases as well. This work was used to show that NK cells can also exhibit memory-like features. Upon mCMV infection, C57Bl/6 mice generate a response dominated by NK cells expressing the NKR Ly49H, which recognizes mCMV encoded m157(293-295). This response allows C57Bl/6 mice to survive mCMV infection. Within 1 week of mCMV infection, $Ly49H^+$ NK cell numbers increased by 2-3-fold in the spleen and 10-fold in the liver (293-295). By 15 to 28 days post infection, the frequency of $Ly49H^+$ NK cells return to pre-infection levels. These $Ly49H^+$ NK cells can be rapidly induced to degranulate and secrete cytokines upon antigen reencounter *in vitro*. Furthermore, adoptive transfer of $Ly49H^+$ NK cells into newborn mice, which are susceptible to mCMV infection because they lack mature NK cells, protected them from infection with mCMV compared to neonatal mice receiving naive NK cells. This showed that NK cells have the ability to remember the previous pathogen by providing protection against subsequent mCMV infections (295) .

1.15.2. NK cell adaptation to human CMV (henceforth CMV)

Adaptive NK (adapNK) cells are also expanded following CMV infection in humans. Despite evidence that NK cells control multiple types of herpesvirus infections, only CMV infection is known to have an effect on the composition of the NK cell repertoire (296). In 2004, Guma et al. was the first to report that CMV infection leaves a durable imprint on the human NK cell repertoire (297). This imprint is reflected by an increased frequency of NK cells expressing the activating NKG2C receptor and CD57, which is a maturation marker and CD16. Since then, expansion of $NKG2C^+$ NK cells have been reported in various human disease settings such as infection with HIV, hantavirus, chikungunya virus, hepatitis B and C viruses (298-301). However, all individuals with these infections who had an expanded population of $NKG2C^+$ NK cells were also CMV co-infected, and it is accepted that the expanded population of these cells was due to CMV infection and not to co-infection with another pathogen. Furthermore, the expanded population of $NKG2C^+$ NK cells persist throughout life in CMV infected individuals, while the frequency of these cells remains low in individuals who are not CMV infected (302). See figure 14.1.

Education by iKIRs appears to be involved in the clonal expansion of adapNK cells as they often express one or more iKIR to self MHC-I (303). AdapNK cells can also express aKIRs in addition to NKG2C (303). Levels of NCRs and NKG2A are reduced (297, 299, 302, 304-307). There is substantial overlap between the population of NK cells co-expressing NKG2C and CD57 with NK cells that have reduced expression of the FcRγ signaling protein, Syk, promyelocytic leukemia zinc finger transcription factor (PLZF) and Ewing's sarcoma's/FLI-1 activated transcript-2 (EAT-2) (268, 269). This association between selective expansion of NK cells bearing the activating receptor NKG2C and CMV infection raised the question as to whether NKG2C might be analogous to Ly49H with its specific recognition of a mCMV encoded protein or peptide that drives the expansion of $NKG2C^+$ NK cells.

The expansion of $NKG2C^+$ NK cells was observed in multiple scenarios, for instance during primary CMV infection in transplant patients, in responses to CMV-infected fibroblasts and in responses to CMV UL-40 derived peptide (284, 297, 306, 308-310). The mechanism by which CMV drives the expansion of $NKG2C^+$ NK cells population is not fully understood. CMV infected cells express HLA-E, which is stabilized by peptides derived from CMV UL40 protein. CMV strain variants carry different UL40 peptides, which display different binding affinities for HLA-E. These binding affinities may affect NK cell proliferation and effector functions. Consequently, the interaction between NKG2C receptors and its HLA-E ligand on the surface of CMV infected cells are thought to be important in NK cell expansion (284, 311). See figure 14.1.

1.15.3. Functional characteristic of adapNK cells in human

$NKG2C^+$ adapNK cells mediate enhanced ADNKA activity (268, 312-314). This adapNK cell population downregulates activating receptors such as NKp46 and NKp30 (315). As a result, these adapNK cells exhibit less effective natural cytotoxicity (312). AdapNK cells also demonstrate higher levels of Ab dependent IFN-γ secretion due to epigenetic remodeling similar to what occur in CMV-specific $CD8^+$ T cells (316). The IFN-γ promoter region is hypomethylated, which leads

to quicker and more accessible stimulation of IFN-γ production. This enables NK cells to adopt adaptive features despite their innate nature (316).

Although NKG2C is an effective marker for NK cell adaptation to CMV infection, its role in *in vivo* protection against CMV has not been confirmed. In fact, in 2008, Kuijpers at al. reported the first case of a T cell-deficient three-month-old girl who had a family history of febrile episodes. The child was admitted to the hospital for gastroenteritis, and she was discharged after the fever resolved. Laboratory results confirmed that the child's clinical symptoms were caused by CMV. Investigators observed three phenomena in this case: 1) the frequency of lymphocyte subsets were abnormal. Ninety percent of the lymphocytes were $CD56^{dim}$ NK cells, 10% were B cells and T cells were absent. 2) The frequency of the NK cells was highest at the peak of CMV VL. 3) high expansion of $NKG2C^{+}$ NK cells with high levels of CD16 expression were observed. This information was interpreted as evidence that $NKG2C^{+}CD16^{+}$ NK cells can effectively control CMV infection in the absence of T cells (317). It should be noted that expansion of $NKG2C^{+}$ adapNK cells does not occur in all CMV seropositive individuals (297, 305, 318). The reason for this is unknown.

1.15.4. CD2 expression on adapNK cells

CD2, which is a co-stimulatory receptor on NK cells is also expressed on adapNK cells. The interaction of CD2 on NK cells with its CD58 ligand, expressed on CMV infected cells contributes to adapNK cell activation (319). See figure 14.1. In a co-culture system, the production of IFN-γ and TNF-α by adapNK cells diminished after blocking this interaction with anti-CD2 or anti-CD58 mAbs (319). Moreover, adapNK cells also expanded in CMV infected $NKG2C^{-/-}$ carriers (307). These adapNK cells from $NKG2C^{-/-}$ carriers had a higher expression of CD2 than those $NKG2C^{+}$ carriers. Although signaling via CD2 has little effect on adapNK cells activation, it synergizes with CD16 signaling leading to NK cell activation to secrete IFN-γ and TNF-α (307).

1.15.5. AdapNK cells express low levels of NKG2A, FcRγ, Syk, and EAT-2

The NKG2C^{+} adapNK cells are mostly cell surface NKG2A negative. AdapNK cells undergo DNA methylation-dependent epigenetic modifications, which distinguish them from conventional NK (cNK) cells and influence their functionality (268, 269). NKG2C^{+} adapNK cells characterized by the downregulation of certain intracellular signaling proteins such as FcRγ, Syk, PLZF and EAT-2 (268, 269, 313, 320). Among these proteins, FcRγ downregulation was the most pronounced in adapNK cells (268). The FcRγ adaptor protein associates with CD16a homodimers or heterodimers with CD3ξ to signal via ITAMs (292, 321). The loss of FcRγ in adapNK cells, has an impact on CD16 signaling resulting in enhanced ADCC and ADNKA activities (313). See figure 14.1.

1.15.6. NKG2C^{+}CD57^{+} adapNK cells and CMV infection

In C57Bl/6 mice, the Ly49H^{+} receptor on adapNK cells subset interacts with the mCMV encoded gene product m157 (322). MCMV infection leads to the expansion and subsequent contraction of these LY49^{+} NK cells (293, 294). Adoptive transfer of these cells to mCMV naïve newborn mice protects them from death upon mCMV infection (295). What is known about whether NKG2C^{+}CD57^{+} adapNK cells exhibit specificity and memory against human CMV? Although the expansion of NKG2C^{+}CD57^{+} adapNK cells was observed in *vivo* and *in vitro* during CMV infection, it is unclear if these human adapNK cells can protect against CMV infection in humans. Guma´ et al. found that the expansion of NKG2C^{+} NK cells was detectable when PBMCs from CMV^{+} subjects were cocultured with CMV infected fibroblasts (299). Hammer et al. found that the interaction of NKG2C with CMV UL40 encoded peptides complexed with HLA-E on CMV infected cells stimulated NKG2C^{+} NK cells to proliferate, secrete cytokines/chemokines and externalize CD107a (284). Co-stimulation through CD2 contributes to NKG2C^{+} NK cell activation, particularly for UL40 peptide variants with a lower avidity for NKG2C (284). While it is still unclear whether adapNK cells control CMV infection the NKG2C receptor on these cells clearly interacts with CMV encoded epitopes (284).

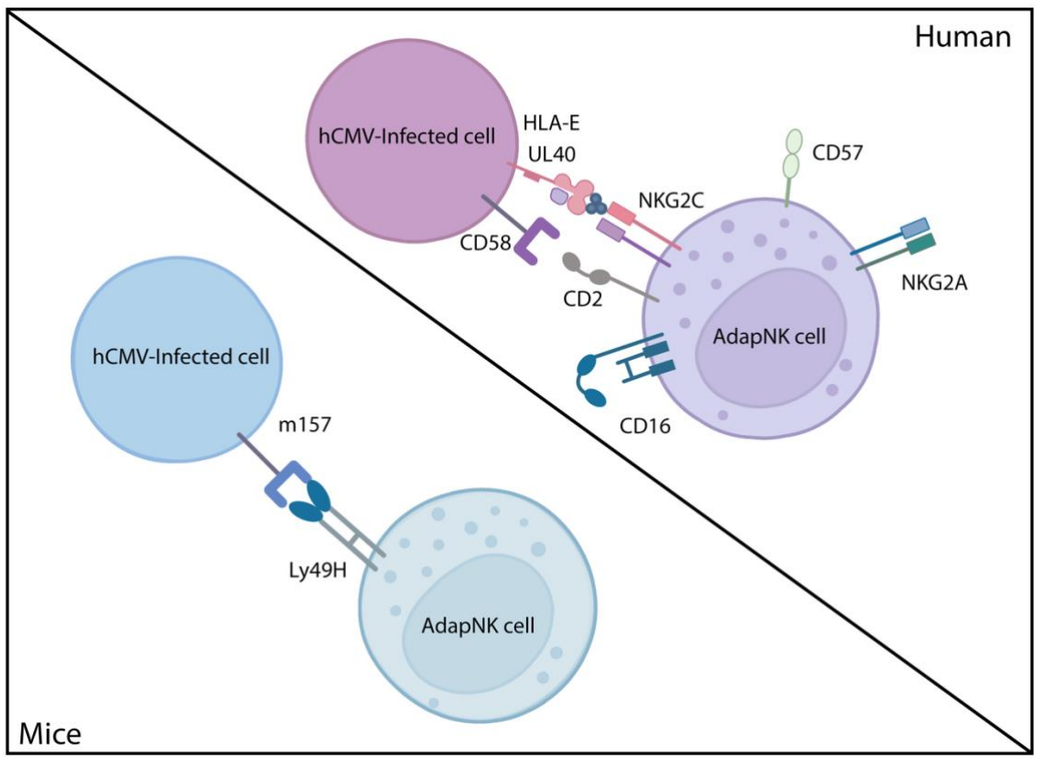

Figure 14.1. Adaption of NK cells in response to mCMV and human CMV

In mice, adapNK cells (bottom left) are generated in response to interactions between mCMV encoded m157 protein and the activating Ly49H receptor on NK cells. In humans (top right), CMV infection increases the frequency of NK cells expressing NKG2C and CD57 possibly through the recognition of peptide derived from CMV encoded UL40 protein complexed to HLA-E. Increased NKG2C expression on adapNK cells is paired with loss of NKG2A. AdapNK cells can be further identified by the loss of the PLZF transcription factor and the FcRγ signaling adaptor molecule, which favors the use of the CD3ξ signaling adaptor molecule in CD16 signaling. (Made by K Alsulami using BioRender.com).

1.16. A ROLE FOR NK CELLS IN HIV PROTECTION AND CONTROL

The Bernard lab has shown that HESN individuals, compared to recently infected (and therefore susceptible) PLWH, have a significantly higher frequency of the *iKIR* allele *KIR3DL1*h* co-carried with *HLA-B*57* (323). KIR3DL1*h receptors are expressed at high levels on NK cells (172). HLA-B*57 binds to KIR3DL1 with a high avidity leading to potent education of NK cells expressing KIR3DL1 (172, 173). The more potently educated an NK cell is, the more activated it will become when it encounters an HIV infected cell that has downmodulated MHC-I antigens through the action of HIV NEF and VPU (46). Activated NK cells secrete IFN-γ, TNF-α, degranulate and secrete chemokines such as CCL3, CCL4 and CCL5 (213). All of these have anti-HIV activity (183, 263, 264, 324-327). HESN, compared to recently infected PLWH, also have a

significantly higher frequency of *KIR3DS1* homozygotes compared to recently HIV infected individuals (261). The ligand for the KIR3DS1 is the activating receptor HLA-F, which is expressed on HIV infected cells (259). The interaction of KIR3DS1 on NK cells with HLA-F on HIV infected cells activates these NK cells (227, 262). As NK cells are primed to respond upon encountering HIV infected $CD4^+$ T cells with reduced ligands for inhibitory receptors on educated NK cells (as is the case for the *KIR3DL1*h+HLA-B*57* combination) or encountering ligands for activating receptor (as is the case for the *KIR3DS1+HLA-F* combination) they can rapidly respond to HIV infected cells by eliciting functions with anti-HIV activity and reduce the chance that a productive HIV infection will become established.

The *KIR2DS4* locus encodes activating KIR2DS4*001-like receptors, or KIR2DS4*003-like receptors that are truncated and not cell surface expressed (328, 329). *KIR3DS1* homozygotes are usually homozygous for a telomeric KIR haplotype B motif known as TB01, which co-carries genes for KIR2DL5A, KIR2DS1 and KIR2DS5 and no locus encoding expressed KIR2DS4 allotypes. This TB01 motif and homozygosity for *KIR3DS1* are associated with protection from HIV infection as they are more frequent in HESNs than in HIV susceptible persons (161). On the other hand, the full length KIR2DS4*001 activating NK cell receptor is associated with more rapid HIV transmission in a Zambian cohort of HIV discordant couples (330). Carriage of *KIR2DS4*001* was also associated with worse prognostic factors (higher HIV VL and lower CD4 counts) in a cohort of HIV-infected, largely African American youth (330, 331). Although absence of KIR2DS4*001 in TB01 motifs is associated with protection from infection while its presence is associated with more rapid HIV transmission and worse prognosis in PLWH, the mechanism underlying these associations are not fully understood.

Another subset of NK cells likely plays a role in the context of HIV infection. Most PLWH are also CMV co-infected (332). CMV infection expands a population of NK cells that express the activating receptor NKG2C (297, 299). Some individuals do not express cell surface NKG2C due to a homozygous deletion in a genomic region that includes *NKG2C*, the gene encoding NKG2C (333, 334). The frequency of this deletion variant of *NKG2C* is around 20% in several populations such that the frequency of the $NKG2C^{-/-}$ genotype is these populations is approximately 4% (307, 334-337). CMV infection also expands the frequency of NKG2C NK

cells having an epigenetic footprint similar to that in NKG2C^{+} NK cells in *NKG2C*$^{-/-}$ individuals (307). Several studies have investigated whether NKG2C^{+} NK cells play a role in protection from HIV infection and slower HIV disease course in those infected. Thomas et al. reported that the frequency of carriers of an *NKG2C*$^{-}$ allele was higher in PLWH than in uninfected individuals with a low risk for HIV infection (338). They also found that a higher percentage of *NKG2C*$^{+}$ homozygotes than carriers of the *NKG2C*$^{-}$ variant had pre-treatment VLs of <30,000 copies/ml of plasma in a small group of 7 *NKG2C*$^{+}$ homozygotes, the frequency of NKG2C^{+} cells was positively correlated with HIV VL (338). Two other studies found that the frequency of NKG2C^{+} cells was negatively correlated with HIV VL in early infection (283, 339).

In chapter II of this thesis, I investigated the influence of NKG2C genotype distribution in PLWH and HESN individuals to determine whether there was a role for NKG2C in protection from HIV infection and outcomes in those infected (340). I investigated the link between *NKG2C* genotypes and 1) HIV susceptibility/protection in 434 PLWH and 157 HESN participants and 2) levels of untreated VL set point in untreated PLWH. The proportion of *NKG2C*$^{+/+}$ and *NKG2C*$^{+/-}$ carriers did not differ between PLWH and HESN subjects. However, while *NKG2C*$^{-/-}$ carriers were present among PLWH, this genotype was absent in HESN subjects suggesting that carriage of this genotype was associated with decreased HIV susceptibility. VL set point in untreated PLWH is a predictor of the rate of treatment-naïve HIV disease progression (134). There was no significant difference in the VL set points among PLWH carrying the 3 *NKG2C* genotypes (340) Thus, the *NKG2C*$^{-/-}$ genotype was associated with higher susceptibility to HIV infection. Although, *NKG2C* copy number was associated with the frequency and intensity of NKG2C expression on NK cells, neither of these parameters correlated with HIV VL set point.

1.17. THE ROLE OF NK CELLS IN CVDS

NK cells have been reported to play a role in CVDs, and to be involved in diverse manifestations of CVD such as atherosclerosis (AS), myocardial infarction (MI) and cardiac fibrosis (341-343). It is important to acknowledge that understanding the role of NK cells in AS is challenging due to the difficulties accessing clinical samples from healthy controls and CVD patients. Mouse CVD models may be worth studying but none of these models have NK cell deficiencies. Although several studies have evaluated the functions of NK cells in experimental animal models of AS, it is not yet clear whether NK cells behave as protective or proatherogenic effectors.

The first report showing that NK cells are present in the carotid artery was published in 2005 by Bobryshev and Lord (344). The carotid artery specimens were obtained from patients aged between 59 and 73. By using anti-CD56 and anti-CD3 Abs, they identified $CD56^{+}CD3^{-}$ NK cells in atherosclerotic plaques. They detected interactions between NK cells and macrophages in plaque using an electron microscope. Hak et al. measured circulating NK cell subset numbers and frequencies and the ability of these cells to respond to the HLA null cell line K562 by secreting IFN-γ and exhibiting cytolytic activity in coronary heart disease (CHD) patients and negative controls (345). Total $CD56^{+}CD3^{-}$ NK cells and $CD56^{dim}CD3^{-}$, but not $CD56^{bright}CD3^{-}$ NK cell frequency, was significantly lower in the circulation of CHD patients than in the negative control group. The frequency of peripheral NK cells with K562 stimulated cytolytic activity was lower in CHD patients than in controls. The frequency of NK cells able to secrete IFN-γ showed a non-significant trend towards being lower in CHD patients than in controls suggesting that circulating NK cells may be dysfunctional in CHD patients (345). Similar results were shown for NK cell frequency and the frequency of NK cells with K562 induced cytolytic activity in patients with AS versus healthy controls (343). Backteman et al. studied AS patients with non-ST-elevation MI (non-STEMI), which is a type of heart attack that occurs due to insufficient blood flow and oxygen supply to the heart (346), patients with stable angina and healthy controls. All patients were receiving aspirin, β-blockers, calcium-antagonists and statins, which are medications to lower blood pressure (BP) and cholesterol, respectively. They found that NK cell frequencies, but not absolute numbers, were significantly lower in both patient groups compared to controls. Neither the frequency of $CD56^{dim}$ and $CD56^{bright}$ NK cells nor the

cytokine profile of stimulated NK cells differed between patients with acute versus stable AS (347). NK cell frequency failed to recover in patients who maintained markers of low-grade systemic inflammation while they did recover to levels similar to those in control patients by 12 mo of follow up in patients with no evidence of systemic inflammation (347). This suggests that a low frequency of circulating NK cell is a marker of poor prognosis in the context of AS.

Bonaccorsi et al. examined NK cells in carotid atherosclerotic plaques (CaAP), obtained by endarterectomy from asymptomatic (aCaAP) and symptomatic (sCaAP) patients. They observed that the frequency of total and $CD56^{bright}$ NK cells and the frequency of those producing IFN-γ were significantly higher in plaque from sCaAP compared to aCaAP patients and controls (348). The NK cells within plaques expressed several activating receptors whose ligands were potentially present on macrophages. In another study of 124 patients diagnosed with AS, there was an increased frequency of NK cells in the patients with complications after carotid endarterectomy (349). The implications of these studies performed on plaques was that NK cells and macrophages interacted in a manner where ligands on macrophages interacted with activating receptors on NK cells, activating NK cells to secrete IFN-γ, which contributes to NK cell/macrophage crosstalk activating both cell types and increasing inflammation and immune activation (IA). Since NK cells were more abundant in plaques from patients with a poor prognosis sCaAP versus aCaAP and patients with complications following an endarterectomy versus not, NK cells may contribute to the AS process.

In conclusion, the role of NK cells in AS is not entirely clear. Further studies, on good animal models and translation into human studies are necessary to clarify the contribution of NK cells to the progression of inflammation in the context of CVDs.

1.18. CARDIOVASCULAR DISEASE (CVD) IN PLWH

With the availability of ART to treat PLWH, HIV infection has become a chronic disease. The life expectancy of treated PLWH now approaches that of the uninfected population. However, PLWH on-ART experience chronic inflammation and IA that drives an elevated risk of several conditions, including CVDs such as AS, MI and stroke (350-353). CVD is an umbrella term that encompasses diseases of the heart and blood vessels. When describing CVD in this thesis, the term will apply mainly to atherosclerotic, cardiovascular/coronary heart disease such as MI, and cerebrovascular disease including ischemic and hemorrhagic strokes, unless otherwise specified. Several studies have investigated the increased risk of CVD in PLWH, but less is known about the exact mechanisms involved in this increased risk. In 2018, a total of 80 studies were included in a meta-analysis of 793,635 PLWH followed 3.5 million person-years (PY). The overall conclusion of this study was that PLWH were twice as likely to develop CVD than HIV uninfected persons (354).

PLWH have a greater risk of MI compared to HIV uninfected people (figure 15.1). The manifestation of MI is even more frequent in PLWH who are receiving ART (355-361). HIV infection can be considered an independent CVD risk factor (359).

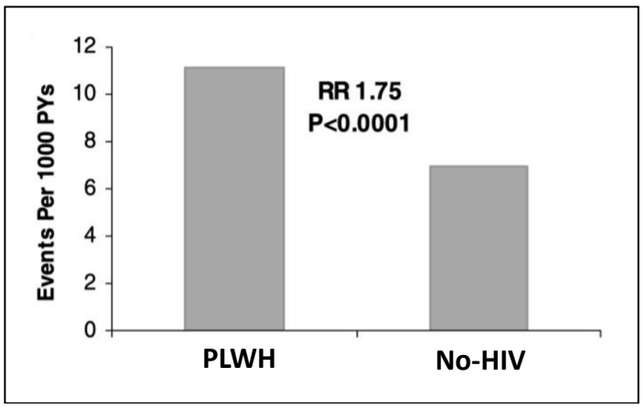

Figure 15.1. Difference in the rate of MI between PLWH and HIV seronegative individuals

The y-axis shows that the number of MI events per 1000 PYs (359)**.** The MI rate is significantly higher in PLWH (relative risk [RR] 11.13, 95% confidence intervals [CI] 9.58-12.68) compared with persons who are not HIV-infected (RR 6.98, 95% CI 6.89-7.06) per 1000 PYs.

MIs, or “heart attacks” or “ischemic heart disease” make up the largest proportion of CVDs. AS is a main cause of MIs. AS occurs when myocardial cells die due to prolonged ischemia, which can occur when a blood clot forms inside the coronary artery and reduces the blood flow to the heart. Lymphocyte and monocyte activation play an essential role in AS. T cell activation is significantly elevated in PLWH and is one of the most important CVD risk factors. (362-364). The term AS comes from the Greek words athero meaning gruel or porridge and sclerosis meaning hardening (365). The hardening comes from fibrosis layers that consist of smooth muscle cells (SMC), leukocytes, and connective tissues (366, 367). AS is a chronic inflammatory disease of large and medium-sized arteries that leads to ischemic heart disease, stroke, and peripheral vascular disease (368). The AS process is initiated when fat accumulates in the inner layers of arteries, forming plaque. Plaque consists of low-density lipoprotein (LDL), connective-tissue elements (fibers and cells), debris and immune cells (369). As a consequence of the growth of these plaques there occurs a bulge inside the arteries ultimately reducing the blood flow (367). Blood clots can then occur, which further block blood flow leading to heart cell damage due to lack of oxygen. Figure 16.1 illustrates conditions that predispose to MIs. Plaque rupture can eventually lead to death (https://www.medline.ca, 2022).

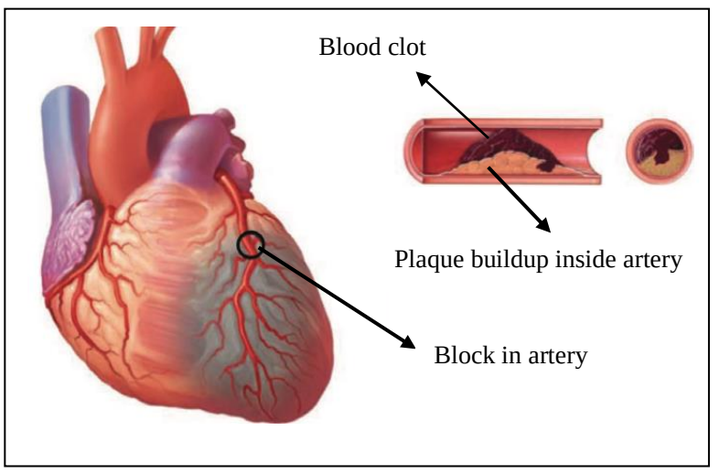

Figure 16.1. Schematic representation of MI or heart attack that occur when the heart coronary artery is blocked

(https://www.elitecardiovascular.com/cardiovascular-conditions/cardiac-conditions/myocardial-infarctions-heart-attacks/, n.d.)

MI can be prevented by controlling diet and lowering traditional CVD risk factors such as smoking, cholesterol, BP, and body weight. One of the immediate treatments to prevent MI is taking aspirin, which inhibits blood clotting and nitroglycerin, which expands blood vessels to supply the heart muscle with more blood (370). According to the European Society of Cardiology, the American

College of Cardiology, and the American Heart Association, MIs can be detected by serum elevations in cardiac enzymes such as troponin, creatine kinase (CK-Mb), heart-type fatty acid binding protein, lactate dehydrogenase, and copeptin. These enzymes are well-established biomarkers due to their high sensitivity and specificity (371).

It is important to highlight that coronary artery disease (CoAD) and carotid artery disease (CaAD) are both atherosclerotic conditions, which appear when plaque builds up inside the heart and brain arteries, respectively. Coronary and carotid arteries are large vessels that are responsible for transporting large blood volumes to the heart and the brain, respectively (372). The biology of the atherosclerotic process is similar in CoAD and CaAD. However, there are differences in plaque morphology and characteristics. Carotid plaques are characterized by a thicker fibrous cap, higher intraplaque hemorrhage, calcified nodules, and lower plaque erosion compared to coronary plaques (373). Plaque rupture in either the coronary or carotid arteries can be determined by fibrous cap thickness. Fibrous cap formation arises from the accumulation of SMCs, connective tissue, lipid, and inflammatory cells such as macrophages and monocytes (374, 375). According to an autopsy study of sudden cardiac death, the average thickness of ruptured caps was 23 μm, and approximately 95% of ruptured fibrous caps were <65 μm thick (376, 377). Furthermore, based on autopsy studies, plaques can be divided into two types: stable plaques, which are less likely to rupture due to the thick fibrous cap and a small lipid core and unstable plaques, which have a thin fibrous cap and substantial core. Unstable plaques have a high risk of rupturing. Information on plaque stability is an important determinant that aids physicians in deciding whether patients need surgery or not (378-380). In chapter III of this thesis, I investigated links between adapNK cell frequencies and the total plaque volume of coronary artery plaques using computed tomography (CT) imaging to measure this parameter inside the heart's arteries.

1.19. IA, INFLAMMATION, AND CVD IN PLWH

Inflammation is a risk factor for CVDs in the general population. IA is a characteristic feature of HIV infection that drives disease pathogenesis. PLWH have higher levels of IA than HIV uninfected people. In untreated PLWH, an IA setpoint can be established from early after infection. This IA setpoint is associated with the rate of CD4 decline (381). Taking ART decreases IA levels through they remained higher in PLWH on-ART than in HIV uninfected people (382). Consequently, PLWH have an elevated risk of CVDs, including AS, MIs and carotid atherosclerosis (CaAS). The mechanism underlying AS in PLWH is complex and not well understood. It likely includes an interplay between HIV-related features such as $CD4^+$ T cells and detectable VL, ART, and traditional cardiovascular risk factors (TCRF). The contribution of these factors to the pathogenesis of cardiomyopathy in PLWH will be discussed in this thesis.

In the literature, low $CD4^+$ T cell counts, low CD4/CD8 ratio and detectable HIV VL are associated with a high risk of MIs in PLWH (360, 383-385). In 2017, Drozd et al. studied PLWH on-ART who were above 40 yrs of age. In this study, MIs were identified by measuring levels of the cardiac enzyme troponin-I, troponin-T, and creatine kinase MB. Increases of one or two of these enzymes in the patient's serum was used as evidence that an MI had occurred (386). Using a Poisson multivariable regression between the rate of MIs and adjusted CVD risk factors, CD4 count, and HIV RNA VL, they found that the rate of MIs in PLWH was associated with low CD4 counts and detectable VL (360). This observation suggested that early ART initiation to suppress VL and increase $CD4^+$ T cell counts would reduce MI risk in PLWH.

AS is also found in ECs. ECs have a higher rate of coronary plaques and IA markers than HIV uninfected individuals, (78% vs 42%), respectively (387). A higher frequency of ECs developed CVDs than did PLWH receiving ART (388). ECs had a higher level of IA as determined by levels of soluble CD163 (sCD163), an activation marker expressed on macrophages and monocytes, than HIV uninfected people (389). High expression of sCD163 is associated with the presence of coronary plaque in PLWH (390). This observation led to the recommendations that ECs be treated to reduce IA, which in turn would reduce complications due to CVDs (387, 391, 392).

In 2019, Subramanya et al. studied the association between inflammation, IA levels, and CaAS in PLWH (393). They investigated inflammation and IA levels by measuring biomarkers such as C-reactive protein (CRP), IL-6, tumor necrosis factor (TNF) receptor 1 (TNFR1) and TNFR2, monocyte activation markers (CCL2, sCD163, sCD14), coagulation markers (fibrinogen, D-dimer), and endothelial dysfunction markers (intracellular adhesion molecule 1 [ICAM-1] in the presence of AS. After adjusting for CVD risk factors, some of these biomarkers were elevated in the presence of CaAS. For example, CCL2, sCD163, CRP, IL-6, and fibrinogen were higher in PLWH than HIV-uninfected people (393, 394). Other studies also demonstrated that D-dimer and CRP were increased in untreated compared to treated PLWH (394, 395). Furthermore, it was shown that lymphocyte and monocyte activation played an essential role in AS. T cell activation is one of the most important hallmarks of CVDs risk factors that are significantly elevated in PLWH.

Certain classes of ART are associated with an increased risk of MIs. ART regimens that include older drugs, particularly NRTIs such as nelfinavir (NFV), lamivudine (3TC), zidovudine (ZDV) and abacavir (ABC), and PIs such as ritonavir (RTV) and lopinavir (LPV) were associated with an increased risk of MIs (357, 396-398). INSTIs have been associated with weight gain but their role in the development of CVDs is as yet unknown (399). These NRTI based regimens induced mitochondrial toxicity that caused mitochondrial dysfunction. Mitochondria are involved in oxidative phosphorylation, which produces adenosine triphosphate (ATP) by using energy released by the oxidation of the food we eat. ATP is an important source of energy for most biochemical and physiological processes including cell function (400). As a consequence of mitochondrial dysfunction, less ATP is generated and reactive oxygen species (ROS) are produced, which can be involved in the development of CVDs such as heart failure (401). PI containing ART regimens that include RTV are associated with hypertriglyceridemia, which is a high level of total cholesterol (TC) and triglycerides (TG) in the blood. Such regimens have been implicated in elevating the risk of AS (396, 402). The risk of MIs in PLWH has decreased following the incorporation of new drugs into ART regimens, including maraviroc (MVC) and dolutegravir (DTG) from the IN and INSTI ART drug classes, respectively (403, 404). Taken together, the current ART regimens, compared to the older regimens, have reduced CVD risk.

Microbial translocation (MT) occurs following the depletion of mucosal $CD4^{+}CCR5^{+}$ by HIV in the acute phase of infection (405). Following this initial assault on the gut mucosa, bacteria, viruses, fungi and other pathogens and their products that should remain in the gut lumen, translocate across the damaged tight junction epithelial barrier into the blood circulation. These pathogens and their products are recognized as foreign by the immune system leading to IA driven pathogenesis (406). In HIV infection, T cells, monocyte and macrophages are activated (407). Markers of MT such as lipopolysaccharides (LPS) and soluble CD14 (sCD14), which are monocyte activation markers are found at higher concentrations in the plasma of PLWH than in uninfected persons. Elevated plasma LPS and sCD14 are associated with higher rates of CVDs and carotid artery intima-media thickness in PLWH (408). Although ART suppresses VL to undetectable levels, LPS levels remain higher in PLWH than HIV uninfected people (405). The GI tract is home to trillions of bacteria, making up the healthy gut microbiome. These bacteria are beneficial to human health because they digest complex carbohydrates into short-chain fatty acids, which are a source of energy. However, some of the bacteria in the GI tract produce trimethylamine-N-oxide, which is a small molecule metabolite that is associated with an increased risk of AS, MI, thrombosis and stroke when present at high levels (409).

In summary, there is a clear association between HIV infection and the development of higher rates of CVDs earlier in life than occurs in HIV uninfected persons. The exact mechanisms that underlie this association are less clear but likely involve an interplay between TCRFs, ART and other HIV related features such as CD4 count and VL. Figure 17.1 summarizes the pathology of CVD in PLWH.

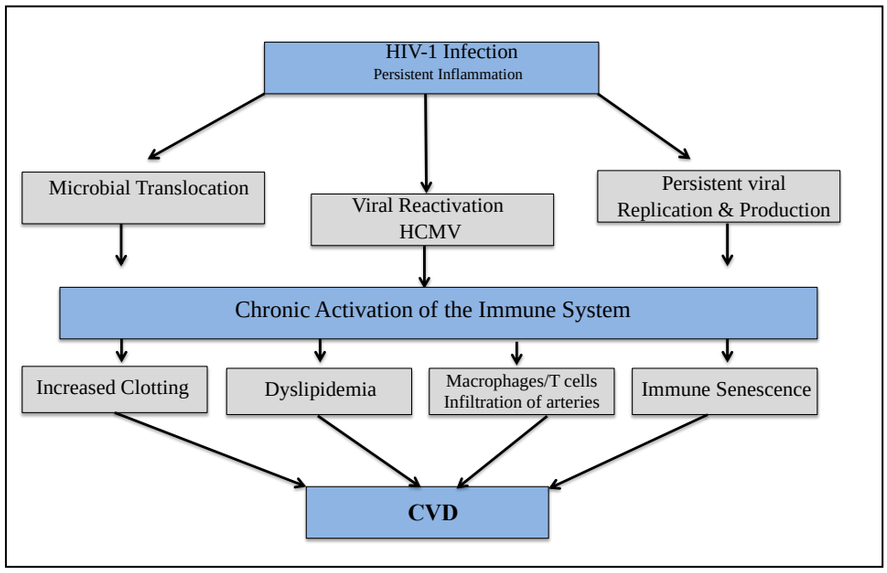

Figure 17.1. The diagram shows the association between HIV-1 disease and CVDs through persistent inflammation

The impact of HIV is multifactorial which includes direct effects of HIV and CMV viral replication, gut microbial translocation due to loss of mucosal integrity as consequences of immunodeficiency. These factors are associated with the chronic inflammation, which leads to increased clotting, dyslipidemia and immune senescence, ultimately causing CVDs. Figure adapted from Hsue et al (410).

Immunohistochemistry and electron microscopy have been used to visualize NK cells in plaques from human atherosclerotic lesions in carotid arteries obtained by endarterectomy (344). These NK cells were in direct contact with macrophages in these lesions. NK cells are a critical component of the innate immune response. Activated NK cells produce cytokines such as IFN-γ, which play a major role in activating monocyte and macrophages (411, 412). It has been suggested that production of cytokines and chemokines in atherosclerotic lesions may be involved in NK cell migration into the atherosclerotic plaque. In turn, macrophages activate NK cells by producing IL-12 and IL-18 or directly through cell-cell contact. These cytokines are NK cell chemo-attractants. Consequently, these cytokines enhance NK cell crosstalk with immune cells such as monocyte, macrophages, and dendritic cells, which promote the atherogenic process (413-416). In summary, NK cells are present in atherosclerosis plaques. However, what their role is in AS and MI is presently unknown as they could be anti-atherogenic or pro-atherogenic. In chapter III of this thesis, I investigated whether the frequency of NK cells, in particularly adaptive NK (adapNK) cells was associated with total plaque volume (TPV) as a pre-clinical marker of AS in CMV infected PLWH.

1.20. TRADITIONAL CARDIOVASCULAR RISK FACTORS (TCRF) IN PLWH

The risk factors contributing to the development and progression of CVDs are categorized into TCRF and non-TCRF factors (417). Modifiable TCRF include smoking, dyslipidemia, diabetes mellitus and hypertension. Non-modifiable TCRF include age, gender and family history. The role of TCRF and non-TCRF have been demonstrated in both PLWH and in HIV uninfected populations. In PLWH, pathogenesis may be accelerated due to increased IA that drives HIV pathogenesis. Althoff et al. studied a large cohort of more than 180,000 PLWH in the US and Canada aged >18 yrs. The study's aim was to investigate the prevalence of modifiable and non-modifiable TCRF in PLWH. They found that participants with MIs were more likely to have hypertension, obesity, diabetes, and hyperlipidemia than those without MIs (418). Touloumi et al. studied the rate of TCRF in 5839 PLWH and 4820 HIV uninfected participants and demonstrated that both groups had comparable rates after adjusting for age and sex. They found that PLWH were more likely to be current smokers and have dyslipidemia than the control group (419). According to the nurse-led intervention to extend the HIV treatment cascade for CVD prevention (EXTRA-CVD) trial, although PLWH knew their VL and $CD4^+$ T cell count, they underestimated their risk of CVDs. It is important to focus on preventing TCRF in PLWH who have achieved viral suppression that would decrease CVDs (420). In addition to the standard TCRF, PLWH have HIV-specific and ART related risk factors. HIV-specific factors include chronic inflammation, IA and CMV co-infection (421, 422). Furthermore, as discussed in section 18.1, certain older ART drugs such as ABC and PIs were associated with increased CVD risk (397). Figure 1.18 shows a list of TCRF and non-TCRF factors.

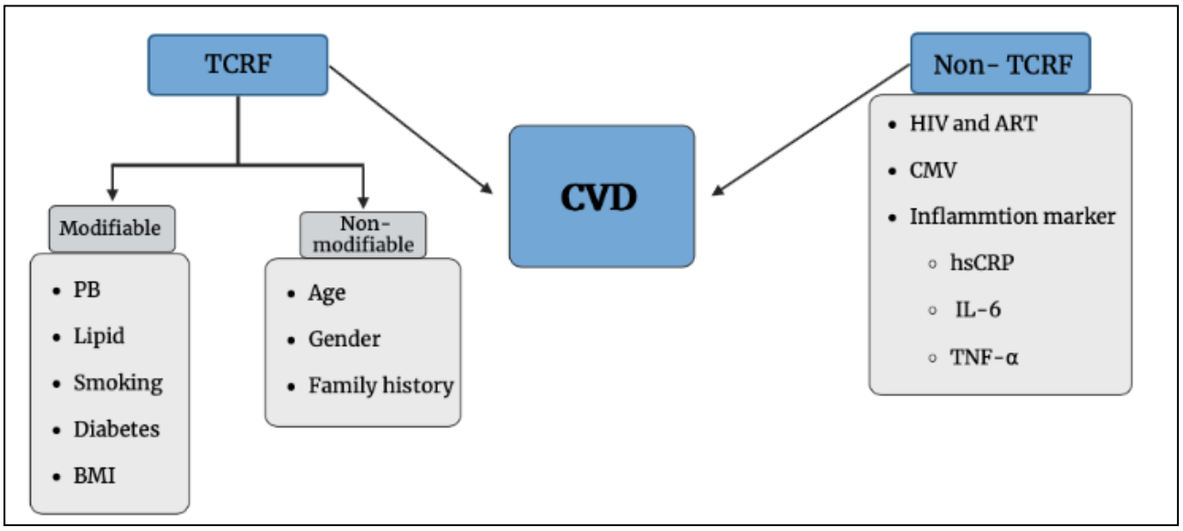

Figure 18.1. Traditional TCRFs and non-traditional TCRFs

This diagram shows TCRFs, which include modifiable and non-modifiable factors, and non-TCRFs. These factors are well recognized risk factor for CVDs such as AS and MI in PLWH (421-423). (Made by K Alsulami using BioRender.com).

1.20.1. Modifiable TCRFs in PLWH

1.20.1.1. Hypertension or high BP

Hypertension and high BP are interchangeable terms. They are a leading cause of mortality (424). PLWH with high BP have a 2-fold higher risk of MIs than PLWH with normal BP (359, 425). When blood flows it puts pressure on artery walls; this is the definition of BP (426). When systolic BP (SBP) is above 140 millimeters of mercury (mmHg) or diastolic BP (DBP) is above 90 mmHg, antihypertensive therapy should be initiated (427). ART is one of the factors contributing to the development of hypertension. Nduka et al. conducted a meta-analysis of 39 studies on 44,903 participants to estimate the impact of ART on risk of hypertension between PLWH on ART compared to those not on ART. They observed that PLWH on ART had higher SPB and/or DBP (428), an observation seen by others (399, 429). The newer ART classes such as INSTIs and PIs are associated with weight gain and progression of hypertension. INSTI-based regimens, particularly those containing DTG are commonly used in sub-Saharan Africa though it is unknown whether there is an association between DTG use and hypertension in this population (430, 431).

A link between hypertension and MT was described in PLWH. MT has been implicated in the pathophysiology of hypertension in PLWH. Manner et al. found that plasma concentration of LPS and sCD14 were strongly correlated with hypertension in PLWH (432). Furthermore, the level of LPS increases in the plasma of PLWH after ART is initiated (433). LPS has been linked to arterial stiffness and endothelial cell apoptosis (76, 434). The directionality of the association of markers of MT with high BP is under consideration. Hypertension has been shown to have a negative impact on MT and gut dysbiosis (435).

1.20.1.2. Dyslipidemia

The impact of lipids on CVDs have been reported in several studies. The American Heart Association introduced the concept of ideal cardiovascular health, which is defined by specific behaviors including not smoking, eating a diet low in saturated fats and regular physical exercise. The prevalence of hyperlipidemia in PLWH is estimated to be 23%-80%, which is associated with high blood sugar (436). Dyslipidemia is characterised by increased TG, decreased HDL, and increased LDL levels, particularly small dense LDL (437). Low-LDL and HDL are the most important types of cholesterol in the body. LDL is the “bad” cholesterol, which promotes CVDs while HDL is the “good” cholesterol, which protects against CVDs (438). High lipid levels in blood serum especially elevated levels of LDL are strongly associated with the development of AS. Circulating LDL and other apo-B lipoproteins, which transport cholesterol and other lipids through the body play a role in the initiation of AS. Lipoproteins are retained in the artery wall over time leading to the development of atherosclerotic plaques. People with high LDL levels retain more particles and experience a faster rate of atherosclerotic plaque growth and vice versa (439).

PLWH on-ART often have a perturbed lipid profile including decreased HDL and increased LDL levels. The association between the alteration in lipid levels and chronic ART-treated HIV infection may be explained by the increase in CVD risk in PLWH compared to uninfected individuals. The newer ART regimens are believed to induce fewer short-term metabolic perturbations than the older ones (440). In conclusion, monitoring lipid levels over time is a necessary component of ideal cardiovascular health that has the potential to reduce the risk of PLWH and HIV uninfected people developing atherosclerotic plagues.

1.20.1.3. Diabetes

Diabetes is a chronic metabolic disorder and inflammatory disease consisting in a deficient insulin activity and subsequent hyperglycemia. The impairment in insulin's physiological action may be due to pancreatic insufficiency or to cellular insulin resistance. Hyperglycemia is associated with long term damage of different organs such as the eyes, kidneys, nerves, heart and blood vessels (441). Hyperglycemia triggers several metabolic signaling pathways that lead to inflammation, cytokine production, cell death, mitochondrial dysfunction and endoplasmic reticulum stress, consequently to diabetic complications (442). The American Diabetes Association (ADA) proposed the classical classification of diabetes into type-1 (T1D) and type-2 (T2D) (443). T1D is characterised by a deficiency in insulin secretion. T2D develops due to a combination of resistance to insulin action and an inadequate compensatory insulin secretory response (443). People with T2D and hypertension have an increased CVD risk compared to with those having only one of these conditions (444).

The diabetes incidence rate in PLWH is higher than HIV uninfected people. The prevalence of diabetes is higher in PLWH on ART than in untreated PLWH and in HIV uninfected people (14%, 7% and 5%, respectively). The prevalence of diabetes is thought to be high in PLWH as a consequence of elevated IA associated with MT, which leads to T cell depletion. (445). Hyperglycemia induces reactive oxygen species that can promote AS formation through vascular smooth muscle cell proliferation and migration to the atherosclerotic lesions (446). Hyperglycemia is associated with reduction of HDL levels and increases of oxidized LDL, which are important

factors associated with AS and CVDs (447). The association between hyperglycemia and dyslipidemia has been shown in several studies to contribute to CVD disease progression.

1.20.1.4. Smoking

Autopsy studies have established smoking as the main TCFR (448). There is a strong link between cigarette smoking and the development of AS, MI, stroke, and HF (449). Exposure to smoke can be divided into mainstream and sidestream exposure. Mainstream refers to smokers inhaling cigarette smoke (CS), while sidestream exposure refers to smoke from the burning end of cigarettes, which is more toxic than mainstream exposure (450, 451). CS consist of more than 7,000 chemicals that are known to mediate the pathophysiology of CVDs including carbon monoxide, nicotine, polycyclic aromatic hydrocarbons that have effects on blood vessels, lipids, and clotting that predispose to CVD events such as AS, MIs, impaired oxygen delivery and endothelial dysfunction (452). The mechanism by which cigarette smoking induces and promotes AS has been well demonstrated in several studies. The process starts when CS damages endothelial cells causing a chronic inflammation, which leads to endothelial dysfunction. Consequently, these inflamed cells secrete substances and attract cholesterol-rich cells that promote plaque formation. As a result of continued inflammation, these plaques rupture, which causes acute narrowing of arteries and thrombosis, ultimately leading to heart attacks and vascular dementia in the brain (452). There is a link between smoking and increased LDL and decreased HDL. Smoking cessation plays an important role in improving HDL levels. Thus, it appears smoking, and dyslipidemia are both related to increased risk of CoAS (453, 454).

Smoking is two to three times more prevalent in PLWH than in the general population. The interaction between the pathophysiology of smoking and physiological basis of HIV infection has a significant negative health impact in PLWH. (455-457). The life expectancy of smokers is at least 10 yrs shorter than in non-smokers. De Socio et al. found that among PLWH the rate of current smokers was 51.6% compared to 25.9% in uninfected persons ($p<0.001$), the rate at which PLWH quit smoking was 27.1% versus 50.1% ($p<0.001$) for the general population. The mean number of cigarettes smoked per day was significantly higher in PLWH, particularly in

those who reported diabetes, high BP and obesity (457). Together, this data shows the urgent need for smoking cessation strategies targeting PLWH that should decrease CVD events.

1.20.1.5. Body mass index (BMI)

Obesity is a chronic metabolic disorder. It has become a worldwide epidemic in the general population. It is an independent risk factor for CVDs and is also associated with an increased CVDs risk in the general population. The World Health Organization (WHO) identifies obesity based on the BMI (kg/m^2) calculation (weight in [kg]/height in [m^2]) using the range of 18.5-24.9 for normal BMI, 25-29.9 for overweight and above 30 for obese (458). Furthermore, several studies have shown that the association of obesity with T2D, high BP, CoAD, MI, stroke and metabolic syndrome (459, 460). Obesity has been categorized into three classes: class I BMI=30-34.9 kg/m^2, class II BMI=35-39.9 kg/m^2 and class III BMI= over 40 kg/m^2 (460, 461).

The pathogenesis of obesity is complex. Weight gain is influenced by several metabolic parameters such as glucose, insulin, fatty acids, adipocytes, genetic factors, and age (462). Obesity and increased adipose tissue influence the pathogenesis of AS. Obese individuals have a two to three-fold increase in the plasma concentration of inflammatory markers such as TNF-α, IL-6, monocyte chemoattractant proteins and hs-CRP. High levels of these immune factors are associated with the AS (463). When inflammatory markers were compared in obese versus non-obese PLWH, obese PLWH had higher level of TNF-α, IL-6 and hs-CRP than non-obese persons (464). In summary, weight loss in overweight and obese PLWH and uninfected persons reduces the risk for diabetes and CVDs.

1.20.2. Non- Modifiable TCRF in PLWH

1.20.2.1. Age

AS was thought to be a problem of the elderly. However, autopsy studies have revealed that CoAS lesions are present in asymptomatic young men as early as in their early twenties (465). In PLWH, aging is the most obvious reason for increased CVD risk. This is due not only to aging itself but also to the appearance of TCRFs with age (466). Onen *et al.* confirmed a higher prevalence of hypertension, and hypertriglyceridemia and lipodystrophy in aging PLWH compared to similarly aged HIV-negative individuals (466). In the large HIV cohort study called Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D), each 5 yrs increase in age was associated with an increase in the RR for MI of 1.32 (95% CI, 1.23-1.41) (467). On one hand, it was suggested that the aging process in PLWH was accelerated since the prevalence of CVDs for a given age equates with that of prevalence in people in the general population who are 10-15 years older. On the other hand, other authors found that the risk of CVDs was similar in persons in both groups at the same age. Other authors have remarked that the most frequent mean age for MIs occurred among PLWH at 48-50 yrs of age and this may be at least partially due to the young age of the HIV community, whose mean age is still younger than that of the general population (468). The wide-spread use of ART leads to demographic changes in PLWH. HIV physicians and cardiologists will need to consider this demographic trend when addressing prevention of and treatment for CVDs in this group of patients.

1.20.2.2. Gender

Male gender is a CVD risk factor, as premenopausal women have a lower risk of CVD than age-matched men. According to the CDC, a higher percentage of American men are diagnosed with HIV than women (81%, 19%, respectively). This suggests that male PLWH are at a greater CVD risk compared with female PLWH. Over the past 20 yrs, the body fat redistribution syndrome called lipodystrophy has been noted in both male and female PLWH (469). Lipodystrophy is characterized by changing body shape including thinning of the face and extremities, truncal obesity and breast enlargement (470). In some HIV cohort studies, women exhibited a higher risk than men. Baldé et al. studied a large cohort of PLWH who were receiving ART. In this study, participants were followed between 2000-2009, and compared for MI incidence in men and women aged between 35 to74. Baldé et al. observed that the RR of MI was 1.99 (1.39-2.75) among

woman whereas in men it was 1.12 (0.99-1.27) (471). Further studies should clarify why this gender difference exists.

1.20.3. Non-TCRF in PLWH

1.20.3.1. Cytomegalovirus (CMV)

Infection with human herpesvirus 5 or CMV causes a persistent life-long infection that is often acquired in childhood or at the time of sexual debut. The prevalence of CMV infection is 40-100% in the general population depending on age and geographical location (472). CMV can be found in urine, PBMCs, stool, the oral mucosa and in the breast milk of PLWH. In addition to all the TCRF that have been shown to be associated to increased risk of CVDs, CMV infection is considered a non-TCRF associated with an increased CVD risk.

Many serological and molecular biological tests have shown that CMV infection of endothelial cells plays an important role in the development of AS. In 1987, Adam et al. were the first to report an association between CMV infection and AS. In this study, patients who had vascular surgery for AS had higher anti-CMV Ab levels than a matched control group with high cholesterol levels (473). In a study of patients with ischemic heart disease, 59% had CMV DNA in the peripheral blood and aortic and venous walls that were associated with AS (474). In another study, the artery vascular tissue was collected from patients with AS, and the presence of CMV IgG and IgM were tested in this tissue. They found that CMV IgG and IgM levels were higher in AS patients compared to controls. This observation suggested that latent and active CMV infection is present in AS patients (475). Taken together these studies suggest that CMV infection is involved in the development of AS. Of note, the role of CMV infection in AS development has been reported in PLWH. The mechanisms underlying how CMV infection is associated with developing AS have been described through different concepts (476):

1- CMV infects epithelial cells causing cellular injury leading to inflammation. CMV from epithelial cells infects SMCs leading to cell proliferation and cholesterol accumulation in the heart arteries ultimately causing AS.
2- In cases of latent CMV infection, when the virus is reactivated it promotes chronic inflammation and damages epithelial cells and SMCs. It can also induce the epithelial cells,

SMCs, foam cells and T cells to produce CCL2, 3, 4, 5 and macrophages to express IL-1, 6, 10, 12 and TNF that are involved in the AS formation process.

3- CMV infection modifies the lipid profile and changes the gene expression pattern to one that is involved in cholesterol metabolism.

4- CMV infection induces the IL-6 expression that triggers epithelial cells to release CCL2 that recruits monocytes and T cells to the heart vessel walls increasing local inflammation leading to AS.

5- CMV infection drives CD8-T cell expansion that would be associated with cytokine production leading increasing inflammation.

BRIDGE PARAGRAPH TO CHAPTER II

CMV infects up to 90% of humans worldwide. The UNAIDS estimates that 38.4 million people are currently living with HIV. Most PLWH are CMV co-infected. CMV infection drives the expansion of a highly differentiated NK cell population with adaptive-like features characterized by expression of the C-type lectin activating NKG2C receptor. High percentages of $NKG2C^+$ NK cell have been noted in PLWH. NKG2C is encoded by a gene that has a deletion variant whose gene product is not cell surface expressed. The allele frequency of the *NKG2C*$^-$ variant is 20% in several populations, including in Caucasians such that the frequency of the homozygous *NKG2C*$^{-/-}$ genotype is 4% in these groups. Thomas et al. questioned whether presence or absence of $NKG2C^+$ NK cells played a role in HIV susceptibility or control (338). They reported that the frequency of carriers of an *NKG2C*$^-$ allele was higher in PLWH than in uninfected individuals with a low risk for HIV infection (338). People at low risk for HIV infection are unlikely to become HIV seropositive making this population an inappropriate one on which to base a conclusion that a low level or lack of NKG2C expression on NK cells is a risk factor for HIV infection. In chapter II, I present comparisons I have made of the frequency of the three possible NKG2C genotypes in PLWH and HESNs who remained HIV seronegative despite exposure to HIV. Thomas et al. and others found conflicting results regarding whether NKG2C genotypes are associated with HIV VL in small populations of treatment naïve PLWH. I had access to longitudinal HIV VL information from 249 untreated PLWH from enough time points to establish a VL set point. This allowed me to determine whether NKG2C genotypes were associated with HIV VL control.

CHAPTER II

2. Influence of NKG2C Genotypes on HIV Susceptibility and Viral Load Set Point

Khlood Alsulami,[a,b,c] Naomi Bolastig,[a,c] Franck P. Dupuy,[a,c] Tsoarello Mabanga,[a,c] Louise Gilbert,[a,c] Zahra Kiani,[a,c] Jean-Pierre Routy,[a,b,c,d,e] Julie Bruneau,[f,g] Réjean Thomas,[h] Cécile Tremblay,[f,i] Christos M. Tsoukas,[a,b,c,j] Jason Szabo,[c,g,h] Pierre Côté,[k] Benoit Trottier,[k] Roger LeBlanc,[l] Danielle Rouleau,[i] Nicole F. Bernard,[a,b,c,e,j] for the investigators in the Montreal Primary HIV Infection cohort

[a]Research Institute of the McGill University Health Centre (RI-MUHC), Montreal, Quebec, Canada
[b]Division of Experimental Medicine, McGill University, Montreal, Quebec, Canada
[c]Infectious Diseases, Immunology and Global Health Program, Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada [d]Division of Hematology, McGill University Health Centre, Montreal, Quebec, Canada
[e]Chronic Viral Illness Service, McGill University Health Centre, Montreal, Quebec, Canada
[f]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, Quebec, Canada
[g]Department of Family Medicine, Université de Montréal, Montreal, Quebec, Canada
[h]Clinique Médicale l'Actuel, Montréal, Quebec, Canada
[i]Department of Microbiology, Infectiology, and Immunology, University of Montreal, Montreal, Quebec, Canada
[j]Division of Clinical Immunology, McGill University Health Centre, Montreal, Quebec, Canada
[k]Clinique de Médecine Urbaine du Quartier Latin, Montréal, Quebec, Canada
[l]Clinique Médicale Opus, Montréal, Quebec, Canada

Corresponding author**:** Nicole F. Bernard, Ph.D. nicole.bernard@mcgill.ca

Research Institute of the McGill University Health Centre, Glen site, Bloc E, 1001 Decarie Blvd., Room EM3.3238, Montreal, Quebec, H3A 3J1, Canada.

Tel: (514) 934-1934 x-44584; Fax: (514) 933-1562

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2.1. ABSTRACT

NKG2C is an activating NK cell receptor encoded by a gene having an unexpressed deletion variant. Cytomegalovirus (CMV) infection expands a population of NKG2C^{+} NK cells with adaptive-like properties. Previous reports found that carriage of the deleted NKG2C^{-} variant was more frequent in people living with HIV (PLWH) than in HIV^{-} controls unexposed to HIV. The frequency of NKG2C^{+} NK cells positively correlated with HIV viral load (VL) in some studies and negatively correlated with VL in others. Here, we investigated the link between *NKG2C* genotype and HIV susceptibility and VL set point in PLWH. *NKG2C* genotyping was performed on 434 PLWH and 157 HIV-exposed seronegative (HESN) subjects. Comparison of the distributions of the three possible *NKG2C* genotypes in these populations revealed that the frequencies of *NKG2C*$^{+/+}$ *and NKG2C*$^{+/-}$ carriers did not differ significantly between PLWH and HESN subjects, while that of *NKG2C*$^{-/-}$ carriers was higher in PLWH than in HESN subjects, in which none were found ($P = 0.03$, x^2 test). We were unable to replicate that carriage of at least 1 *NKG2C*$^{-}$ allele was more frequent in PLWH. Information on the pre- treatment VL set point was available for 160 *NKG2C*$^{+/+}$, 83 *NKG2C*$^{+/-}$, and 6 *NKG2C*$^{-/-}$ PLWH. HIV VL set points were similar between NKG2C genotypes. The frequency of NKG2C^{+} CD3^{-} CD14^{-} CD19^{-} CD56^{dim} NK cells and the mean fluorescence intensity (MFI) of NKG2C expression on NK cells were higher on cells from CMV^{+} PLWH who carried 2, versus 1, *NKG2C*$^{+}$ alleles. We observed no correlations between VL set point and either the frequency or the MFI of NKG2C expression.

2.2.IMPORTANCE

We compared *NKG2C* allele and genotype distributions in subjects who remained HIV uninfected despite multiple HIV exposures (HESN subjects) with those in the group PLWH. This allowed us to determine whether *NKG2C* genotype influenced susceptibility to HIV infection. The absence of the $NKG2C^{-/-}$ genotype among HESN subjects but not PLWH suggested that carriage of this genotype was associated with HIV susceptibility. We calculated the VL set point in a subset of 252 *NKG2C*-genotyped PLWH. We observed no between-group differences in the VL set point in carriers of the three possible *NKG2C* genotypes. No significant correlations were seen between the frequency or MFI of NKG2C expression on NK cells and VL set point in cytomegalovirus-coinfected PLWH. These findings suggested that adaptive NK cells played no role in establishing the in VL set point, a parameter that is a predictor of the rate of treatment-naive HIV disease progression.

2.3.INTRODUCTION

Natural killer (NK) cells are cytotoxic lymphocytes that generate early immune responses to virus-infected and cancer cells (1). The activation state of NK cells is determined by the integration of signals received from activating and inhibitory receptors (2, 3). Among the types of receptors present on NK cells are the NKG2 receptors, which belong to the C-type lectin family. The genes encoding these receptors are located in the 12p13 region of chromosome 12, within the NK receptor complex (4, 5). The NKG2C activating receptor, like its inhibitory counterpart NKG2A, is expressed as a heterodimer with CD94 (6). The ligand for NKG2C and NKG2A is HLA-E, a nonclassical major histocompatibility complex class Ib (MHC-Ib) molecule, stabilized by peptides derived from classical MHC-I antigens and HLA-G (7, 8). HLA-E molecules complexed with epitopes from the human cytomegalovirus (CMV)-encoded viral protein UL40 leader sequences are ligands for NKG2C (9–12). Among $CD56^{dim}$ NK cells, $NKG2C^{+}$ NK cells are typically $NKG2A^{-}$ (13, 14). The interaction of NKG2C with its ligands transmits signals that activate cells bearing this receptor (7, 15).

Although NK cells are traditionally thought to be part of the innate immune system, $NKG2C^{+}$ NK cells, which often coexpress CD57, can undergo clonal expansion in response to CMV infection (9, 13, 16, 17). Because the expansion of $NKG2C^{+}$ cells resemble that seen in adaptive immune responses, these NK cells are called adaptive NK cells. Expanded adaptive NK cells frequently lack the signaling proteins Ewing's sarcoma's/FLI-1 activated transcript-2 (EAT-2), spleen tyrosine kinase (SYK), and FcεRγ, as well as the transcription factor promyelocytic leukemia zinc finger (PLZF) (18, 19). This is due to DNA methylation-dependent epigenetic modifications, which distinguish adaptive from conventional NK cells. Adaptive $NKG2C^{+}$ cells exhibit enhanced CD16- dependent cytokine secretion due to epigenetic remodeling of the gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) promoter regions (20–22).

Some individuals do not express NKG2C at the NK cell surface due to homozygous deletion of an ~16-kb genomic region that includes the *nkg2c* (also called *klrc2*) gene that encodes NKG2C (23, 24). In several Caucasian, Japanese and a Tanzanian population, the frequency (%) of the *NKG2C* deletion haplotype is close to 20% with a homozygous deletion frequency of approximately 4% (22, 24–26). However, frequencies of the *NKG2C* deletion haplotype were found to be as low as 10.3% in a Mexican mestizo group and as high as high as 36.8% in west African populations from the Gambia and Guinea-Bissau (26–28). NK cells expressing NKG2C have been shown to play a role in the immune surveillance of CMV (17).

CMV infection also drives the expansion of NKG2C$^-$ NK cells in people who are *NKG2C*$^{-/-}$ (29). NKG2C$^-$ NK cells having an epigenetic footprint characteristic of NKG2C$^+$ adaptive NK cells are observed in *NKG2C*$^{-/-}$ carriers (22). Comparisons of the phenotype and function of adaptive NK cells from *NKG2C*$^{-/-}$ with those from *NKG2C*$^{+/+}$ and *NKG2C*$^{+/-}$ carriers found few differences, suggesting that the contribution of NKG2C to NK cell adaptation to CMV infection can be compensated for in NK cells from *NKG2C*$^{-/-}$ carriers in CMV mono-infected as well as in HIV$^+$CMV$^+$ co-infected subjects (22, 30). In adaptive NK cells from *NKG2C*$^{-/-}$ carriers, CD2 co-stimulation plays an important role in compensating for the absence of NKG2C in antibody-dependent responses (22).

HIV/CMV co-infection has been reported by many to drive the expansion of NKG2C$^+$ NK cells over that seen in CMV mono-infected persons (31, 32). Several studies have questioned whether NKG2C$^+$ cells play a role in protection from HIV infection or in slower disease course in those infected. Supporting a role for the NKG2C$^-$ variant in susceptibility to HIV infection was the observation that the % of carriers of a *nkg2c*$^-$ allele either in homozygous or heterozygous form was higher in HIV infected, than in an HIV uninfected, individuals with no history of HIV exposure (33). Whether NKG2C$^+$ NK cells play a role in HIV control is unclear. Thomas et al. showed that among HIV-infected persons, the proportion of individuals with a pre-treatment plasma viral load (VL) <30,000 copies/ml was higher in carriers of the *NKG2C*$^{+/+}$ genotype than among those carrying a *nkg2c*$^-$ allele (33). Contrasting with the notion that the *NKG2C*$^{+/+}$

genotype was associated with lower VL control was the finding that the % of NKG2C^{+} NK cells from seven *NKG2C*$^{+/+}$ carriers was positively correlated with a single pre-treatment HIV VL (33). However, in two other studies, the % of NKG2C^{+} NK cells was negatively correlated with VL in early infection (34, 35).

Here, we compared *NKG2C* genotypes in people living with HIV (PLWH) enrolled in the Montreal HIV primary infection (PI) cohort with HIV exposed seronegative (HESN) subjects who remained HIV uninfected despite multiple high-risk HIV exposures. We found that carriage of the *NKG2C*$^{-/-}$ genotype, was associated with increased HIV susceptibility. However, neither the *NKG2C*$^{+/+}$, *NKG2C*$^{+/-}$ nor the combination of both *NKG2C*$^{+/-}$ and *NKG2C*$^{-/-}$ genotypes, was associated with changes in HIV susceptibility. We observed no differences in VL set point between HIV-infected carriers of the three possible *NKG2C* genotypes. We also observed no correlation between VL set point and the % of NKG2C^{+} NK cells or the intensity of NKG2C expression. Thus, carriage of an *nkg2c*$^{-}$ allele does not appear to affect HIV VL set point, which is a determinant of the rate of HIV disease progression.

2.4.MATERIALS AND METHODS

Ethics statement

This research study was approved by the Institutional Review Board of the Research Ethics Committee of the McGill University Health Centre (study identification code 2018-4501). It was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent for the collection of each individual's specimens and subsequent analyses using these samples was obtained from all study subjects.

Study population

The study population included 591 individuals: 434 were PLWH enrolled in the Montreal PI study, and 157 were HESN subjects (61). Persons at high risk of being sexually exposed to HIV, which we will designate here are sexually exposed (SE) HESN (n = 78), included HIV-uninfected men who have sex with men (MSM) recruited from the Clinique Médicale l'Actuel (n = 40) and subjects en- rolled in the Ipergay Pre-Exposure Prophylaxis (PrEP) on-demand study followed in Montreal (n=21) (62). These MSM SE HESN subjects answered "yes" to the question "Have you had unprotected receptive anal intercourse with a partner of unknown HIV serostatus or known to be HIV-infected, at least 5 times in the last 6 months or at least 50 times in your lifetime before starting PrEP?" An additional 17 SE HESN subjects were HIV-negative partners in HIV-discordant couples who remained HIV uninfected despite multiple exposures that occurred before the availability of antiretroviral treatment (ART). These included 9 men and 8 women; 6 of the men were MSM (63). We also recruited HIV-negative injection drug user (IDU) HESN subjects from the St. Luc cohort (n = 79) (64). All IDU HESN subjects answered "yes" to the question "Have you shared needles and/or injection equipment with partners known to be HIV-infected at least 5 times?" Clinic visits for St. Luc cohort participants occurred approximately every 6 months, at which time information was collected regarding the frequency of their at-risk behavior for HIV exposure. All HESN subjects provided a blood sample from which peripheral blood mononuclear cells (PBMCs) and plasma were isolated and stored frozen until use. HIV serostatus was assessed using HIV enzyme immunoassays (EIAs) (65). Subjects enrolled in the Montreal PI cohort included individuals recruited within the first 6 months of HIV infection, who were then followed an average of every 3 months for up to 4 years (65). At

each clinic visit, CD4 and CD8 counts and plasma VL were measured, ART status was recorded, and blood was drawn for isolation of PBMCs and plasma, which was stored frozen until use. For one experiment comparing the expression of NKG2C on cells from HIV^{-} CMV^{+} persons, 11 additional subjects who had minimal HIV exposure, were included.

NKG2C genotyping

Genomic DNA was extracted from the PBMCs of all study subjects using the QIAamp DNA Blood Mini Kit (QIAGEN, Inc., Toronto, ON, Canada) according to the manufacturer's instructions. Full length *nkg2c* (*nkg2c*$^{+}$) and the deleted variant (*nkg2c*$^{-}$) are alleles at the same locus (24). NKG2A is encoded at a separate locus. The presence of *nkg2c*$^{+}$, *nkg2c*$^{-}$ alleles and the *nkg2a* locus, as a positive control present in all subjects, was determined by sequence specific PCR. Three sets of forward and reverse sequence-specific primers for *nkg2c*$^{+}$, *nkg2c*$^{-}$ and *nkg2a* were used to amplify the allele groups at the *nkg2c* and *nkg2a* loci. The forward and reverse primers for amplification of the *nkg2c*$^{+}$ allele were NKG2CT/F (5'-ATCAATTATTGAAATAGGATGC-3') and NKG2CT/R (5'-CGCAAAGTTACAACCATCACCAT-3') (24). Those amplifying the *nkg2c*$^{-}$ allele were BREAK-F (5'-ACTCGGATTTCTATTTGATGC-3') and BREAK-R (5'-ACAAGTGATGTATAAGAAAAAG-3') (24). Twenty-five ng/μl of genomic DNA from each participant was amplified with Platinum Taq (Thermo Fisher Scientific, Burlington, ON, Canada) in a T100^{TM} Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA) using the following conditions: Denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec, followed by a 5 min extension at 72°C. Amplicons were visualized by gel electrophoresis on a 2% agarose gel in 0.5X TBE (45mM Tris Base; 45mM boric acid; 1 mM EDTA) buffer run at 125 V for 30 min in Fluo-DNA Loading Buffer (6x, Zmtech Scientifique, Montreal, QC, Canada) and imaged with an Omega Lum^{TM} C imaging system (Gel Company, Inc., San Francisco, CA). Band sizes of 300-bp corresponded to *nkg2c*$^{+}$ alleles, the 400-bp band to *nkg2c*$^{-}$ alleles, and the 800-bp band to *nkg2a* (29). Samples were classified as homozygous for the presence of the *nkg2c*$^{+}$ allele when only the 300-bp band was present (*NKG2C*$^{+/+}$), homozygous for *nkg2c*$^{-}$ (*NKG2C*$^{-/-}$) when only

the 400-bp band was present and heterozygous for *nkg2c*$^{+}$ and *nkg2c*$^{-}$ when bands of both sizes (NKG2C$^{+/-}$) were present (29).

Flow cytometry analysis of the frequency of NKG2C^{+} cells and the intensity of NKG2C expression

PBMCs from 32 *NKG2C*$^{+/+}$, 20 *NKG2C*$^{+/-}$ and 6 *NKG2C*$^{-/-}$ HIV^{+}CMV^{+} subjects were stained with an antibody cocktail that allowed for gating on live NK cells as CD3^{-}CD14^{-}CD19^{-}CD56^{dim} lymphocytes. We also stained PBMC from 43 *NKG2C*$^{+/+}$ and 18 *NKG2C*$^{+/-}$ HIV^{-}CMV^{+} subjects with this antibody cocktail; all belonged to the HESN group except for 11 HIV^{-}CMV^{+} low risk controls. These were examined for differences in the % of NKG2C^{+}CD56^{dim}, NKG2C^{+}CD57^{+}CD56^{dim}, and NKG2A^{+}CD56^{dim} NK cells and the intensity of NKG2C expression on NKG2C^{+}CD56^{dim} NK cells from subjects carrying each *NKG2C* genotype. The intensity of NKG2C staining was assessed by measuring the mean fluorescence intensity (MFI), the median fluorescence intensity and the fold-change in NKG2C MFI over background staining. Cryopreserved PBMCs were thawed and resuspended in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS); 2mM L-glutamine; 50 IU/ml penicillin; and 50 mg/ml streptomycin (R10) (all from Wisent, St Jean Baptiste, QC, Canada). PBMCs, (10^{6} in 100 µl of R10) were cell surface stained for 25 min at 4°C with previously optimized concentrations of fluorochrome conjugated antibodies to the following cell surface markers: CD3-BV785 (clone OKT3), CD19-BV785 (HIB19), CD14-BV785 (M5E2), CD56-BV605 (HCD56) (all from Biolegend, San Diego, CA), CD16-APC-Cy7 (3G8, BD Biosciences, Baltimore, MD), NKG2C-PE-Cy7 (REA250), NKG2A-APC (REA110) (from Miltenyi Biotec, Auburn, CA), CD57-PE (TB01, Life Technologies, Burlington, ON, Canada) and Indo-Violet live/dead (L/D) stain (Fisher Scientific, Waltham, MA). Cells were then washed twice with FACS buffer (phosphate buffered saline [PBS]; 4% FBS; 0.05% NaN_3) and fixed in 2% paraformaldehyde (Santa Cruz Biotechnology, Santa Cruz, CA). Between 5 and 7 x 10^{5} cells were acquired using an LSRFortessa^{TM} X-20 flow cytometer (BD Biosciences, San Jose, CA). Results were analyzed using FlowJo v10.6.2 software (Tree Star, Ashland, OR).

VL set point determination

VL set points were calculated for 160 *NKG2C*$^{+/+}$, 83 *NKG2C*$^{+/-}$, and 6 *NKG2C*$^{-/-}$ HIV^{+}carriers. The average of the VLs from all treatment-naive time points 6 months after the estimated date of infection to the end of their follow-up in the Montreal PI cohort were used to calculate the VL set point.

Statistical analysis

Statistical analysis and graphical presentation of results were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA) and Statistical Analysis System (SAS) software version 9.4 (SAS Institute, Cary, NC). The statistical significance of differences in the racial/ethnic composition of the HIV^{+} and HESN populations and deviations in the distributions of NKG2C genotype from Hardy-Weinberg equilibrium (HWE) was assessed using x^2 tests. Between-group differences in the frequency of *NKG2C* genotypes in PLWH and HESN populations were determined using two-tailed Fisher's exact tests with Haldane's correction. The statistical significance of between-genotype differences in the percentage of NKG2C^{+} NK cells, the intensity of NKG2C expression on CD56^{dim} NK cells, and VL set point in ART-naive PLWH was assessed using Kruskal-Wallis tests with Dunn's post tests. The significance of correlations between the percentages of NKG2C^{+}, NKG2C^{+} CD57^{+}, and NKG2A^{+} CD56^{dim} NK cells and intensity of NKG2C expression and VL set point in ART-naive CMV^{+} PLWH was assessed using Spearman's correlation tests. P-values of < 0.05 were considered significant.

2.5.RESULTS

PLWH and HESN populations differ in NKG2C$^{-/-}$ genotype frequency

Table 1 provides information on the racial/ethnic composition of the study population. Both populations were composed mainly of Caucasians (92.9% and 88.5% for PLWH and HESN participants, respectively) living in the same geographical region (Montreal, QC, Canada). There were no significant between-group differences in their ethnic/racial composition (p>0.11, for comparisons of Caucasians, Asians, Latinos and American/African Blacks, two-tailed χ^2 tests). The number and % of PLWH and HESN subjects carrying the wild type (*nkg2c*$^{+}$) and deleted (*nkg2c*$^{-}$) alleles and the three *NKG2C* genotypes is shown in Table 2. The allele % was similar in both populations. The distribution of the three *NKG2C* genotypes: *NKG2C*$^{+/+}$, *NKG2C*$^{+/-}$ and *NKG2C*$^{-/-}$ at this locus did not deviate statistically from the HWE in PLWH (p=0.09, χ^2 test) while it did in the HESN subjects (p=0.003, χ^2 test). When the proportion of *NKG2C*$^{+/+}$, *NKG2C*$^{+/-}$ and *NKG2C*$^{-/-}$ genotypes was compared in PLWH and HESN subjects, there was a significantly higher frequency of *NKG2C*$^{-/-}$ individuals among PLWH (odds ratio [OR] 8.60, 95% confidence interval [CI] 0.50 to 146, p=0.04, two-tailed Fisher's exact test), while the proportion of individuals carrying the *NKG2C*$^{+/+}$ and *NKG2C*$^{+/-}$ genotypes in these two populations did not differ significantly (Table 2). Thomas et al. previously reported that HIV uninfected persons at low risk for infection were significantly more likely than PLWH to carry the *NKG2C*$^{+/+}$ genotype, suggesting that carriage of at least 1 *NKG2C*$^{-}$ variant was associated with higher HIV susceptibility (33). Comparisons of the PLWH and HESNs revealed no between-population differences for either the *NKG2C*$^{+/+}$ or the combined *NKG2C*$^{+/-}$ and *NKG2C*$^{-/-}$ genotypes. In summary, carriage of the *NKG2C*$^{-/-}$ genotype was associated with higher susceptibility to HIV infection.

Risks for HIV transmission include sexual exposure to, and needle sharing with, PLWH. As the PLWH and HESN populations included individuals who were at risk for sexual exposure to HIV, as well as IDUs, we investigated whether there was evidence that carriage of *NKG2C*$^{-/-}$ genotype was linked to HIV susceptibility by mucosal or parental exposure. Of the SE subjects, 371 were PLWH and 78 belonged to the HESN group with genotype distributions shown in Table 2**.** The *NKG2C* genotype distributions diverged from HWE in both the SE PLWH and HESNs (p=0.045 and p=0.001, respectively, χ^2 tests). When the proportion of *NKG2C*$^{+/+}$, *NKG2C*$^{+/-}$ and *NKG2C*$^{-/-}$

genotypes were compared in SE PLWH and HESN subjects, no significant between-group differences were observed. Of the 63 PLWH and 79 HESN IDU evaluated for *NKG2C* genotypes, the genotype distribution in both populations was in HWE (p=0.9 and p=0.07, respectively, χ^2 tests). The proportion of $NKG2C^{-/-}$ genotypes was significantly higher among IDU PLWH than HESNs (12, 0.6 to 277.7, p=0.04, Fisher's exact test); the proportion of $NKG2C^{+/+}$ and $NKG2C^{+/-}$ genotypes carriers in the IDU PLWH and HESN subpopulations did not differ significantly from each other (Table 2**)**. In summary, carriage the $NKG2C^{-/-}$ genotype was significantly associated with higher HIV susceptibility in IDU but not in SE subjects.

NKG2C cell surface expression is genotype dependent

We next compared the % of $NKG2C^+$ cells and the intensity of NKG2C expression on $CD56^{dim}$ NK cells from carriers of the three *NKG2C* genotypes. As CMV infection drives the expansion of $NKG2C^+$ NK cells (9,13,31), for this analysis, we included only subjects who were CMV^+. Since these results were to be correlated with VL set point measures this analysis included HIV^+CMV^+ participants for whom a VL set point could be calculated. Cells from 32 $NKG2C^{+/+}$, 20 $NKG2C^{+/-}$ and 6 $NKG2C^{-/-}$ PLWH and 43 $NKG2C^{+/+}$and 18 $NKG2C^{+/-}$ HIV^- subjects were tested. Figure 1A shows the strategy used to gate on live, singlet, $CD3^-CD14^-CD19^-CD56^{dim}$ NK cells, which is the predominant populations expressing NKG2C (36). From these, $NKG2C^+$ cells were gated on. Figure 1B shows that HIV^+CMV^+ subjects who were $NKG2C^{+/+}$ and $NKG2C^{+/-}$ had a higher % of $NKG2C^+$ NK cells than did $NKG2C^{-/-}$ carriers with a median interquartile range (IQR) of 45.5% (33.5, 67.5), 30.1% (7.41, 44.63) for $NKG2C^{+/+}$ and $NKG2C^{+/-}$ carriers, respectively and background levels of 2.7% (1.04, 5.14) for $NKG2C^{-/-}$ carriers ($p<0.001$ and $p<0.05$ for comparisons of $NKG2C^{+/+}$ and $NKG2C^{+/-}$ carriers with $NKG2C^{-/-}$ carriers, Dunn's post-tests). In a sub-analysis comparing $NKG2C^{+/+}$ with $NKG2C^{+/-}$ carriers, we found that the % of $NKG2C^+CD56^{dim}$ NK cells was significantly higher in HIV^+CMV^+ $NKG2C^{+/+}$ than in $NKG2C^{+/-}$ carriers ($p<0.05$, Mann-Whitney test). For intensity measurements we examined the MFI, the median fluorescence intensity and the NKG2C MFI's fold-change over background staining. The later measure controls for between-experiment variations in MFI. Since values for Mean and Median fluorescence intensity did not differ substantially, we only report MFI values here. Figure 1C shows the results for MFI of NKG2C expression on $CD56^{dim}$ NK cells while

Supplemental Figure 1A shows intensity of NKG2C expression using the fold-change in MFI over background measurements. The MFI of NKG2C expression from *NKG2C*$^{+/+}$, *NKG2C*$^{+/-}$ and *NKG2C*$^{-/-}$ carriers was 4562 (2813, 7175), 1870 (671, 3061) and 269.6 (212.8, 325.3), respectively (Figure 1C). NKG2C expression was higher on CD56^{dim} NK cells from *NKG2C*$^{+/+}$ and *NKG2C*$^{+/-}$ than on those from *NKG2C*$^{-/-}$ carriers ($p<0.001$ and $p<0.05$ for each comparison, Dunn's post-tests). NK cells from *NKG2C*$^{+/+}$ carriers expressed higher levels of NKG2C than those from NKG2C$^{+/-}$ carriers ($p<0.05$, Dunn's post-test). The fold-change in MFI over background for NKG2C expression intensity was also significantly higher on NK cells from *NKG2C*$^{+/+}$ than *NKG2C*$^{+/-}$ carriers who were CMV^{+} PLWH (Supplemental Figure 1A), We also investigated the % of NKG2C^{+}CD56^{dim} NK cells and the intensity of NKG2C expression on these cells from HIV^{-}CMV^{+} subjects. Although the % of NKG2C^{+} NK cells was higher on cells from *NKG2C*$^{+/+}$ than *NKG2C*$^{+/-}$ carriers (11.8% [3.77, 28.7] and 7.23% [4.08, 15.75], respectively) as was the MFI of NKG2C expression on CD56^{dim} NK cells (719 [247, 1569] and 657.5 [247, 1556], respectively), the difference did not achieve statistical significance (Figure 1B, C). The difference in the fold-change in NKG2C MFI over background on NKG2C cells from *NKG2C*$^{+/+}$ and *NKG2C*$^{+/-}$ carriers who were CMV mono-infected was not significant (Supplemental Figure 1B). Figure 1B, C also show that the % of NKG2C was significantly higher among CD56^{dim} NK cells from *NKG2C*$^{+/+}$ and *NKG2C*$^{+/-}$ carriers who were CMV^{+} PLWH than among these cells from CMV mono-infected persons as was the MFI of NKG2C expression on NK cells from *NKG2C*$^{+/+}$ carriers ($p<0.006$ for all, Mann-Whitney tests). Using the fold-change in NKG2C MFI over background measure, the level of NKG2C on *NKG2C*$^{+/+}$ carriers from CMV+ PLWH was higher than on both *NKG2C*$^{+/+}$and *NKG2C*$^{+/}$ carriers from CMV mono-infected individuals ($p<0.001$ and $p<0.0001$, respectively) (Supplemental Figure 1A, B).

NKG2C genotypes and HIV VL set point

VL set points in pretreatment PLWH are measures of HIV progression associated with time to AIDS, CD4 counts of < 200 copies/ml of plasma, and death (37, 38). When all *NKG2C*-genotyped PLWH for whom information on the VL set point was available were included, we found no significant differences between NKG2C genotypes in the VL set points (P = 0.26 by Kruskal-Wallis test) (Fig. 2). We next investigated whether the percentage of $NKG2C^{+}$ $CD56^{dim}$ NK cells and/or the intensity of NKG2C expression correlated with the pretreatment VL set point. Forty-three NKG2C-genotyped CMV^{+} PLWH with a known HIV VL set point were included in this analysis: 21 $NKG2C^{+/+}$, 16 $NKG2C^{+/-}$, and 6 $NKG2C^{-/-}$ HIV^{+} CMV^{+} subjects. Neither the percentage nor the intensity of NKG2C expression (MFI or fold change over background in NKG2C MFI) was significantly correlated with VL set point when all observations were considered together or when results were stratified according to *NKG2C* genotype (Spearman's correlation tests) (Fig. 3A to L) As adaptive NK cells are typically also $CD57^{+}$, we also tested whether there was a correlation between the percentage of $NKG2C^{+}$ $CD57^{+}$ $CD56^{dim}$ NK cells and HIV VL set point.

Figure 4A shows the strategy used to gate on $NKG2C^{+}$ $CD57^{+}$ $CD56^{dim}$ NK cells. No significant correlation was observed between these parameters for all NKG2C genotypes or for results stratified by NKG2C genotype (Spearman's test) (Fig. 4B to E).

As others have shown, the percentage of $NKG2C^{+}$ NK cells was significantly negatively correlated with the percentage of $NKG2A^{+}$ NK cells. This was the case for all genotypes together and for the $NKG2C^{+/+}$ and $NKG2C^{+/-}$ genotypes specifically (Fig. 5A). Figure 5B shows the strategy used to gate on $NKG2A^{+}$ $NKG2C^{-}$ $CD56^{dim}$ NK cells. As for $NKG2C^{+}$ and $NKG2C^{+}CD57^{+}$ $CD56^{dim}$ NK cells, the percentage of $NKG2A^{+}$ $NKG2C^{-}$ $CD56^{dim}$ NK cells did not correlate with the VL set point when results from all subjects were examined together or when results from $NKG2C^{+/+}$ and $NKG2C^{-/-}$ carriers were examined separately. For $NKG2C^{+/-}$ carriers, a negative correlation was observed (r = -0.49, P=0.04) (Fig. 5D). However, application

of a Bonferroni correction for multiple correlations reduced the significance of the correlation below the level of significance.

2.6.DISCUSSION

In this report, we assessed whether the *NKG2C* genotype distributions differed in a population of recently HIV-infected individuals compared to subjects who remained uninfected despite multiple documented exposures to HIV. We found that the *NKG2C*$^{-/-}$ genotype was more frequent among PLWH than HESN subjects. None of the 157 HESN subjects tested carried this genotype, which was present in 11 of 434 (2.53%) of PLWH. The distributions of *NKG2C* genotypes did not differ in the PLWH and HESN subpopulations who were exposed to HIV mucosally, while the *NKG2C*$^{-/-}$ genotype was more frequent in parenterally exposed PLWH than in HESN individuals. These findings suggest that the *NKG2C*$^{-/-}$ genotype is associated with a higher risk of HIV infection. The PLWH population included individuals who remained treatment naive long enough to calculate a post-acute infection, pre-treatment plasma VL set point. When this parameter was compared in carriers of the three *NKG2C* genotypes, we found no between-genotype differences in VL set point. Furthermore, neither the percentage of NKG2C^{+} NK cells, MFI, nor fold change over background of the MFI of NKG2C expression on these cells correlated with VL set point in the CMV^{+} PLWH.

There exists a variation in chromosome 12 where a 16-kb genomic region that includes the *nkg2c* gene is either present or entirely absent (23, 24). Genotyping of the mainly Caucasian study population described in this article found that the frequency of the *nkg2c*$^{-}$ variant was close to 20% in both the PLWH and HESN populations and the frequency of the homozygous *NKG2C*$^{-/-}$ genotype was 2.53% in PLWH. The *nkg2c*$^{-}$ allele frequency and the distribution of *NKG2C* genotypes in the PLWH are in line with those reported for several populations of European extraction, as well as in a Japanese population and an East African Tanzanian population (22, 24–26, 33). The allele frequency of *nkg2c*$^{-}$ was lower (10.3%) in a population of Mexican mestizos and higher (29.3 to 36.7%) in West African populations from the Gambia and Guinea-Bissau (26–28) In contrast with what we found in PLWH, we observed no *NKG2C*$^{-/-}$ carriers

among 157 HIV-uninfected persons at risk for HIV exposure, a difference that was statistically significant. The non-Caucasian ethnic composition of the study populations was balanced between PLWH and HESN subjects. However, if only Caucasians were included in the analysis, proportional between-group differences in the percentage of the *NKG2C*$^{-/-}$ genotype fell below the level of significance (P = 0.1). This may be due to the smaller sample sizes. It was not possible to compare the proportional between- group differences in the percentages of the *NKG2C*$^{-/-}$ genotype for the other ethnicities included in the study populations due to the small numbers of subjects in these subgroups.

The *NKG2C* genotype distributions in the PLWH and the uninfected population described here differed from those reported by Thomas et al. (33). They compared the *NKG2C* genotype distribution in 433 PLWH with that in 280 controls who had no history of HIV exposure (33). They found NKG2C$^{-/-}$ subjects among their HIV-uninfected population, while we did not. They reported a significant association between carriage of a *nkg2c*$^{-}$ allele (i.e., combined NKG2C$^{+/-}$ and *NKG2C*$^{-/-}$ carriers) with HIV infection and that there was a higher proportion of *NKG2C*$^{+/+}$ carriers among uninfected controls than among PLWH. The main difference between the population reported by Thomas et al. and the one described here was the composition of the HIV-uninfected population. In the study by Thomas at al., the control population was not HIV exposed and thus was at a low risk for HIV infection. While it is possible that some of the people at high risk for HIV exposure studied here remained HIV uninfected by chance, they represent a group that is likely to have a higher level of resistance to HIV infection than the HIV-uninfected population described by Thomas et al. The inclusion of HESN participants allowed us to explore more directly whether *NKG2C* genotypes were associated with HIV susceptibility. This may account for the discrepancy between our results and those reported by Thomas et al. regarding which *NKG2C* genotypes were associated with HIV susceptibility.

We stratified both PLWH and HESN subjects into those whose route of HIV infection/exposure was mucosal (SE) versus parenteral (IDU). When SE and IDU PLWH and HESN subjects were compared separately, we observed that the frequency of the NKG2C$^{-/-}$ genotype was significantly higher in the IDU PLWH than HESN subjects, while this frequency did not differ significantly between SE PLWH and those at risk for sexual exposure to HIV. Many factors influence the per-act risk of HIV transmission, including the VL of the transmitting partner, the route of exposure, the presence of genital ulcers, circumcision, and the frequency of exposure, among others (39, 40). The SE PLWH and high risk for HIV exposure subpopulations were mainly men who have sex with men (MSM). Of these, all reported unprotected receptive (where the receptive partner was HIV seronegative) anal intercourse. This route of exposure averages at least a 10-fold higher risk of transmission per act than unprotected insertive anal or vaginal intercourse and a per-act risk that is close to that of injection drug use (41–44).

What accounts for the frequencies of the *NKG2C*$^{-/-}$ genotype not differing significantly between SE PLWH and those at risk for mucosal HIV exposure is unknown. The level of exposure to HIV may be a factor if a higher proportion of HIV-transmitting partners of SE than IDU HESN populations are on antiretroviral treatment (ART). In the context of *NKG2C*$^{+}$ cells, the biology of HIV transmission by injection versus sexual exposure may be a factor. Parenteral exposure involves the introduction of needles contaminated with HIV-infected cells and/or virions into the circulation. Transmitted HIV- infected cells will express HLA-E, the ligand for NKG2C, and downmodulate HLA-A, -B, and -C, the ligands for inhibitory killer immunoglobulin-like receptors also present on NKG2C^{+} cells (2, 3, 7, 8, 36). The integration of these signals promotes NKG2C^{+} NK cell activation that may contribute to HIV clearance prior to the establishment of a productive infection. In this setting, the absence of NKG2C^{+} cells in *NKG2C*$^{-/-}$ carriers may be linked to heightened HIV susceptibility in those who became infected. In the case of sexual exposure, HIV-infected cells or virions must cross mucosal barriers to access the NKG2C^{+} NK cells in the circulation. Our knowledge of NKG2C^{+} NK cells at mucosal genital/anal sites is limited. NK, tissue-resident NK (TrNK), and NK-like innate lymphoid cells are present in tissues, including in the female genital tract (45, 46).

The NK receptor profile of these cells differs from that of circulating NK cells, making it challenging to evaluate their stage of maturity, their ability to interact with HIV-infected cells, and the consequences of such an interaction in the context of what is known about circulating NK cells. Whether NK-like cells at portals of HIV entry express NKG2C is unknown. A study of the transcriptomic and protein expression patterns of TrNK cells in lung mucosal tissue did not report expression of NKG2C, while this receptor was shown to be expressed on adaptive NK cells in the liver, although these NK cells had distinct NK cell receptor profiles from those in the circulation (46, 47). If NKG2C^{+} NK cells were absent at the portals of HIV entry, it would reduce the relevance of *NKG2C* genotypes in modulating infection risk through a mucosal route. In sum, more information on the NK cell landscape at mucosal portals of HIV entry would aid in understanding the discrepancy between the percentage of *NKG2C*$^{-/-}$ carriers in SE versus IDU PLWH and HESN subjects.

The reason underlying why none of the 157 HESN subjects carried the *NKG2C*$^{-/-}$ genotype and how this may contribute to the maintenance of seronegative status despite multiple HIV exposures are unknown. CMV infection drives the expansion of adaptive NK cells (17, 29, 48). It is notable that NKG2C^{-} adaptive NK cells also expand in CMV-infected *NKG2C*$^{-/-}$ carriers (22, 36, 49, 50). Adaptive NKG2C^{-} and NKG2C^{+} NK cells are found at similar frequencies in those who do not and those who do carry a *nkg2c*$^{+}$ allele, and these cells share phenotypic, epigenetic, and functional properties that distinguish them from conventional NK cells (22, 30, 36, 49, 50). One of the differences between adaptive and conventional NK cells is that the former is more likely to express CD2. CD2 is a major coactivating receptor found on NK cells and T cell subsets, whose ligand is CD58 (LFA-3), which is expressed on many tissues (22, 51). CD2 is present on a higher percentage of adaptive NK cells from *NKG2C*$^{-/-}$ than *NKG2C*$^{+}$ carriers (51, 52). It compensates for the absence of NKG2C on adaptive NK cells from *NKG2C*$^{-/-}$ carriers in a manner that contributes to the activation of these cells. Although signaling through CD2 alone has little effect on adaptive NK cell activation, it synergizes with CD16 signaling, to potently activate NK cells to secrete IFN-g and TNF-a (22). It is tempting to speculate that CMV infection provides the costimulatory signals (i.e., CD16 cross-linking by anti-CMV antibody Fc

regions and CD2-CD58 interactions) to activate adaptive NK cells. CMV has tropism for epithelial cells, fibroblasts, myeloid cells, and endothelial cells, all of which express CD58 and thus have the potential to be adaptive NK cell-interacting partners (53). CMV infection is a common infection, with a prevalence close to 40% in HIV-uninfected Canadians that increases with age (54–56). In ART-naive PLWH enrolled in the Montreal PI cohort, the prevalence of CMV coinfection is 84% (57). It would be interesting to investigate whether the higher frequency of the *NKG2C*$^{-/-}$ genotype in PLWH than in HESN subjects is linked to differential activation of these *NKG2C*$^{-}$ adaptive NK cells in PLWH than in HESN subjects due to factors such as differential levels of CMV infection or other factors that affect NK cell activity in a manner that influences HIV susceptibility.

We observed that the percentages of NKG2C^{+} NK cells in CMV^{+} PLWH and in CMV-monoinfected persons differed according to NKG2C genotype. CMV infection drives the expansion of NKG2C^{+} NK cells (9, 13, 31). This was the rationale for confining this analysis to PLWH and HIV-uninfected subjects who were CMV seropositive. Cell surface NKG2C percentage, MFI, and intensity of fold change over background in the MFI of NKG2C expression results reported by others did not test for CMV serostatus, which if negative, would preclude the expansion of NKG2C^{+} NK cells (33). In CMV-monoinfected subjects, differences in the percentages and intensities of NKG2C expression between *NKG2C*$^{+/+}$ *and NKG2C*$^{+/-}$carriers were not significant. However, these values in CMV^{+} PLWH compared to CMV-monoinfected persons were higher for cells from carriers of both *NKG2C*$^{+/+}$ and *NKG2C*$^{+/-}$ genotypes, as has been seen by others (31, 32, 58).

Treatment-naive VL set point is associated with the rate of HIV disease progression, as measured by time to CD4 counts of ,200/mm^3, AIDS, and death (37, 38). We found no significant correlations between either the percentage of NKG2C$^+$ CD56^{dim} NK cells or the intensity of NKG2C expression on NK cells and the VL set point. This was also the case for correlations between the percentage of NKG2C$^+$ CD57$^+$ and NKG2A$^+$ CD56^{dim} NK cells and the VL set point. These results differ from those of others who correlated the percentage of NKG2C$^+$ cells with single VL measures in ART-naive individuals. Thomas et al. found a positive correlation between these parameters, although their analysis only included 7 untreated subjects in the chronic phase of infection (33). In contrast, Ma et al. found a negative correlation between the percentage of NKG2C$^+$ NK cells and concurrent VL in 22 treatment-naive PLWH infected at least 120 days, which corresponded to the VL set point (34). Gondois-Rey et al. also found a negative correlation between the percentage of NKG2C$^+$ NK cells and concurrent VL in 18 treatment-naive subjects tested at time points in acute/early infection (35). The analysis performed here was done on a larger group of 43 HIV$^+$ and CMV$^+$ individuals together and stratified by *NKG2C* genotype. To our knowledge this is the first report investigating correlations between the intensity of NKG2C expression on NK cells and VL set point. Overall, we found no evidence that NKG2C$^+$ NK cell parameters influenced VL set point, which is a determinant of the rate of HIV disease progression.

γδ T cells also express NKG2C and have been shown to respond to HIV-infected cells (59, 60). Future studies should explore the link between *NKG2C* genotype, CMV infection, and frequency of NKG2C-expressing γδ T cells at the level of susceptibility/resistance to HIV infection and at the level of HIV control.

In summary, our results support that carriage of the *NKG2C*$^{-/-}$ genotype is associated with higher susceptibility to HIV infection, particularly by the parenteral infection route. Although, *NKG2C* copy number was associated with percentage and intensity of NKG2C expression on NK cells, these parameters did not correlate with HIV VL set point.

2.7.ACKNOWLEDGMENTS

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2.8. FIGURE AND LEGENDS

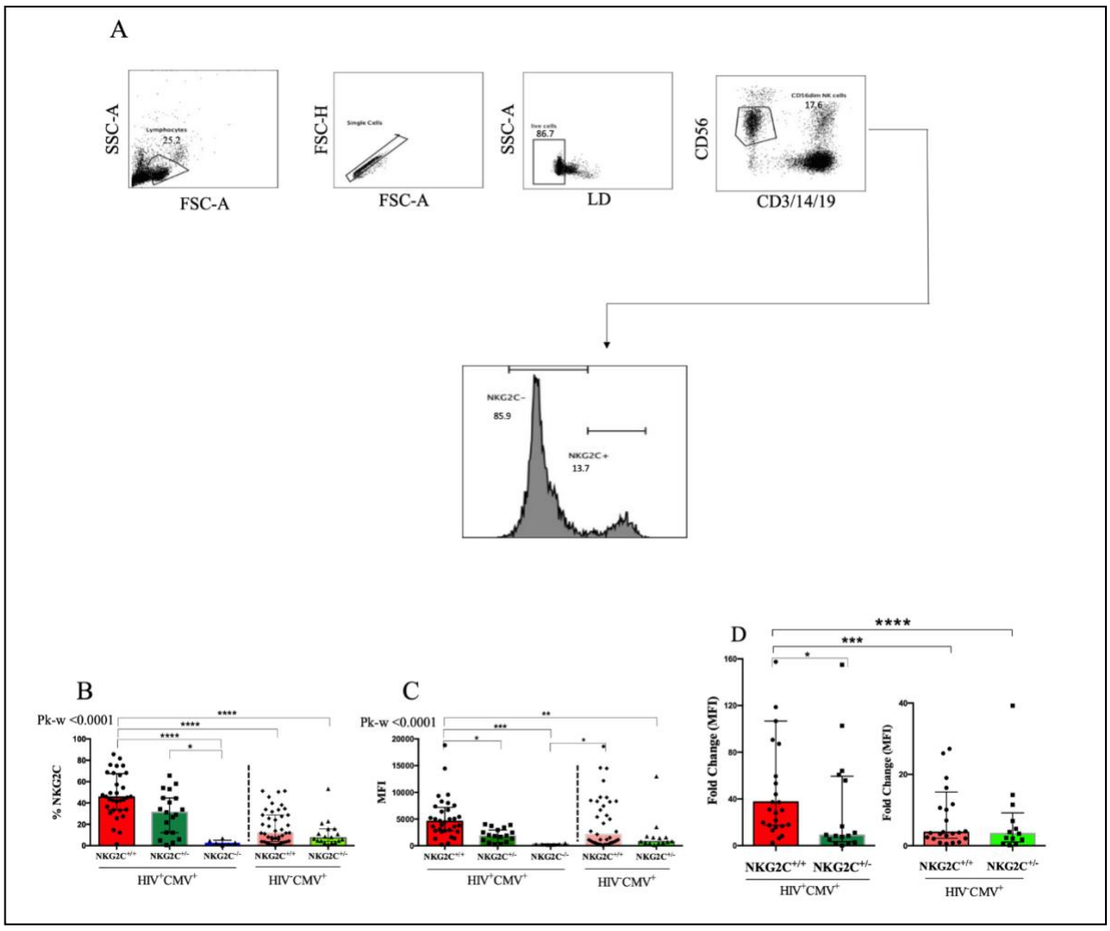

Figure 1.2. Evaluation of the frequency of NKG2C^{+}NK cells and mean fluorescence intensity (MFI) of NKG2C^{+} expression

(A) Shown is the gating strategy used to detect the frequency and MFI of NKG2C expression. Peripheral blood mononuclear cells were stained for viability and cell surface CD3, CD56, CD14, CD19 and NKG2C. CD3^{-}CD14^{-}CD19^{-}CD56^{dim} NK cells were gated on from the live, singlet, lymphocyte gate. From these, we determined the frequency NKG2C^{+} CD56^{dim} NK cells and MFI of NKG2C expression on NK cells. The frequency (B) and MFI (C) of NKG2C expression is shown for cells from HIV^{+}CMV^{+} and HIV^{-}CMV^{+} individuals carrying the *NKG2C*$^{+/+}$, *NKG2C*$^{+/-}$ and *NKG2C*$^{-/-}$ genotypes. Each point represents a single individual. Bar graph heights and error bars represent medians and interquartile ranges for the group. FSC-A, forward scatter area; SSC-A, side scatter area; LD, live/dead; FSC-H, forward scatter height, Pk-w, p-value for the Kruskal-Wallis test used to analyze the significance of differences between groups; "*", $p < 0.05$; "***", $p < 0.001$; "****", $p < 0.0001$.

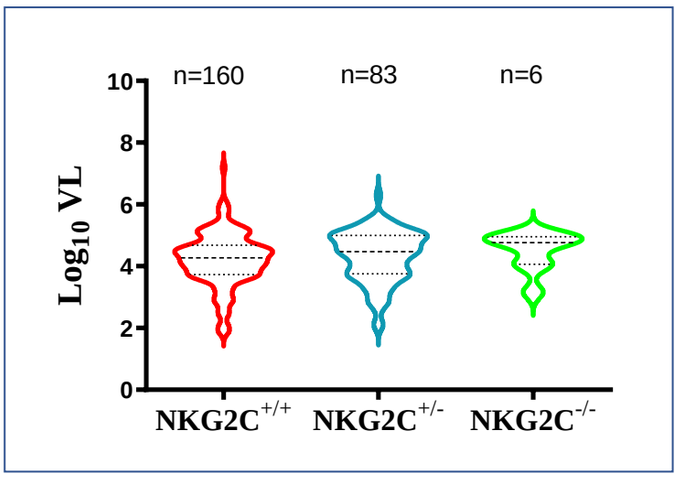

Figure 2.2. Log10 viral load (VL) set points in people living with HIV (PLWH) carriers of the *NKG2C*$^{+/+}$, *NKG2C*$^{+/-}$, and *NKG2C*$^{-/-}$ genotypes

Violin plot showing the median and interquartile range of the treatment naïve $\log_{10}$ VL set point in each *NKG2C* genotype group. The number of subjects included in each group is shown above each data set. A Kruskal-Wallis test was used to assess the significance of between-group differences in $\log_{10}$ VL set point.

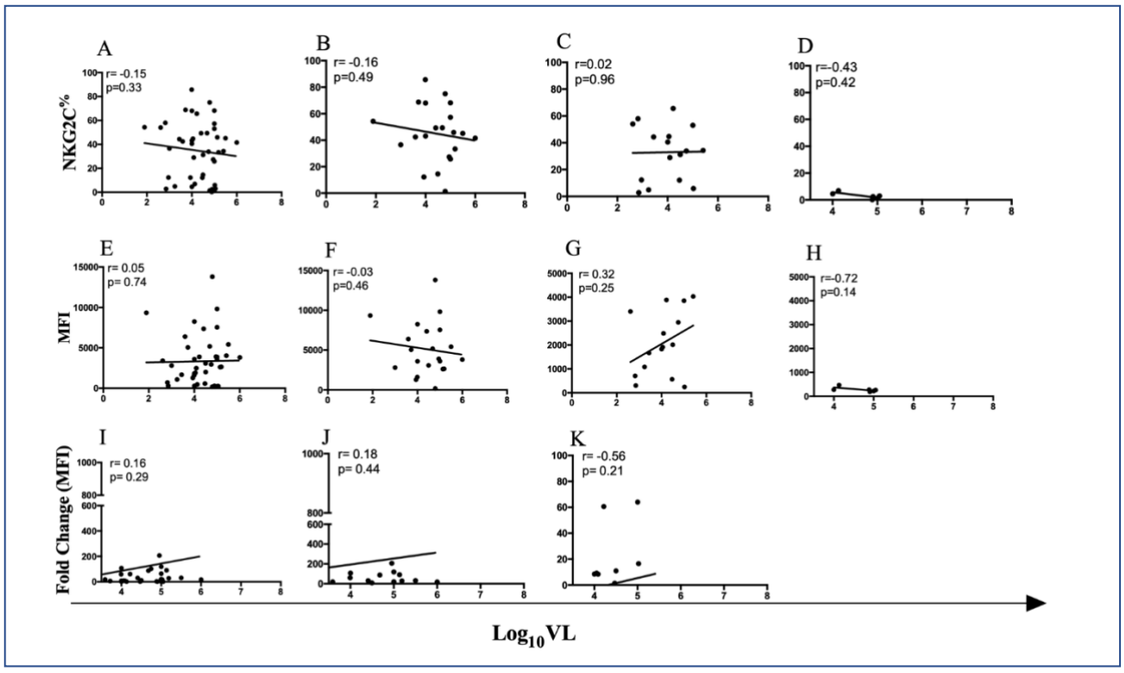

Figure 3.2. Correlation between log10 VL set point and frequency of NKG2C^{+} NK cells and mean fluorescence intensity (MFI) of NKG2C expression in cells form HIV^{+}CMV^{+} NKG2C$^{+/+}$, NKG2C$^{+/-}$, and NKG2C$^{-/-}$ carriers

Correlations between the frequency (A-D) and MFI (E-H) of NKG2C expression on NK cells from CMV^{+} PLWH with $\log_{10}$ VL for carriers of all *NKG2C* genotypes tested (A, E) and stratified by *NKG2C*$^{+/+}$ (B, F), *NKG2C*$^{+/-}$ (C, G) and *NKG2C*$^{-/-}$ (D, H) genotypes. The number of subjects tested, the correlation coefficients (r) and the p-values for each correlation are shown in the inset at the top left corner of the graphs.

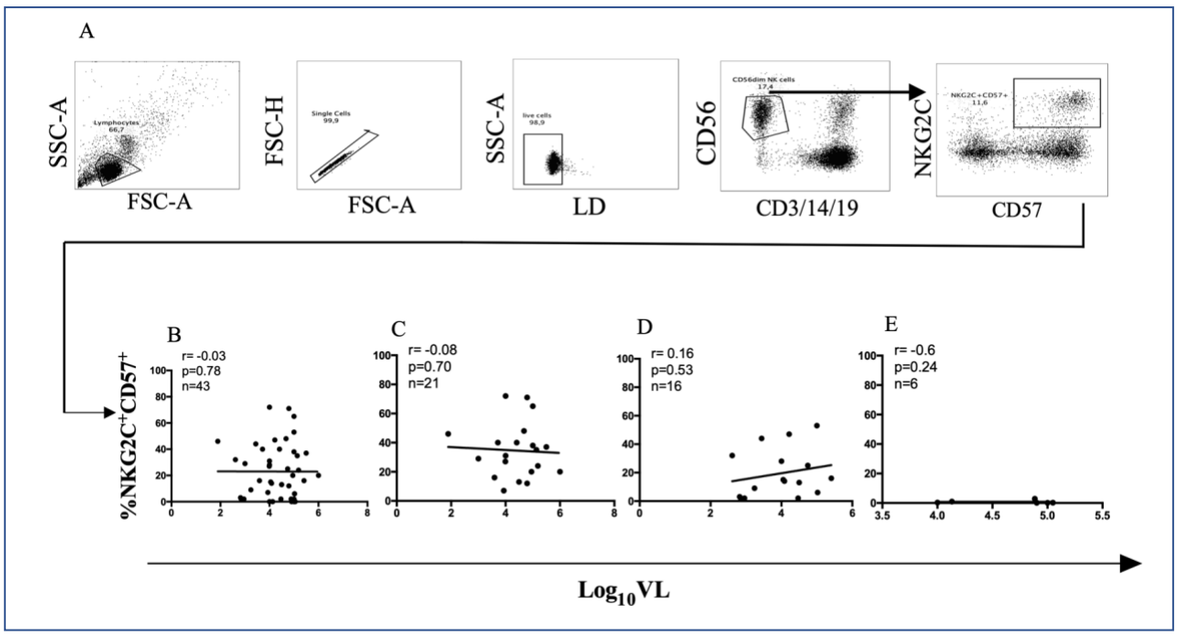

Figure 4.2. Correlation between log10 (VL) viral load set point and frequency of NKG2C^{+}CD57^{+} cells from CMV^{+}PLWH carrying the three possible NKG2C genotypes

(A) From the live singlet lymphocytes gated on in Figure 1, CD56^{dim} CD3^{-}CD14^{-}CD19^{-} NK cells were gated on (left panel). From these NKG2C^{+}CD57^{+} NK cells were gated onto assess the frequency of these cells among CD56^{dim} NK cells. Correlations between the frequency of NKG2C^{+}CD57^{+} (B-E) CD56^{dim} NK cells with log_{10} viral load set point from for CMV^{+} PLWH carrying all *NKG2C* (B), *NKG2C*$^{+/+}$ (C), *NKG2C*$^{+/-}$ (D) and *NKG2C*$^{-/-}$ (E) genotypes. The number of subjects tested, the correlation coefficients (r) and the p-values for each correlation are shown in the inset at the top left corner of the graphs.

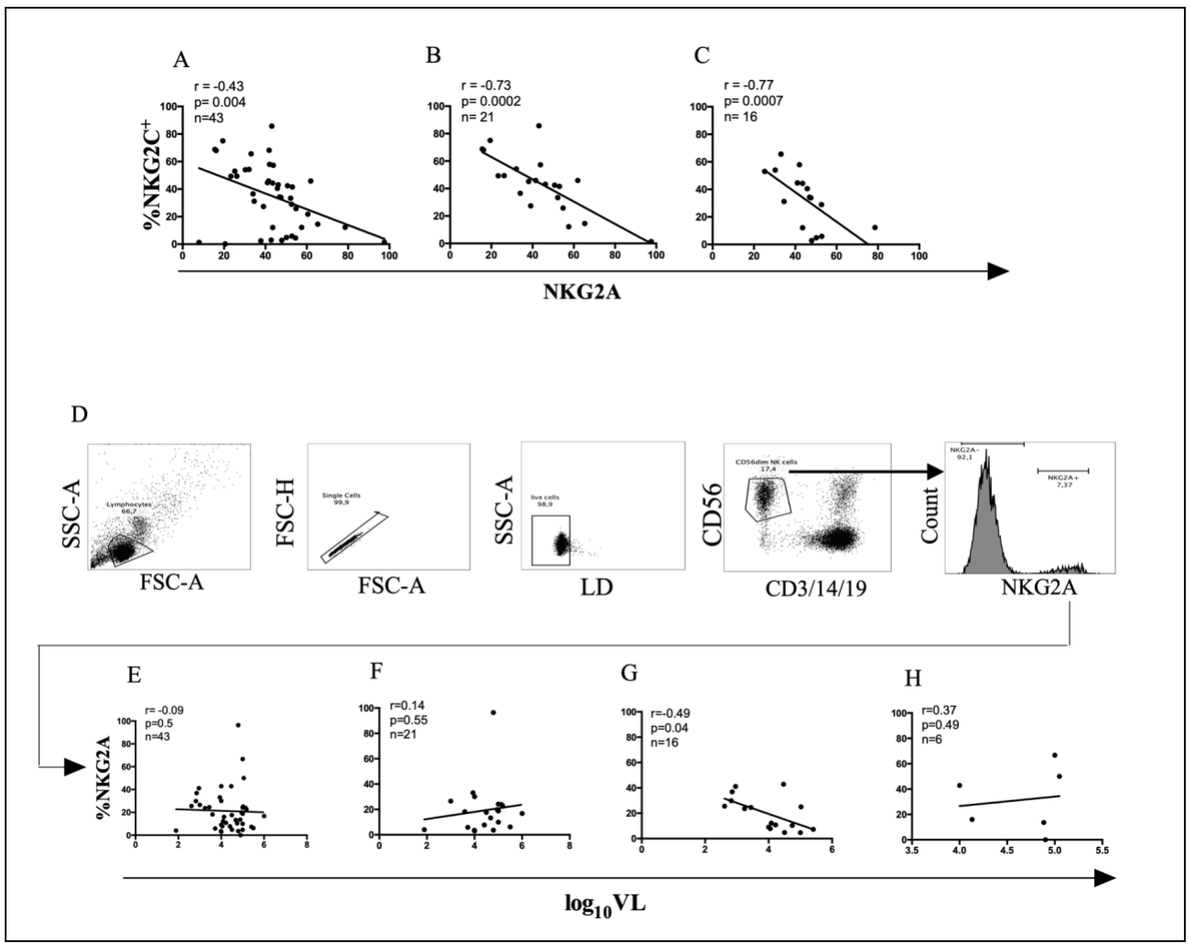

Figure 5.2. Correlation between log10 VL set point and frequency of NKG2A^{+}NKG2C^{-} CD56dim NK cells from CMV^{+}PLWH carrying the three possible *NKG2C* genotypes

Correlations between the frequency of NKG2C^{+} CD56^{dim} and NKG2A^{+} CD56^{dim} NK cells from CMV^{+} PLWH for carriers of all NKG2C (A), NKG2C$^{+/+}$(B), and NKG2C$^{+/-}$ (C) genotypes. The number of subjects tested, the correlation coefficients (r) and the P values for each correlation are shown in the inset at the top left corner of each graph. (D) Shown is the strategy used to gate on CD56^{dim} CD3^{-} CD14^{-} CD19^{-} NK cells, from which NKG2A^{+} NKG2C^{-} cells were gated onto assess their frequency among CD56^{dim} NK cells. Correlations between the frequency of CD56^{dim} NKG2A^{+} NKG2C^{-} NK cells with $\log_{10}$ VL set point from for CMV^{+} PLWH carrying all NKG2C (E), NKG2C$^{+/+}$(F), NKG2C$^{+/-}$ (G), and NKG2C$^{-/-}$ (H) genotypes. The numbers of subjects tested, the correlation coefficients (r), and the P values for each correlation are shown in the top left corner of the graphs.

Table 1.2. Study population demographics

Population	PLWH[1] N=434	HIV- N=157	P-value
Males	408 (94.0)[2]	141 (89.8)	
Females	25 (5.8)	23 (14.6)	
HIV Exposure Risk Group			
Sexually exposed	371	78	
MSM[3]	337	67[4]	
Heterosexually exposed men	9	3	
Heterosexually exposed women	25	8	
IDU[5]	63	79	
Low risk control		11	
Ethnicity			
Caucasian	384 (88.5)	146 (92.9)	0.11
American/African Black	19 (4.5)	4 (2.5)	0.25
Latino	27 (6.2)	5 (3.2)	0.19
Asian	4 (0.9)	2 (1.3)	0.76

[1]People Living with HIV; [2]N (percent); [3]Men who have Sex with Men; [4]At risk for sexual exposure [5]Injection Drug User.

Table 2.2. NKG2C allele and genotype frequencies in People Living with HIV and HIV Exposed Seronegative subjects

NKG2C allotype/genotype	PLWH[1]	HESN[2]	OR[3]	95% CI[4]	P-value
All	**N = 434**	**N = 157**			
nkg2c^{+}	80.5[5]	80.93	1.0	0.5-2.1	1.00
nkg2c^{-}	19.5	19.1			
NKG2C$^{+/+}$	276 (63.6)[6]	97 (61.8)	1.1	0.7-1.6	0.70
NKG2C$^{+/-}$	147 (33.9)	60 (38.2)	0.84	0.58-1.24	0.38
NKG2C$^{-/-}$	11 (2.53)	0	8.6**	0.5-146.0**	0.04*
NKG2C$^{+/-}$ + *NKG2C*$^{-/-}$	158 (36.4)	60 (38.2)	0.9	0.6-1.5	0.70
SE[7]	**371**	**78**			
nkg2c^{+}	81.4	78.2	1.21	0.6-2.4	0.72
nkg2c^{-}	18.6	21.9			
NKG2C$^{+/+}$	240 (64.7)	44 (56.4)	1.4	0.9-2.3	0.20
NKG2C$^{+/-}$	124 (33.4)	34 (43.6)	0.6	0.4-1.1	0.09
NKG2C$^{-/-}$	7 (1.9)	0	3.2**	0.2-57.2**	0.61*
IDU[8]	**63**	**79**			
nkg2c^{+}	75.4	83.5	0.6	0.3-1.2	0.21
nkg2c^{-}	24.6	16.5			
NKG2C$^{+/+}$	36 (57.1)	53 (67.1)	0.6	0.3-1.3	0.29
NKG2C$^{+/-}$	23 (36.5)	26 (32.9)	1.2	0.6-2.3	0.72
NKG2C$^{-/-}$	4 (6.3)	0	12.0**	0.6-277.7**	0.04*

[1]People Living with HIV; [2]HIV Exposed Seronegative; [3]Odds Ratio; [4]95% Confidence Intervals; [5]Allele frequency; [6]NKG2C genotype N (frequency); [7]Sexually Exposed; [6]Injection Drug User.
*Fisher exact test; ** Haldane's correction

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BRIDGE FROM CHAPTER II TO CHAPTER III

In chapter II, I assessed whether *NKG2C* genotype distribution differed in PLWH compared to a group of HESN who remained HIV seronegative despite multiple exposures to HIV. These HESN individuals would have a higher likelihood of being resistant to HIV infection than people not exposed to HIV. I also examined whether there was a link between *NKG2C* genotype and HIV control in those infected. This works was done to address whether certain *NKG2C* genotypes were associated with susceptibility/resistance to HIV infection or HIV control in those infected. In chapter I, I reviewed what is known regarding the role of NK cells in the development of CVD in PLWH and in HIV uninfected individuals. Martínez-Rodríguez et al. reported that NKG2C^{+} adapNK cells were more frequent in patients with carotid atherosclerotic plaque (CAP) that was advanced enough to require an endarcterectomy. I had access to peripheral blood samples from the CHACS, a cohort of aging PLWH who have a high frequency of CMV co-infection. A subset of CHACS participants underwent a CT scan that measured TPV, a preclinical marker of coronary AS and CVD. This provided an opportunity to investigate whether adapNK cells were implicated in early stages of CVD. To our knowledge, the involvement of NKG2C^{+} adapNK cells in CVD pathogenesis among CMV^{+}PLWH compared to the CMV mono-infected subjects, is currently unclear. In chapter III, I tested the hypothesis that the frequency of NKG2C^{+} adapNK cells would be associated with TPV in CMV^{+}PLWH and CMV mono-infected subjects.

Chapter III

3. High Frequencies of Adaptive NK Cells are Associated with Absence of Coronary Plaque in Cytomegalovirus Infected People Living with HIV

Khlood Alsulami, MSc[1, 2, 3], Manel Sadouni, MD[4], Daniel Tremblay-Sher MD[4], Jean-Guy Baril, MD[5], Benoit Trottier, MD[5], Franck P. Dupuy, PhD[1, 3], Carl Chartrand-Lefebvre, MD[4,6], Cécile Tremblay, MD[4,7], Madeleine Durand, MD[4,7], Nicole F. Bernard, PhD[1, 2, 3, 8,*]

[1]Research Institute of the McGill University Health Centre (RI-MUHC), Montreal, QC, Canada.
[2]Division of Experimental Medicine, McGill University, Montreal, QC, Canada.
[3]Infectious Diseases, Immunology and Global Health Program, Research Institute of the McGill University Health Centre, Montreal, QC, Canada.
[4]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montréal, QC, Canada.
[5]Clinique de Médecine Urbaine du Quartier Latin, Montréal, QC, Canada.
[6] Département de Radiologie, Radio-oncologie et Médecine Nucléaire, Faculté de Médecine, Université de Montréal, Montré, QC, Canada.
[7]Department of Microbiology Infectiology and Immunology, University of Montreal, Montreal, QC, Canada.
[8]Division of Clinical Immunology, McGill University Health Centre, Montreal, QC, Canada

*Corresponding author: Nicole F. Bernard, PhD nicole.bernard@mcgill.ca

Research Institute of the McGill University Health Centre, Glen site, Bloc E, 1001 Decarie Blvd., Room EM3.3238, Montreal, Quebec, H3A 3J1, Canada.

Tel: (514) 934-1934 x-44584; Fax: (514) 933-1562

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Keywords: HIV, Cytomegalovirus, Aging, Atherosclerosis, Natural Killer cells, Adaptive Natural Killer cells

3.1. ABSTRACT

Objective: To evaluate whether adaptive NKG2C^{+}CD57^{+} natural killer (adapNK) cell frequencies are associated with pre-clinical coronary atherosclerosis in participants of the Canadian HIV and Aging Cohort Study (CHACS).

Design: This cross-sectional study included 194 CHACS participants aged ≥40 yrs of which 128 were CMV^{+} people living with HIV (PLWH), 8 were CMV^{-}PLWH, 37 were CMV mono-infected and 21 were neither HIV nor CMV infected. Participants were evaluated for the frequency of their adapNK cells and total plaque volume (TPV).

Methods: TPV was assessed using cardiac computed tomography. Participants were classified as free of, or having, coronary atherosclerosis if their TPV was "0" and >0", respectively. The frequency of adapNK cells was categorized as low, intermediate or high if they constituted <4.6%, between ≥4.6% and 20% and >20%, respectively, of the total frequency of CD3^{-}CD56^{dim} NK cells. The association between adapNK cell frequency and TPV was assessed using an adjusted Poisson regression analysis.

Results: A greater proportion of CMV^{+}PLWH with TPV=0 had higher adapNK cell frequencies than those with TPV>0 [61.90% versus 39.53%, p=0.03] with a similar non-significant trend for CMV mono-infected participants [46.15% versus 34.78%]. The frequency of adapNK cells was negatively correlated with TPV. A high frequency of adapNK cells was associated with a relative risk of 0.75 (95% confidence intervals 0.58, 0.97, p=0.03) for presence of coronary atherosclerosis.

3.2. INTRODUCTION

Antiretroviral therapy (ART) has transformed human immunodeficiency virus (HIV) infection into a treatable, chronic disease. ART stops progression to the acquired immunodeficiency syndrome in most people living with HIV (PLWH), diminishes morbidity, lengthens survival and prevents HIV transmission (1). However, PLWH have higher levels of immune activation than uninfected individuals, leading to the development of non-AIDS comorbidities including cancer, kidney, liver and cardiovascular disease (CVD)(2) . Long term ART treated PLWH have a 1.5 to 2 times higher risk of developing various manifestations of CVD (3-5). Aside from classic risk factors for CVD, co-infection with herpesviruses may have a substantial effect on the progression of atherosclerosis and cardiovascular risk. Infection with cytomegalovirus (CMV) is thought to be involved in the development of atherosclerosis based on clinical, epidemiological and experimental studies and has been proposed to contribute to the progression of atherosclerotic plaque to heart disease and stroke (6).

CMV infection drives the persistent expansion of a peripheral blood natural killer (NK) cell subset that expresses the cell surface activating NK receptor (NKR) NKG2C (7, 8). NKG2C, which forms a heterodimer with CD94 are members of the C-type lectin receptor family (9). The ligand for NKG2C/CD94, like its inhibitory counterpart, NKG2A/CD94 is the non-classical major histocompatibility complex 1b antigen HLA-E bound to epitopes derived from the leader sequence of several HLA antigens (10, 11). Peptides originating from the UL40 gene product of human CMV complexed with HLA-E together with pro-inflammatory signals control the expansion and differentiation of $NKG2C^+$ NK cells (12). $NKG2C^+$ NK cells are called adaptive NK (adapNK) cells because they have properties ascribed to adaptive immune cells such as the ability to expand following acute CMV infection in patients receiving transplants from CMV infected donors (13-16), ability to expand upon CMV reactivation (14, 16, 17), persistence and epigenetic regulation of enhanced effector functions that are similar to those seen in memory $CD8^+$ T cells (18). Most $NKG2C^+$ NK cells also express CD57, which is a marker of mature NK cells (7, 19-22).

Since CMV infection drives the expansion of $NKG2C^+CD57^+$ adapNK cells, we questioned whether the frequency of these cells was associated with subclinical CVD. We found that CMV^+PLWH and CMV-mono-infected persons without subclinical coronary atherosclerosis had higher frequencies of $NKG2C^+CD57^+$ adapNK cells than those with coronary atherosclerosis.

3.3. MATERIAL AND METHODS

Ethics statement

This research study was approved by the Research Ethics Boards of the Centre Hospitalier de l'Université de Montréal and the McGill University Health Centre (Project Identification Code 2019-4605). It was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from each study subject for the collection of specimens, subsequent analyses and publication of results obtained from these analyses.

Study subjects

The current study is a cross sectional analysis, nested within the Canadian HIV and Aging Cohort Study (CHACS), which has been described previously (23). Briefly, CHACS inclusion criteria were to be ≥40 yrs of age, or to have lived with HIV for at least 15 yrs. Consecutive participants from CHACS who were free of clinically overt CVD at baseline and presented a Framingham risk score ranging between 5-20% were invited to participate in the cardiovascular imaging sub-study (24). Of the 219 participants in the imaging sub-study, the 194 participants with complete data and available blood samples were included in our analyses. Of these, 128 were CMV^{+}PLWH, 8 were CMV^{-}PLWH, 37 were CMV mono-infected and 21 individuals were negative for both HIV and CMV infection. Participants were recruited from HIV and sexually transmitted disease clinics in Montreal, QC, Canada. Most were men who have sex with men. The PLWH had been on ART for a median of 15 yrs and had viral loads <50 copies/ml of plasma. Data on all traditional cardiovascular risk factors were collected prospectively as part of the CHACS study visits.

Definition of subclinical coronary atherosclerosis

Subclinical coronary atherosclerosis was defined by the presence of atherosclerotic plaque in the coronary arteries, measured using a 256-slice cardiac computed tomography (CT) scanner (Brilliance iCT; Philips Healthcare, Cleveland, OH) with injection of contrast media. Details of the imaging procedure are published elsewhere (24). Briefly, every atherosclerotic plaque in the coronary arteries were identified. Their volume (in mm^3) was measured using advanced software and summed to obtain the total plaque volume (TPV). Therefore, TPV represent the total burden of coronary atherosclerosis for every participant. For this analysis, TPV was dichotomised as 0 (absence of subclinical coronary atherosclerosis) or greater than 0 (presence of coronary atherosclerosis) (24).

All imaging studies were performed at the Centre Hospitalier de l'Université de Montréal, Montreal, QC, Canada, and interpreted by a board-certified cardiothoracic radiologist (CC-L). All radiology personnel performing image interpretation and postprocessing were blinded to HIV and CVM status.

Laboratory measurements

HIV infection was diagnosed by quantifying HIV-1 p24 antigen/antibody in plasma and confirmed by Western blot as previously reported (25). Blood samples collected into vacutainers containing ethylenediaminetetraacetic acid (EDTA) anticoagulant were obtained from CHACS participants at each study visit. Blood was processed into plasma, which was stored frozen at -80^{o}C until use. Peripheral blood mononuclear cells (PBMC) were isolated from blood by centrifugation over a ficoll-hypaque gradient at 400xg for 30 min. Cells were then washed and frozen in 90% fetal bovine serum (FBS); 10% dimethyl sulfoxide. Cryovials containing cells were stored in liquid nitrogen until use. CD4 and CD8 T cells counts were measured using 4-color flow cytometry. All participants enrolled in this study were tested for CMV serostatus using commercially available ELISA kits measuring the presence of anti-CMV specific IgG (Abcam, Waltham, MA).

Staining PBMC for adapNK cells

Frozen PBMCs were thawed and resuspended in RPMI 1640; 5% FBS; 2mM L-glutamine; 50 international units/ml penicillin; 50 mg/mL streptomycin (R5) (all from Wisent, Inc., Saint-Jean-Baptiste, QC, Canada). LIVE/DEAD fixable dead cell stain (Invitrogen, Saint Laurent, QC, Canada) was added to the PBMCs as per manufacturer's directions before surface staining cells using a panel that included the following fluorochrome conjugated antibodies to CD3-BV785 (clone OKT3), CD14-BV785 (M5E2), CD19-BV785 (HIB19), CD56-BV605 (HCD56) (all from BioLegend, San Diego, CA), CD57-PE (TB01) (Life Technologies, Burlington, ON, Canada), CD16-APC-Cy7 (3G8) (BD Bioscience, Baltimore, MD), NKG2C-PE-Vio770 (REA205) and NKG2A-APC (REA110) (Miltenyi Biotec, Auburn, CA).

Flow cytometry

A total of $1.5x10^6$ to $1.8x10^6$ events were acquired for each sample using an LSR Fortessa instrument (BD Bioscience, San Jose, CA). Results were analyzed using FlowJo^{TM} software v10.3 (BD, Ashland, OR). NK cells were identified as live, singlet, $CD3^-CD14^-CD19^-CD56^{dim}$ lymphocytes. AdapNK cells were distinguished from conventional NK (cNK) cells based on expression of cell surface NKG2C. AdapNK cells were defined as $CD3^-CD14^-CD19^-CD56^{dim}$ $NKG2C^+CD57^+$ while cNK cells were defined as $CD3^-CD14^-CD19^-CD56^{dim}$ $NKG2C^-$ cells. Florescence minus one staining was used to set gates for each experiment. Single-stained beads (BD^{TM} CompBead, BD Bioscience) were used to set compensation. The mean plus 2 standard deviations (SD) frequency of $NKG2C^+CD57^+$ NK cells in CMV seronegative individuals was 4.6%. CMV seropositive donors having a frequency of adapNK cells <4.6% were classified as having a "low" level of adapNK cell expansion. Those with frequencies of adapNK cells of between 4.6% and <20% and ≥20% were classified as having "intermediate" and "high" levels of adapNK cell expansion, respectively.

Statistical Analysis

GraphPad Prism 6 (GraphPad Software, La Jolla, CA) was used for data analysis and graphical presentation. The significance of difference in the same variable between two, or more than two groups, were tested using non-parametric Mann-Whitney or Kruskal-Wallis tests with Dunn's post-tests, respectively. The significance of proportional between-group differences in sex, high blood pressure, statin use, anti-platelet use, diabetes, exercise and body mass index (BMI) was assessed using Chi-square tests. Exercise frequency was categorized into two groups. Participants who exercised 30 min every day or 3 times per week were considered physically active while those who exercised 30 min weekly or less, were classified as not physically active. Smoking status categories included non-smokers and smokers. Smokers were categorized according to smoking intensity measured as total number of pack-years smoked (with one pack-year representing one year of smoking one pack a day). The significance of correlations between two variables were assessed using non-parametric Spearman tests. Results were considered significant when p-values were <0.05 (two-tailed).

We used separate univariable and multivariable models of modified Poisson regression with robust variance to assess the association of the frequency of adapNK cells with the presence/absence of subclinical coronary atherosclerosis. A parsimonious approach was used to build the multivariable models. Potential confounders were identified based on a priori knowledge and included HIV status and CVD risk factors (age, high blood pressure, smoking exposure, LDL cholesterol, statin use and BMI). Potential confounders were entered into the multivariable model if they showed a univariable association with TPV with a p-value ≤ 0.1 and kept in the final model if they were independently associated with TPV or if they modified the point estimate for other predictors by more than 10%. Effect modification by HIV was assessed by inclusion of an interaction term to the fully adjusted model. Although considered potential confounders, sex and diabetes were not included into the final model due to the small number of participants who had diabetes and who were women, when prevented adjusting for these variables. Adjusted odds ratios (OR) and prevalence ratios were reported with 95% confidence intervals (CIs). No adjustments were made for multiple comparison. Statistical analyses were performed using R version 3.4.3 software (R Foundation for Statistical Computing, Vienna, Austria).

3.4. RESULTS

Study participant characteristics

One hundred and ninety-four CHACS participants were included in the imaging sub-study and in the investigations presented here. These individuals were classified into four groups based on their HIV and CMV infection status. The number of individuals in each of the 4 groups, their median (interquartile range [IQR]), age and sex distribution as well as their clinical characteristics are provided in Table 1. Between-group differences in sex and age were significant ($p=0.002$ and $p=0.03$, Chi-square and Kruskal-Wallis tests, respectively). Participants were more likely to be male. Compared to CMV mono-infected participants, CMV^+PLWH had a history of higher smoking intensity, lower levels of low-density lipoproteins (LDL) and lower D-dimer levels, ($p<0.05$ for all, Dunn's post-tests), fewer had high blood pressure and were physically active ($p=0.03$ and $p<0.0001$, respectively, Chi-square tests). Other variable listed in Table 1 did not differ significantly between groups or between CMV^+PLWH and CMV^-PLWH for HIV related clinical characteristics. $CD4^+$ T and $CD8^+$ T cell counts and CD4/CD8 ratios did not differ significantly between CMV^+PLWH and CMV^-PLWH participants (Table 1).

Frequency of $NKG2C^+CD57^+$adapNK cells in CHACS participants

All participants were tested for the frequency of their $NKG2C^+CD57^+CD56^{dim}$ adapNK cells. The strategy used to gate on these cells is shown in Figure 1A. The frequency of adapNK cells did not differ significantly between CMV^+PLWH (16.8 [6.6-38.5]) and CMV mono-infected (16.3 [8.9-33.9]) participants (Figure 1B). The frequency of adapNK cells was significantly higher in CMV^+PLWH and CMV mono-infected persons than in CMV^-PLWH (2.5 [1.0 -4.9]) and CMV^-HIV^- subjects (2.0 [0.9-2.9]) ($p<0.0001$, for all four comparisons, Dunn's post-tests). The distribution of adapNK cell frequencies in CMV^+PLWH and CMV mono-infected individuals categorized as having low, intermediate and high levels of adapNK cells is shown in Supplemental Figure 1.

The frequency of adapNK cells was higher in CMV^+ participants without subclinical coronary atherosclerosis

Supplementary Figure 2 shows the distribution of TPV in 128 CMV^+PLWH (A) and 36 CMV mono-infected participants (B) having low, intermediate and high frequencies of adapNK cell. The median TPV tended to decrease (showing a smaller burden of coronary atherosclerosis) as the frequency of adapNK cells increased, with differences being significant for comparisons between participants with low versus high adapNK cell frequencies in CMV^+PLWH only.

Table 2 shows the association of presence/absence of coronary atherosclerosis in CMV^+ participants (both PLWH and CMV mono-infected) with adapNK cell frequency categories and other variables. A significantly higher frequency of CMV^+ persons without coronary atherosclerosis had high frequencies of adapNK cells (p=0.01, Chi-square test). CMV^+ persons without, compared to, with coronary atherosclerosis were younger (p=0.006, Mann-Whitney test), had a lower exposure to smoking (p=0.02, Mann Whitney test) and lower D-dimer levels (p=0.004, Mann-Whitney test). The other variables in Table 2 did not differ significantly between CMV^+ participants without and with coronary atherosclerosis.

A significantly greater proportion of CMV^+PLWH without, compared to with, coronary atherosclerosis (TPV = 0 versus TPV > 0) had a high frequency of adapNK cells (p=0.03, Chi-square test) (Figure 2 upper pie chart graphs). In CMV mono-infected participants, there was a non-significant trend towards a higher frequency of adapNK cells in persons without compared to with coronary atherosclerosis (p=0.19, Chi-square test) (Figure 2 lower pie chart graphs).

For all CMV^+ individuals, there was a weak, though significant negative correlation between the frequency of adapNK cells and TPV (Figure 3A). Correlations between these 2 parameters were significant for CMV^+PLWH (Figure 3B) but only trended towards significance for CMV mono-infected participants (Figure 3C). Together, these results show that increasing frequencies of adapNK cells were associated with less subclinical coronary atherosclerosis in all CMV^+ persons, a finding that was also apparent when CMV^+PLWH and CMV mono-infected participants were considered separately.

CMV^+PLWH and CMV mono-infected participants with high frequencies of adapNK cells have a reduced risk of coronary atherosclerosis

The proportion of participants with coronary atherosclerosis did not differ significantly between CMV^+PLWH and CMV mono-infected participants (p=0.71, Chi-square test) (Figure 4A). There were also no between-group differences in the distribution of TPV when analysed as a continuous variable (p=0.37, Mann-Whitney test) (Figure 4B). Finally, among CMV^+ participants, we did not observe effect modification by HIV status of the association between adapNK cells frequency and presence of coronary atherosclerosis. The p-value of the interaction term between HIV status and adapNK cell frequency was 0.28.

This prompted us to combine results for 128 CMV^+PLWH and 36 CMV mono-infected participants for the purpose of carrying out an independent multivariable Poisson regression after adjusting for traditional cardiovascular risk factors (Table 3). In this adjusted analysis, a high frequency of adapNK cells was associated with a relative risk (RR) of 0.75 (95% CI, 0.58-0.97, p=0.03) for presence of coronary atherosclerosis, independently of other factors. Each 10-year increase in age was associated with an increased RR for coronary atherosclerosis of 1.23 (1.06-

1.44, p=0.006). Smoking intensity was also associated with a RR of increased coronary plaque of 1.09 (1.05-1.13, p< 0.001) for each additional pack-year of exposure. On the other hand, there was no evidence of an association between HIV and coronary atherosclerosis (RR 1.08 [0.81-1.42], p=0.58). There was no evidence of interaction by HIV status in these analyses.

3.5. DISCUSSION

Although ART is effective at controlling HIV replication, aging in PLWH, is associated with greater non-AIDS morbidities, such as CVDs than in age-matched uninfected persons (26). Increasing morbidity is associated with immune dysfunction, which persists despite treatment that suppresses HIV viral loads below the limit of detection (27). Persistent co-infections are common in PLWH and likely contribute to this HIV disease related immune dysfunction (28) Indeed, 94% of PLWH in the CHACS were CMV co-infected. Both HIV and CMV infections are independently associated with inflammation, morbidities related to inflammation and CVD risk, particularly in the elderly (29). A hallmark of CMV infection is the expansion of a population of adapNK cells (16, 17). In this report, we investigated whether the frequency of these adapNK cells was associated with pre-clinical atherosclerosis measured by TPV in CMV^+PLWH and CMV mono-infected individuals with frequencies of adapNK cells that varied widely from 0.19% to 78.6% of $CD3^-CD14^-CD19^-CD56^{dim}$ NK cells. The median (IQR) frequency of adapNK cells was similar in CMV^+PLWH and CMV mono-infected individuals who were ≥40 yrs of age, suggesting that CMV seropositivity, rather than HIV, drives adapNK cell frequency. In this study, CMV^+ participants with no subclinical atherosclerosis had higher levels of adapNK cells. This association remained after adjusting for traditional cardiovascular risk factors; CMV^+ subjects with high frequencies of adapNK cells had an RR (95% CI) of 0.75 (0.58-0.97, p=0.03) for presence of coronary atherosclerosis, indicating a significantly reduced risk.

Atherosclerosis is an inflammatory process in which immune cells and their mediators are important determinants of the disease process (30-34). HIV infection, even in successfully treated individuals, is characterized by higher immune activation levels than in uninfected persons (35). This likely predisposes PLWH to the development of atherosclerosis. Using coronary CT angiography, PLWH had a higher prevalence of subclinical atherosclerosis, particularly of non-calcified plaque, which is more vulnerable to rupture, than HIV seronegative persons with similar risk factors (24). This was the case even for PLWH with low Framingham scores and no evidence of CVD (36).

CMV infection reconfigures the immune system by driving the expansion of CMV-specific $CD8^+$ T cells that are pathogenic and independently related to higher levels of carotid intima-media thickness in PLWH (37). Up to 30% of all $CD8^+$ T cells in CMV^+ persons can be CMV-specific (38). When activated, these T cells can contribute to CVD pathogenesis by recognizing CMV epitopes present in plaque where CMV antigens and nucleic acids have been detected (39-41). AdapNK cells can control CMV infection (14, 18, 42). These cells may also regulate CMV-specific $CD8^+$ T cells, which express higher levels of HLA-E, the ligand for NKG2C, than do $CD8^+$ T cells of other specificities (43). CMV viremia activates CMV-specific $CD8^+$ T cells, further increasing their expression of HLA-E/CMV peptides, which, in turn, activates adapNK cells expressing NKG2C, the receptor for HLA-E/CMV peptide complexes. Once activated, adapNK cells have the potential to kill CMV-specific $CD8^+$ T cells to limit the CD8 T cell inflation observed in CMV^+ persons (43).

NKG2C and NKG2A are activating and inhibitory NKRs, respectively that are covalently associated with CD94 (44). They share sequence homology and ligands such as HLA-E complexed with epitopes derived from the leader sequence of HLA-A, B, C and G and epitopes from the UL40 CMV protein (10, 12). The interaction of these ligands with NKG2A is more avid than that with NKG2C and inhibitory signals tend to predominate those of activating signals (45, 46). Thus, if $NKG2C^+CD57^+$ NK cells co-expressed NKG2A, inhibition of $NKG2C^+$ NK cell functions could ensue. Others have reported that in $CD56^{dim}$ NK cells, expression of NKG2C generally excludes expression of NKG2A (47, 48). NK cell staining of samples from the CHACS studied here showed that among the $NKG2C^+CD57^+$ $CD56^{dim}$ NK cells <0.3% co-expressed NKG2A. This finding makes it unlikely that NKG2A expression on $NKG2C^+CD57^+$ NK cells, dampens the function of these cells. The titer of IgG antibodies to CMV was measured in a subset of 83 study participants. While there was a significant positive correlation between anti-CMV IgG titers and the frequency of $NKG2C^+CD57^+$ $CD56^{dim}$ NK cells, these antibody titers did not correlate with TPV.

Upon expansion, $NKG2C^+CD57^+$ NK cells acquire epigenetic changes that that distinguish them from cNK cells and regulate their effector functions (49, 50). AdapNK cells mediate higher levels of antibody dependent cellular cytotoxicity (ADCC) activity and higher levels of IFN-γ and TNF-α secretion upon activation than cNK cells (18, 50). CMV infection also induces ADCC competent CMV-specific antibodies, which can opsonize CMV antigens present in plaque. Higher frequencies of CD16 expressing adapNK cells would have an advantage over low frequencies of these cells to bind opsonized anti-CMV antibodies and activate adapNK cells to eliminate CMV infected cells within plaques by ADCC. These cells can also limit $CD8^+$ T cell inflation and damage caused by these cytolytic cells. This may explain why CMV^+PLWH with the highest levels of adapNK cells are more likely to have no subclinical atherosclerosis.

In addition to their role in anti-tumor and anti-viral responses, NK cells play a crucial role in repairing damaged tissues and maintaining tissue homeostasis (51). Following myocardial infarction, NK cell expansion from c-kit$^+$ bone marrow derived cells protected the heart by reducing cardiomyocyte apoptosis, deposition of collagen and subsequent fibrosis (52). In an experimental model of acute myocarditis, activated NK cells accumulated in the heart and released perforin, granzyme B and IFN-γ resulting in decreased fibrosis by inhibiting eosinophil activation and inducing eosinophil apoptosis (53). While this information is not specific to pre-clinical atherosclerotic plaques, it illustrates the potential of NK cells to repair damaged tissues and limit fibrosis.

Others reported that CMV driven expansion of NKG2C$^+$ NK cells was related to carotid atherosclerotic plaque (CAP) instability (54). They found higher frequencies of NKG2C$^+$ NK cells in persons with high-risk CAP than in those with non-high-risk CAP. This finding was interpreted as evidence that expansion of NKG2C$^+$ cells in subjects with CAP was associated with an increased risk of plaque destabilization (54). There is no clear explanation for the discrepancy between our results and those of Martinez et al. in terms of the role of NKG2C$^+$ NK cells in CVDs. The protective versus pathogenic role of adapNK cells may differ at different stages of the atherosclerotic process. In our investigations, pre-clinical atherosclerosis was assessed in persons with no clinical manifestations of CVD while in Martinez et al., plaque in carotid arteries was symptomatic and severe enough to warrant surgical removal of the CAP. Another possibility that may explain these discrepant results is that Martinez et al. enumerated the frequency of NKG2C$^+$ NK cells while we investigated the frequency of NKG2C$^+$CD57$^+$ NK cells. The CD57 marker improves the detection of NK cells with adaptive properties (16). Although the biology of the atheroscolerotic process is similar in coronary and carotid arteries there exist differences in plaque morphology and characteristics between the two sites. For example, the coronary atherosclerotic plaques are charecterized by a thiner fibrous cap, more intra-plaque hemorrhage and calcified nodules compared to plaque in carotid arteries (55). Further investigation is needed to explain the discrepancy between our results and those of Martinez et al.

This study had some limitations. The study population size, particularly for CMV mono-infected subjects was small, which may have precluded achieving statistical significance for some of the analyses in which only CMV mono-infected individuals were included. No adjustments were made for multiple comparisons. Therefore, results must be regarded with caution and duplication of these results is desirable. Sex and diabetes were considered as potential confounders due to previous epidemiological knowledge and were investigated as such in the model building strategy, and the text has been modified to reflect this adequately. However, most of the sample were men, and diabetes cases were very rare. Sex and diabetes were not associated with the outcome in univariable analysis, and as such, they could not be confounders of the associations,

so they were not included into the final adjusted models. Indeed, due to small cell issues (i.e.few observations available in the comparison groups), it would not have been possible to include them into the final models, as it would make estimations unstable. Investigating whether NK cells or adapNK cells or whether CMV antigens were present in atherosclerotic plaques was not feasible in this population with no CVD symptoms being investigated for subclinical CVD. Additionally, the cross-sectional nature of the study limits causal interpretations.

In summary, high frequencies of adapNK cells were associated with a reduced prevalence of coronary atherosclerotic plaque in CMV seropositive individuals, both in the PLWH and in CMV mono-infected groups. Further investigations should focus on determining the directional causality of this link in the setting of pre-clinical atherosclerosis and in other stages in the development of atherosclerosis and other manifestations of CVD.

3.6. ACKNOWLEDGEMENTS

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Ethics statement. This research study was approved by the Research Ethics Boards of the Centre Hospitalier de l'Université de Montréal and the McGill University Health Centre (Project Identification Code 2019-4605). It was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from each study subject for the collection of specimens, subsequent analyses and publication of results obtained from these analyses.

3.7. FIGURE AND LEGENDS

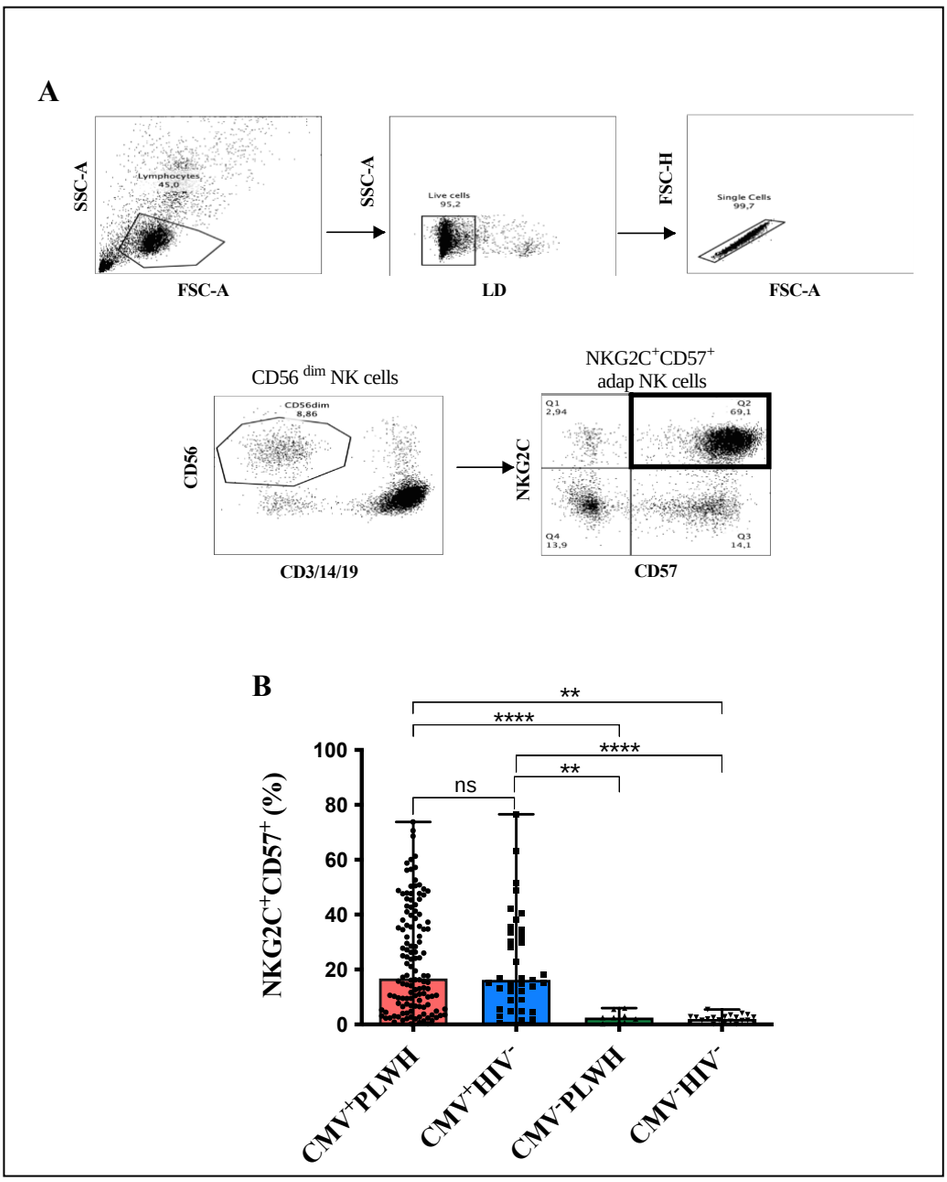


Figure 1.3. Evaluation of the frequency of NKG2C^{+}CD57^{+} NK cells in HIV$^{+/-}$CMV$^{+/-}$ participants.

(A) Shown is the gating strategy used to detect the frequency of NKG2C^{+}CD57^{+} expressing NK cells. Peripheral blood mononuclear cells were stained for viability and cell surface CD3, CD14, CD19, CD56, CD57 and NKG2C. CD3^{-}CD14^{-}CD19^{-}CD56^{dim} NK cells were gated on from the live, singlet, lymphocyte gate. From these, we determined the frequency NKG2C^{+}CD57^{+}CD56^{dim} NK cells. (B) The frequency of NKG2C^{+}CD57^{+} NK cells is shown for cells from CMV^{+}PLWH, CMV mono-infected (CMV^{+}HIV^{-}), CMV^{-}PLWH and HIV CMV uninfected persons). Each point represents a single individual. Bar graph heights and error bars represent medians and interquartile ranges for the subject groups. A Kruskal-Wallis test with Dunn's post tests were used to analyze the significance of differences between groups. FSC-A, forward scatter area; SSC-A, side scatter area; LD, live/dead; FSC-H, forward scatter height, CMV, cytomegalovirus; PLWH, people living with HIV.; "*", $p<0.05$; "***", $p<0.001$; "****", $p<0.0001$.

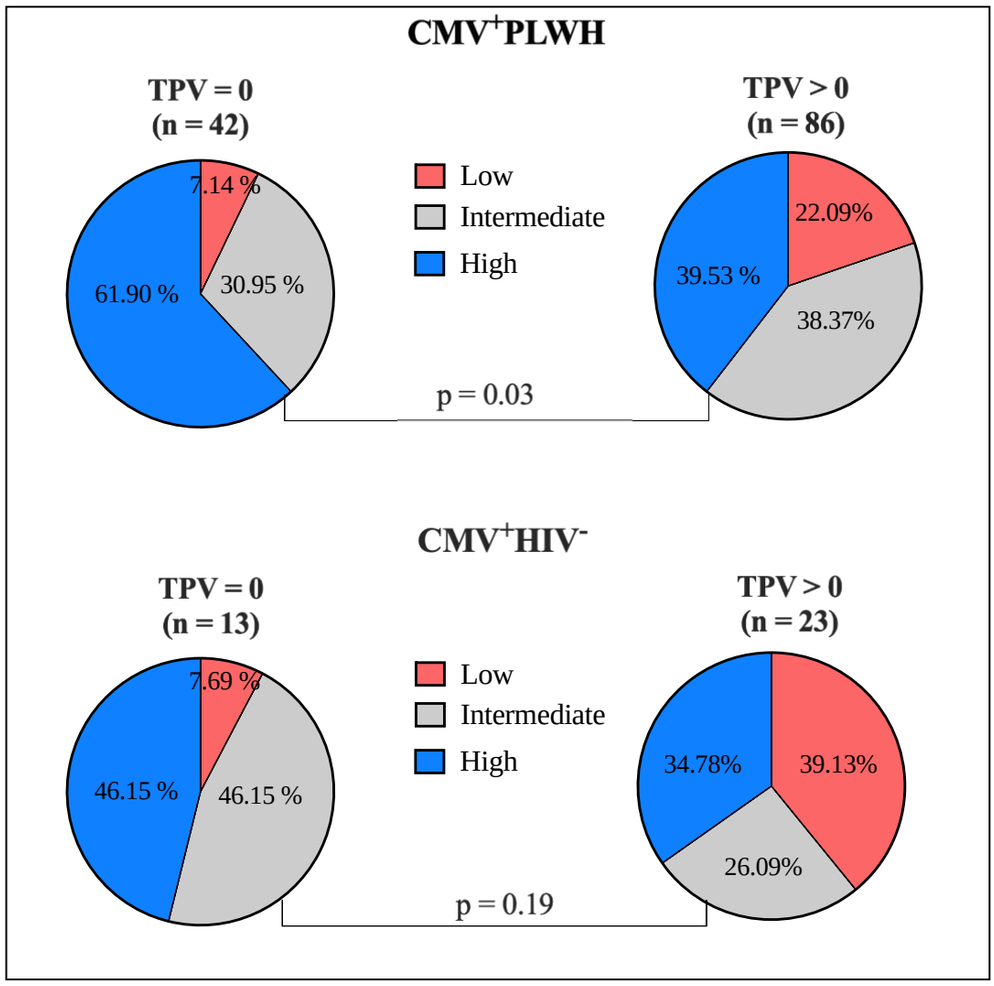


Figure 2.3. The proportion of NKG2C$^+$CD57$^+$ adaptive NK (adapNK) cell frequency categories in total plaque volume (TPV) negative and positive CMV infected people living with human immunodeficiency virus (CMV$^+$PLWH) and CMV mono-infected

The proportion of NKG2C$^+$CD57$^+$ adapNK cell frequency categories (low, intermediate, and high) was compared in participants with negative (left-hand pie charts) versus positive (right-hand pie charts) TPV in CMV$^+$PLWH (n=128) (upper pie charts) and CMV mono-infected (CMV$^+$HIV$^-$) individuals (n=36) (lower pie charts). Chi-square tests were used to test the significance of proportional between-group differences in adapNK cells frequency categories between participants with negative (TPV=0) versus positive (TPV>0) for subclinical atherosclerosis.

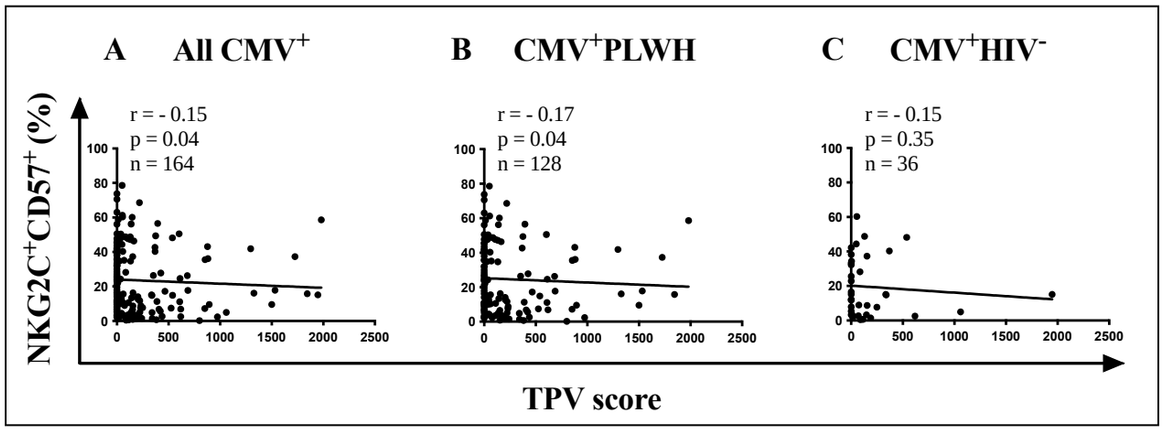


Figure 3.3. Correlation between NKG2C$^+$CD57$^+$ adaptive NK (adapNK) cell frequency and total plaque volume (TPV) in CMV$^+$PLWH and CMV mono-infected (CMV$^+$HIV$^-$) individuals

(A) The y-axes show the frequency of adapNK cells and the x-axes the TPV in (A) CMV$^+$ (n=164), (B) CMV$^+$ PLWH (n=128), and (C) CMV mono-infected (n=36) individuals. The number of subjects tested, the correlation coefficients (r) and the p-values for each correlation are shown in the inset at the top left corner of each graph. The statistical significance of the correlations was tested using non-parametric Spearman correlation tests.

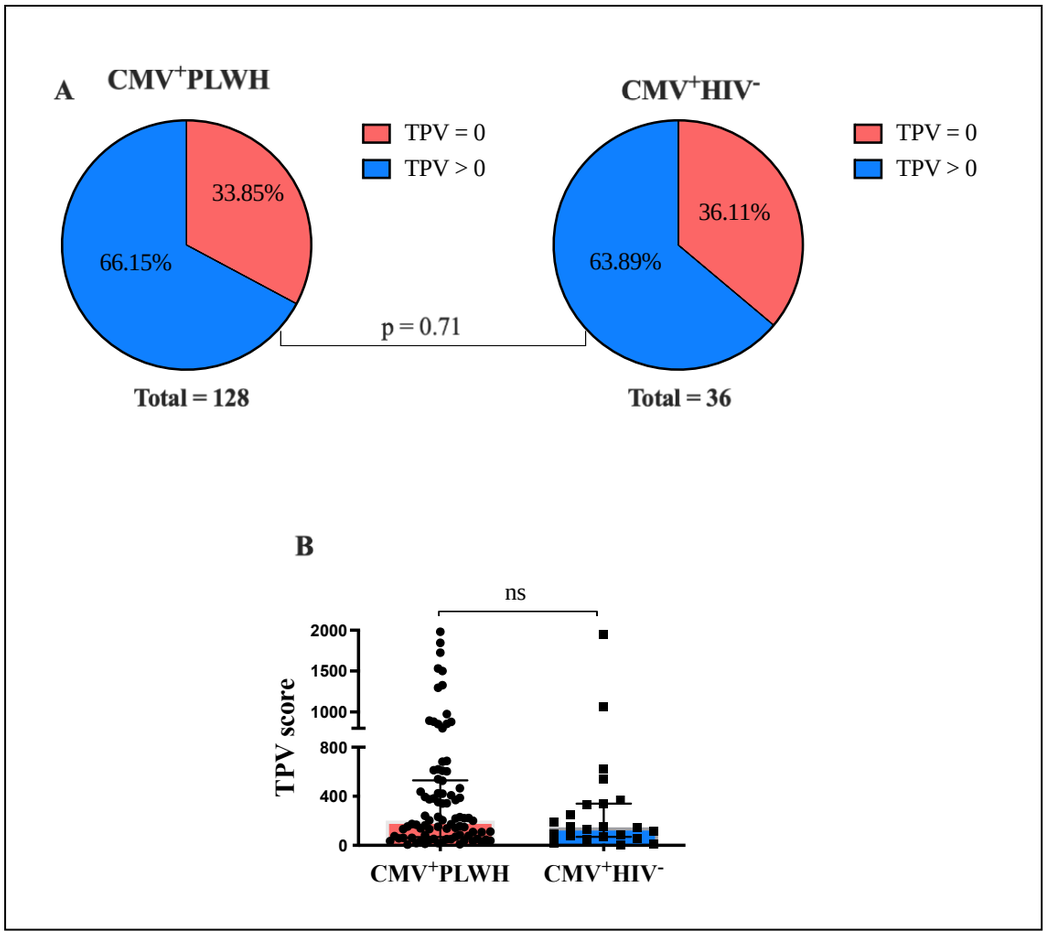


Figure 4.3. Comparison of CMV$^+$PLWH and CMV mono-infected participants for the proportion with negative versus positive total plaque volume (TPV) and the distribution of these scores

(A) The proportion CMV$^+$PLWH (left-hand panels) and CMV mono-infected individuals (right-hand panels) with negative (TPV=0) versus positive (TPV>0) TPV scores. A Chi-square tests was used to determine the statistical significance of proportional between-group differences in positive and negative TPV. (B) Shown on the y-axis are the TPV scores for CMV$^+$PLWH and CMV mono-infected individuals with positive TPV scores. A Mann-Whitney test was used to assess the statistical significance of differences in the distribution of TPV scores in CMV$^+$PLWH versus CMV mono-infected individuals.

Table 1.3. Demographic and Clinical Parameters of the Study Population (N=194)

Characteristic	CMV^+PLWH **n= 128**	CMV^+HIV^- **n= 37**	CMV^-PLWH **n= 8**	CMV^-HIV^- **n= 21**	P value
Sex, n (%)					
Males	119 (92.9)	28 (75.7)	8 (100)	15 (71.4)	**0.002**
Females	9 (7.0)	9 (24.3)	0 (0)	6 (28.6)	
Age (years), median, IQR	55.0 (50.8-60.3)	58.9 (53.0-65.7)	55.6 (51.3-57.4)	58.6 (52.8-63.9)	**0.03**
Hypertension, n (%)	39 (30.5)	11 (29.7)	2 (25.0)	3 (15.0)	**0.04**
Diabetes mellitus, n (%)	8 (6.1)	0/ (0)	0 (0)	0 (0)	0.13
LDL cholesterol (mmol/L), median (IQR)	2.7 (2.2-3.4)	3.2 (2.5 -3.9)	2.1 (1.9-3.4)	3.3 (2.8-4.1)	**0.01**
HDL cholesterol (mmol/L), median (IQR)	1.3 (1.0-1.5)	1.3 (1.1-1.7)	1.2 (1.0-1.4)	1.4 (1.1-1.6)	0.21
Markers of Inflammation					
- D-dimer, n (%)	46 (35.9)	16 (43.2)	5 (62.5)	7 (33.3)	
ng/ml, median (IQR)	270.0 (173.8-340)	333.5 (290.3-484.0)	310 (110.1-495.0)	192 (174.0-382.0)	**0.02**
- Hs-CRP, n (%)	91 (71.1)	15 (40.5)	5 (62.5)	16 (76.2)	
mg/L, median (IQR)	5 (5-5)	5 (5-5)	5 (4-17.3)	5 (5-5)	0.49
Lipid-lowering medication use					
- Statin, n (%)	35 (27.3)	7 (18.9)	3 (60)	2 (11.8)	0.11
Anti-platelet, n (%)	29 (22.7)	2 (5.4)	1 (25)	2 (11.8)	0.09
Smoking exposure (pack/year)					
- Smokers, n (%)	89 (69.5)	17 (45.9)	4 (50)	14 (66.6)	
packs/year, median (IQR)	18.0 (6.0- 30.8)	10.4 (3.9- 21.50)	27.8 (9.0-40.0)	7.7 (3.4 – 20.2)	**0.03**
- Non-smokers, n (%)	36 (28.1)	19 (51.4)	4 (50)	7 (33.3)	
packs/year, median (IQR)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	
Exercise, n (%)					
Physical activity	39 (30.4)	32 (86.5)	3 (37.5)	15 (71.4)	**<0.0001**
No Physical activity	62 (48.4)	4 (10.8)	5 (62.5)	5 (23.8)	
BMI					
n (%)	120 (93.8)	35 (94.6)	8 (100)	21 (100)	
Kg/m^2, median (IQR)	24.3 (21.9-27.5)	25.6 (23.7-32.0)	26.6 (22.8-32.8)	27.1(24.0-30.2)	**0.01**
Waist circumference (cm), median (IQR)	93 (86.0-101)	93 (89.0-100)	97.5 (88.0-110)	96.0 (89.0-104)	0.35
CD4 current (cells /ml), median (IQR)	576.0 (406.5- 726.0)	-	693 (324.0-1087)	-	0.44
CD8 current (cells /ml), median (IQR)	693.5 (554.7-1020)	-	874 (384-924)	-	0.29
CD4/CD8 ratio (cells/ml), median (IQR)	0.9 (0.55-1.1)	-	0.8 (0.59-0.95)	-	0.69
Total years on ART, median (IQR)	15.1 (13.4-22)	-	12 (3.8-20.4)	-	0.88
Years HIV-infected, median (IQR)	18.1 (14.3-28.9)	-	15.2 (4.8-23.3)	-	0.38
Undetectable HIV-1 RNA, n (%)	125 (98)	-	7 (87.5)	-	0.22

Abbreviations: IQR, interquartile range; LDL, Low-density lipoprotein cholesterol; HDL, High-density, BMI, Body mass index, lipoprotein cholesterol; BMI, body mass index; Hs.CRP, high sensitivity C-reactive protein. Note: Kruskal-Wallis tests were used to assess the significance of differences in continuous variables between groups; Chi-square tests were used assess to assess the significance of differences in discrete variables between groups.

Table 2.3. Univariable and Multivariable Analysis of Association of AdapNK cell frequency with positive total plaque volume score in CMV Seropositive Participants

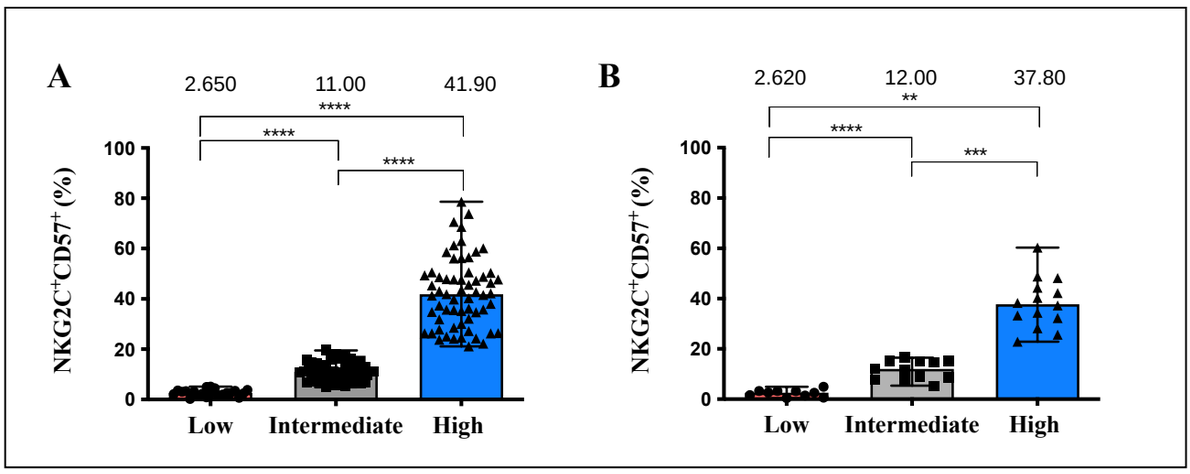
Characteristic	Univariable analysis		Multivariable analysis	
	RR (95% CI)	P-value	RR (95% CI)	P-value
AdapNK cells frequency				
High (>20%)	0.74 (0.56– 0.95)	**0.019**	0.75 (0.58 – 0.97)	**0.030**
Intermediate (4.6-20%)	0.90 (0.72 – 1.14)	0.410	0.92 (0.72-1.17)	0.509
Low (<4.6%)	1.0 (ref)		1.0 (ref)	
HIV status				
positive	1.07 (0.81-1.40)	0.620	1.08 (0.81 – 1.42)	0.582
negative	1.0 (ref)		1.0 (ref)	
Age (per 10 years increase)	1.26 (1.09- 1.45)	**0.001**	1.23 (1.06 – 1.44)	**0.006**
High blood pressure			-	
Present	1.07 (0.85- 1.33)	0.56		-
Absent	1.0 (ref)			
Smoking exposure (per each increase in 10 pack-years)	1.09 (1.05-1.13)	**<0.001**	1.09 (1.05-1.13)	**<0.001**
LDL-cholesterol (1 mmol/l)	1.00 (0.91 - 1.11)	0.888	-	-
Statin use				
yes	1.21 (0.98 - 1.48)	0.07	-	-
no	1.0 (ref)			
BMI (1kg/m2)	0.98 (0.96 - 1.01)	0.36	-	-

AdapNK cells, adaptive NK cells; LDL-cholesterol; Low density lipoprotein cholesterol; BMI, body mass index; RR, relative risk; 95% CI, 95% confidence intervals.

RR per 10-year increase in age.

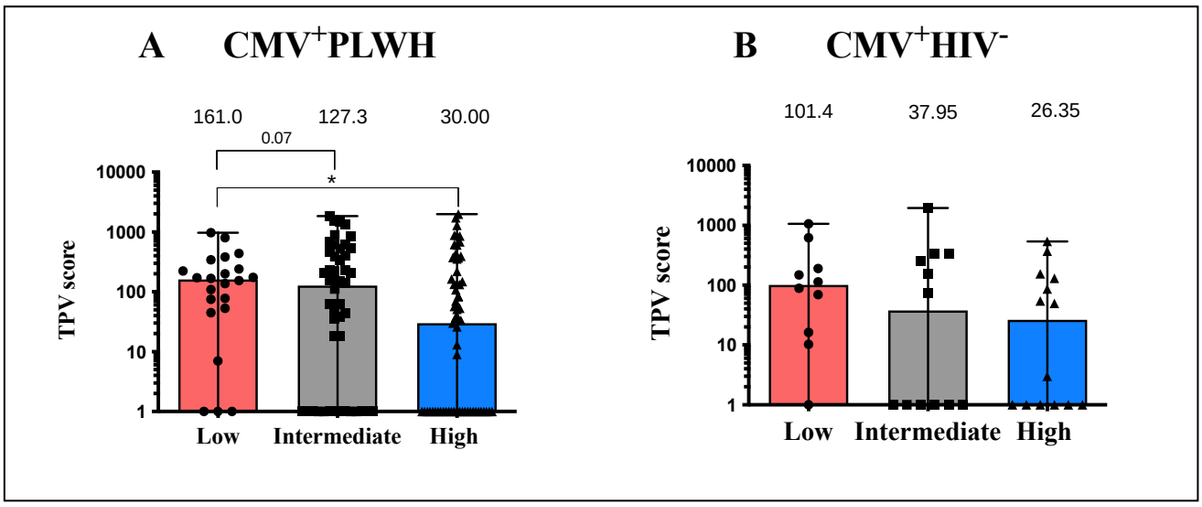
RR per 10 pack per year increase in smoking.

3.8. SUPPLEMENTAL FIGURES AND LEGENDS



Supplemental figure 1.3. Distribution of NKG2C$^+$CD57$^+$ adaptive NK (adapNK) cell frequency in CMV infected people living with human immunodeficiency virus (CMV$^+$PLWH) and CMV mono-infected (CMV$^+$HIV$^-$) individuals

The distribution of NKG2C$^+$CD57$^+$ adapNK cells frequency was categorized into those with low, intermediate and high NKG2C$^+$CD57$^+$ adapNK cell frequencies. in (A) CMV$^+$PLWH and (B) CMV-mono-infected individuals. Each point represents a single individual. Bar graph heights and error bars represent medians and interquartile ranges for the group. A Kruskal-Wallis test with Dunn's post tests were used to analyze the significance of differences between groups. CMV, cytomegalovirus; PLWH, people living with HIV.; "**", $p<0.01$; "***", $p<0.001$; "****", $p<0.0001$.



Supplemental figure 2.3. Total plaque volume (TPV) in CMV infected people living with human immunodeficiency virus (CMV$^+$PLWH) and CMV mono-infected (CMV$^+$HIV$^-$) individuals according to the level of NKG2C$^+$CD57$^+$ adaptive NK cell (adapNK) expansion

(A) The y-axes show the TPV in CMV$^+$PLWH and (B) CMV mono-infected (CMV$^+$HIV$^-$) individuals based on adapNK cell frequency level categories (low, intermediate and high). A Kruskal-Wallis test with Dunn's post tests were used to analyze the significance of differences between groups; "*", $p<0.05$. The "0" value for TPV was changed to 1 for this graph whose y-axis is plotted using a log scale.

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BRIDGE FROM CHAPTER III TO CHAPTER IV

In chapter III, I determined the frequency of NKG2C^{+}CD57^{+} adapNK cells in CMV^{+}PLWH and CMV^{+}HIV^{-} participants enrolled in the CHACS. I found that the frequency of adapNK cells did not differ significantly between the two CHACS groups. This was a surprising result as many other investigators had previously reported that the frequency of adapNK cells was significantly higher in CMV^{+}PLWH than in CMV^{+} mono-infected subjects. A possible difference between the study subjects tested by others and those enrolled in the CHACS was that CHACS participants may have been older and on ART for longer than the study groups reported by others, though this information was not always clearly provided. In chapter IV, I investigated whether the discrepancy between our results and those of others with respect to the difference in the frequency of adapNK cells in CMV^{+}PLWH compared to in CMV^{+}HIV^{-} subjects was due to a difference in age or time on ART. I hypothesized that the absence of a significant difference in the frequency of adapNK cells between CMV^{+}PLWH and CMV^{+}HIV^{-} subjects was due to their older age but also considered whether the length of their time on ART could be contributing to lower frequencies of adapNK cells in CHACS participants.

CHAPTER IV

4. The Frequency and Function of NKG2C^{+}CD57^{+} Adaptive NK cells in Cytomegalovirus Co-infected People Living with HIV decline with Duration of Antiretroviral Therapy

Khlood Alsulami[1,2,3], Franck P. Dupuy[1,3], Louise Gilbert[1,3], Marc Messier-Peet[4], Madeleine Durand[4] Cécile Tremblay[4,5], Jean-Pierre Routy[1,3,6,7], Julie Bruneau[4,8], Jean-Guy Baril[9], Benoit Trottier[9] and Nicole F. Bernard[1,2,3,7, 10]

[1]Research Institute of the McGill University Health Centre (RI-MUHC), Montreal, QC, Canada.
[2]Division of Experimental Medicine, McGill University, Montreal, QC, Canada.
[3]Infectious Diseases, Immunology and Global Health Program, Research Institute of the McGill University Health Centre, Montreal, QC, Canada.
[4]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, QC, Canada.
[5]Department of Microbiology Infectiology and Immunology, University of Montreal, Montreal, QC, Canada.
[6]Division of Hematology, McGill University Health Centre, Montreal, QC, Canada.
[7]Chronic Viral Illness Service, McGill University Health Centre, Montreal, QC, Canada.
[8]Department of Family Medicine and Family Medicine, Université de Montréal, Montreal, QC, Canada.
[9] Clinique de Médecine Urbaine du Quartier Latin, Montreal, QC, Canada
[10]Division of Clinical Immunology, McGill University Health Centre, Montreal, QC, Canada
*Corresponding author**:** Nicole F. Bernard, Ph.D. nicole.bernard@mcgill.caResearch Institute of the McGill University Health Centre, Glen site, Bloc E, 1001 Decarie Blvd., Room EM3.3238, Montreal, Quebec, H3A 3J1, Canada.Tel: (514) 934-1934 x-44584; Fax: (514) 933-1562

Corresponding author**:** Nicole F. Bernard, Ph.D. nicole.bernard@mcgill.ca

Research Institute of the McGill University Health Centre, Glen site, Bloc E, 1001 Decarie Blvd., Room EM3.3238, Montreal, Quebec, H3A 3J1, Canada.

Tel: (514) 934-1934 x-44584; Fax: (514) 933-1562

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Keywords: People living with HIV (PLWH), HIV, CMV, NK cells, adaptive NK cells, aging.

4.1. INTRODUCTION

Human natural killer (NK) cells are involved in immune responses to viruses and tumor cells [1]. NK cells express germline encoded inhibitory and activating receptors that tune NK cell responses to target cells based on the latter's expression of stress ligands, activating receptor ligands and loss of major histocompatibility complex (MHC) class I antigens, the ligands for inhibitory NK receptors (NKRs) [2]. NK cells, as part of the innate immune system, are primed to respond rapidly, before T and B cells can expand and differentiate into effector cells [3]. Mature NK cells comprise 10 to 15% of peripheral lymphocytes and can be divided into $CD56^{bright}$ and $CD56^{dim}$ subsets [4]. $CD56^{bright}$ NK cells make up approximately 10% of circulating NK cells and are thought to be precursors of the more abundant peripheral $CD56^{dim}$ NK cells [4-7]. As NK cells differentiate from $CD56^{bright}$ to $CD56^{dim}$ cells, they lose their cell surface expression of the inhibitory NKG2A NKR, sequentially acquire inhibitory Killer Immunoglobulin-like Receptors (KIRs) and begin to express the maturation marker CD57 [8, 9]. Most $CD56^{dim}$ NK cells also express the activating NKR, FcγRIIIa or CD16 [1, 2]. In the setting of untreated HIV infection, a poorly functional NK cell subset that is $CD56^{null}CD16^{+}$ emerges [10].

Human cytomegalovirus (CMV) infection is caused by a wide-spread β-herpesvirus with a prevalence of between 40 and 100% depending on age, socio-demographic factors, and geographic location [11, 12]. Most HIV infected persons are also CMV co-infected [13]. Prior to the availability of antiretroviral treatment (ART), CMV co-infection of People Living with HIV (PLWH) was considered to be an opportunistic infection associated with important morbidity and mortality [14, 15]. In the era of ART availability, CMV and HIV co-infections worsen each other's disease course by contributing to systemic inflammation, cardiovascular disease and immune senescence [16-22]. CMV infection reshapes the NK cell repertoire by driving the expansion of a subset of NK cells expressing NKG2C and CD57 [21-26]. NKG2C is an activating receptor belonging to the C-type lectin receptor family, which is expressed as a heterodimer with CD94 [27]. The ligand for NKG2C/CD94 is HLA-E whose cell surface expression is stabilized by epitopes derived from the leader sequence of HLA-A, -B, -C and G antigens or the UL40 CMV gene product [22, 24, 27-31]. These $NKG2C^{+}CD57^{+}$ NK cells have memory like features such as antigen-specific clonal expansion following CMV infection and

form long-lived memory cells, which are features of adaptive lymphocytes. These cells also exhibit epigenetic changes similar to those observed in cytotoxic CD8 T cells, including DNA methylation-dependent silencing of the promyelocytic leukemia zinc transcription factor (PLZF) and stochastic loss of expression of signalling molecules such as FcεRIγ, spleen tyrosine kinase and EWS/FLI1-activated transcript 2 [32-35]. These $NKG2C^{+}CD57^{+}$ NK cells are called adaptive NK cells. Adaptive NK cells differ from conventional NK cells by expressing lower frequencies of the Natural Cytotoxicity Receptors (NCRs) NKp30 and NKp46, CD161, higher frequencies of inhibitory KIRs, particularly those using HLA-C as ligands and leukocyte immunoglobulin-like receptor family, member 1 (LILRB1) and similar levels of CD57, NKG2D and CD16 [23, 36, 37].

Several studies have compared the frequency of $NKG2C^{+}CD57^{+}$ adaptive NK cells in CMV^{+}PLWH and in CMV mono-infected ($CMV^{+}HIV^{-}$) individuals [24, 38-40]. While three of these studies found that HIV infection further accentuated adaptive NK cell expansion observed in CMV mono-infected persons, Guma et al. found no between-group differences in the frequency of adaptive NK cells. The possibility that the age of the study subjects or how long they had been on ART may account for the presence or absence of differences in adaptive NK cell frequencies between groups has not been addressed and could not be assessed as precise information on the mean/median age and age range of the study populations analyzed was only provided in two of these studies [24, 38]. Information on how long they had been on ART was often not specified.

Herein, we screened participants of the Canadian HIV and Aging Cohort Study (CHACS), all aged >40 yrs and on ART a median of 16 yrs who were CMV^{+}PLWH and CMV-mono-infected individuals for the frequency of their adaptive NK cells. We then compared the frequency of these cells in CHACS participants with those in CMV^{+}PLWH and CMV-mono-infected individuals who were younger than 40 yrs of age. We also investigated the capacity of $NKG2C^{+}CD57^{+}$ adaptive NK cells from CMV^{+}PLWH and CMV mono-infected subjects who were below versus above 40 yrs of age to degranulate and produce cytokines/chemokines following antibody dependent (AD) stimulation. The frequency of adaptive NK cells did not differ significantly between CMV^{+}PLWH and CMV mono-infected populations who were ≥40

yrs of age and treated a median of 16 yrs but was higher in CMV^+PLWH than in CMV^+HIV^- individuals who were <40 yrs of age and treated a median of 1.4 yrs consistent with an inverse correlation between the frequency of adaptive NK cells and either age or time on ART in CMV^+PLWH. When the frequency of adaptive NK cells was compared in CMV^+PLWH who were <40 versus >40 yrs of age on ART for a similar length of time were compared, no significant differences were observed, suggesting that time on ART was more important than age as a determinant of NK cell frequency. Older age, which was highly correlated with length of time on ART, was associated with a lower frequency of adaptive NK cells secreting IFN-γ and TNF-α following AD stimulation.

4.2. MATERIALS AND METHODS

2.1. Ethics statement

This research study was approved by the Research Ethics Boards of the Centre Hospitalier de l'Université de Montréal and the McGill University Health Centre (Project Identification Code 2019-4605). It was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from each study subject for the collection of specimens and subsequent analyses.

2.2. Study population

The study population included 229 CMV seropositive individuals. Study population characteristics are shown in Table 1. Of the 50 individuals who were below 40 yrs of age, 28 were CMV^+PLWH enrolled in the Montreal Primary Infection (PI) cohort and 22 were HIV uninfected CMV mono-infected individuals. All of the 164 individuals enrolled in the CHACS were ≥40 yrs of age, which was one of the inclusion criteria for this cohort. Of these, 127 were CMV^+PLWH, and 37 were CMV mono-infected individuals. An additional 15 individuals, enrolled in the Montreal PI cohort were >40 yrs of age. The study design and protocol of the Montreal PI Cohort and the CHACS have been previously reported [41, 42]. Of the subjects enrolled in the CHACS 84% were male and 85% were Caucasian. In the Montreal PI cohort 96% were male and 89% were Caucasian. Subjects enrolled in the Montreal PI cohort included individuals recruited within the first 6 mos of HIV infection, who were then followed an average

of every 3 mos for up to 4 years. At each clinic visit, blood was drawn for isolation of plasma and peripheral blood mononuclear cells (PBMC), which were stored frozen until use [43]. The PI cohort samples used in this study were from time points collected during the chronic phase of infection, a median (interquartile range [IQR]) of 2.2 (1.5, 2.9) yrs after their presumed date of infection at which time participants had been on ART for a median of 1.7 (1.2, 2.05) yrs with viral loads <50 copies/ml of plasma. The younger group of CMV mono-infected individuals were 33.2 (30.8, 38.2) yrs of age. CHACS participants were recruited from HIV and sexually transmitted disease clinics in Montreal, Quebec, Canada. Most were men who have sex with men. All CMV^{+}PLWH were on ART for a median of 16 (8.6, 19.1) yrs and had viral loads of <50 copies/ml of plasma.

2.3. CMV testing

Plasma samples collected either at the same time point or at a time before the time point used for the phenotypic and functional assessments described in this report were collected from each participant to assess their CMV serological status. This was done by using CMV IgG enzyme immunoassays (EIA) from either CD Creative Diagnostics (Shirley, NY), Abcam (Cambridge, MA), GenWay, Biotech LLC (San Diego, CA) or Abbott Diagnostics (AxSym CMV or Architect CMV IgG, Abbott Park, IL). Testing for CMV antibodies was done according to manufacturers' directions.

2.4. Staining PBMC for adaptive NK cells

Frozen PBMCs were thawed and resuspended in RPMI 1640; 5% fetal bovine serum (FBS); 2mM L-glutamine; 50 international units/ml penicillin; 50 µg/mL streptomycin (R5) (all from Wisent, Inc., Saint Jean-Baptiste, QC, Canada). LIVE/DEAD fixable dead cell stain (Invitrogen, Saint Laurent, QC, Canada) was added to the PBMCs as per manufacturer's directions before surface staining cells using a panel that included the following fluorochrome conjugated antibodies to CD3-BV785 (clone OKT3), CD14-BV785 (M5E2), CD19-BV785 (HIB19), CD56-BV605 (HCD56) (all from BioLegend, San Diego, CA), CD57-PE (TB01) (Life Technologies, Burlington, ON, Canada), CD16-APC-Cy7 (3G8) (BD Bioscience, Baltimore, MD), NKG2C-

PE-Vio770 (REA205) and NKG2A-APC (REA110) (Miltenyi Biotec, Auburn, CA) for 20 min at 4ºC.

2.5. AD NK cell activation (ADNKA) assay

The ADNKA assay was used to assess the frequency of NK cells producing the degranulation marker CD107a, the chemokine CCL4 and the cytokines IFN-γ and TNF-α by adaptive and conventional NK cells from study subjects following stimulation of PBMC with HIVIG opsonized, sorted, infected CEM.NKR.CCR5 (siCEM) cells. HIVIG is a pool of purified IgG from asymptomatic PLWH with $CD4^+$ counts above 400 cells/μl, (HIVIG was obtained from the National Agri-Food Biotechnology Institute (NABI) and the National Heart, Lung, and Blood Institute (NHLBI) through the NIH AIDS Reagent Program, Division of AIDS (DAIDS), National Institutes of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH) [44]. HIVIG contains antibodies recognizing the HIV Envelope expressed on HIV infected cells [45, 46]. CEM cells were obtained from the NIH AIDS Reagent Program, DAIDS, NIAID, NIH as CEM.NKR.CCR5 cells from Dr. Alexandra Trkola [47, 48].

SiCEM cells are HIV infected CEM cells. The virus used to infect CEM cells is an NL4.3 based HIV virus pseudotyped with Bal-Envelope and engineered to express the murine Heat Stable Antigen (HSA or mCD24) [49]. After infection, these cells were sorted for HSA expression and expanded in culture. SiCEM cells were virtually 100% HIV infected as determined by expression of HSA and intreacellular HIV p24 and expressed no cell surface CD4, as it was downmodulated on infected cells by wild type Nef and Vpu [50, 51]. The absence of cell surface CD4 precludes cell surface HIV Envelope from adopting its open conformation [45]. Thus, siCEM cells expose HIV Env in a closed conformation analogous to what would be present of genuinely HIV infected cells.

Study subject PBMCs were used as responder (R) cells in the ADNKA assay. Frozen PBMCs were thawed and rested for two hrs in R5 media. One million stimulatory (S) siCEM cells were opsonized with 50 ug/ml of HIVIG in a volume of 100 μl of RPMI 1640; 10% FBS; 2mM L-

glutamine; 50 international units/ml penicillin; 50 µg/mL streptomycin (R10) for 20 min at a room temperature (RT) in V-bottomed 96-well tissue culture plates. Control siCEM S cells remained unopsonized. One hundred µl of PBMCs (R) at a concentration of 10^7 cells/ml of R10 were added to the wells containing HIVIG or unopsonized siCEM cells and co-cultured at 37^{o}C, in a humidified, 5% CO_2 incubator for 6 hrs with BV711 conjugated anti-CD107a (clone M4A3: BioLegend) Ab. After 1 hr, Golgi stop (monensin) and Golgi plug (brefeldin A) (both from BD Bioscience) were added according to manufacturer's instructions for the remaining 5 hrs of culture. Cells washed in RPMI 1640; 2% FBS before staining for the same cell surface markers as used for quantifying the frequency of adaptive and conventional NK cells. After cell surface staining, cells were permeabilized (Fixation/Permeabilization Kit, BD Bioscience) as per manufacturer directions and stained intra-cellularly with antibodies to CCL4-AF700 (clone D21-1351), IFN-γ-BV510 (clone B27) and TNF-α-BV650 (clone Mab11, all from BD Bioscience) for 30 min at RT in the dark. Cells were then washed and resuspended in 2% paraformaldehyde.

2.6. Flow cytometry

A total of 1.5 x 10^6 to 1.8 x 10^6 events were acquired for each sample using an LSR Fortessa instrument (BD Bioscience, San Jose, CA). Results were analyzed using FlowJo^{TM} software v10.3 (BD, Ashland, OR). NK cells were identified as live, singlet, $CD3^-CD14^-CD19^-CD56^{dim}$ cells. Adaptive NK cells were defined as $NKG2C^+CD57^+CD56^{dim}$ NK cells; conventional NK cells were defined as $NKG2C^-CD56^{dim}$ NK cells. Florescence minus one staining was used to set gates for each experiment. Single-stained beads (BD^{TM} CompBead, BD Bioscience) were used to set compensation. Functional markers were gated on within the adaptive NK or conventional NK cell gates.

2.7. Statistical analysis

Statistical analysis and graphical presentation of results were performed using GraphPad Prism 9.3.1 (GraphPad Software Inc., La Jolla, CA). The statistical significance of differences between two unmatched and two matched groups was assessed using two-tailed Mann-Whitney tests and Wilcoxon tests, respectively. The significance of correlations between the frequency of $NKG2C^{+}CD57^{+}$ adaptive NK cells and age or ART duration was assessed using Spearman's correlation tests. P-values of <0.05 were considered significant. In cases where multiple comparisons were made, Bonferroni corrections were applied.

4.3. RESULTS

Study population characteristics

Of the 229 individuals screened for the frequency of their adaptive NK cells, 170 (74.2%) were PLWH and 59 (25.8%) were CMV mono-infected. All PLWH were receiving ART. Two hundred and two (88.2%) of the study participants were male. Table 1 provides information on the age, sex distribution, duration of infection for the 2 groups of subjects enrolled in the PI cohort, VL, CD4 count CD4:CD8 and yrs on ART for the groups who were PLWH. The significance of between-group differences in these parameters are provided in the table.

Table 1.4. Study Population Characteristics

	CHACS		PI cohort		HIV uninfected	Significance of Between Group Comparisons		
	CMV^+PLWH (n =127) Group 1	CMV^+HIV^- (n=37) Group 2	CMV^+PLWH (n=28) Group 3	CMV^+PLWH (n=15) Group 4	CMV^+HIV^- (n=22) Group 5	1 vs 2[2] 1 vs 3[3] 1 vs 4[4] 1 vs 5[5]	2 vs 3[6] 2 vs 4[7] 2 vs 5[8]	3 vs 4[9] 3 vs 5[10] 4 vs 5[11]
Age[1]	55.7 (51.1, 59.3)	57.7 (50.8, 62.4)	33.2 (30.8, 38.2)	54.1 (52.9, 60.1)	31.5 (25.2, 36.6)	p>0.99[2] p<0.0001[3] p>0.99[4] p<0.0001[5]	p<0.001[6] p>0.[7] p>0.0001[8]	p<0.0001[9] p>0.99[10] p<0.0001[11]
Sex, n (%) • Male • Female	117 (92.1) 10 (7.9)	28 (75.77) 9 (24.3)[4]	26 (92.8) 2 (7.1)	14 (93.3) 1 (6.7)	17 (77.3) 5 (22.7)	p=0.016[2] p>0.99[3] p>0.99[4] p=0.05[5]	p<0.1[6] p>0.25[7] p>0.99[8]	p>0.99[9] p=0.22[10] p=0.47[11]
Duration of infection (years)[1]	Unknown	N.A.	2.2 (1.4, 2.6)	2.2 (2.1, 2.5)	N.A.			p=0.15[9]
Viral load (HIV RNA copies/ml of plasma)	<50	N.A.	<50	<50	N.A.			
$CD4^+$ count ($cells/mm^3$)[1]	575 (411, 723)	N.D.	650 (500, 926.5)	700 (600.5, 814)	N.D.	p=0.14[3] p=0.053[4]		p>0.99[9]
$CD4^+/CD8^+$ ratio[1]	0.8 (0.54, 1.1)	N.D.	1 (0.7, 1.3)	1.1 (0.9, 1.5)	N.D.	p=0.12[3] p=0.006[4]		p=0.6[9]
Years on ART[1]	16.0 (8.6, 19.1)	N.A.	1.4 (1.0, 2.2)	1.94 (1.8, 2.0)	N.A.	p<0.0001[3] p<0.0001[4]		p>0.99[9]

CHACS = Canadian HIV and Aging Cohort Study; PI = Primary Infection; N.A. = Not Applicable; N.D. = Not Determined; CMV^+PLWH = Cytomegalovirus positive People Living with HIV; CMV^+HIV^- = Cytomegalovirus mono-infected; vs = versus.[1] Median (interquartile range). The significance of differences in age, CD4 count, CD4:CD8 ratio and yrs on ART was assessed using Kruskal-Wallis tests with Dunn's post tests. The significance of the difference in duration of infection between the two groups from the PI cohort was assessed using a Mann-Whitney test. The significance of proportional between group differences in sex distribution was assessed using Fishers exact tests.

The frequency of NKG2C^{+}CD57^{+}CD56^{dim} adaptive NK cells is dependent on ART duration

We first evaluated the frequency of NKG2C^{+}CD57^{+}CD56^{dim} adaptive NK cells in CMV^{+}PLWH and CMV mono-infected individuals enrolled in the CHACS. Figure 1A shows the strategy used to gate on adaptive NK and conventional NK cells, which were identified as live, singlet, lymphocytes, which were CD3^{-}CD14^{-}CD19^{-}CD56^{dim}. There was no significant between-group difference in the median (interquartile range [IQR]) frequency of NKG2C^{+}CD57^{+}CD56^{dim} adaptive NK cells in CMV^{+}PLWH and CMV mono-infected CHACS subjects aged >40 yrs (15.9% (5.8, 39.4] vs 13.7% (3.7, 33.9], respectively, p = 0.36, Mann-Whitney test) (Figure 1B). Some previous studies had reported that CMV^{+}PLWH had higher frequencies of adaptive NK cells than CMV mono-infected individuals [38, 39, 52]. In two of these reports, the age range of the populations studied was not reported [39, 52] while in Heath *et al*., the median [IQR] age of the CMV^{+}PLWH and CMV mono-infected populations overlapped that of the participants in the CHACS [38]. We questioned whether age may affect the frequency of adaptive NK cells and whether the differences between our results and those generated by others with respect to between-group differences in adaptive NK cell frequencies was due to differences in age. When we screened CMV^{+}PLWH and CMV mono-infected individuals aged <40 yrs for the frequency of their adaptive NK cells, we found that their frequency was significantly higher in CMV^{+}PLWH than in CMV mono-infected subjects (39.8% (18.8, 58.2) vs 15.6% (2.5, 33.3), p = 0.003, Mann-Whitney test) (Figure 1C). Furthermore, the frequency of adaptive NK cells was inversely correlated with age for all CMV^{+}PLWH (Groups 1 and 3) (r = -0.16, p = 0.04, Spearman correlation test) (Figure 1D). While a similar inverse correlation between the frequency of adaptive NK cells and age was noted for CMV mono-infected individuals (Groups 2 and 5), this observation did not achieve statistical significance (Figure 1E).

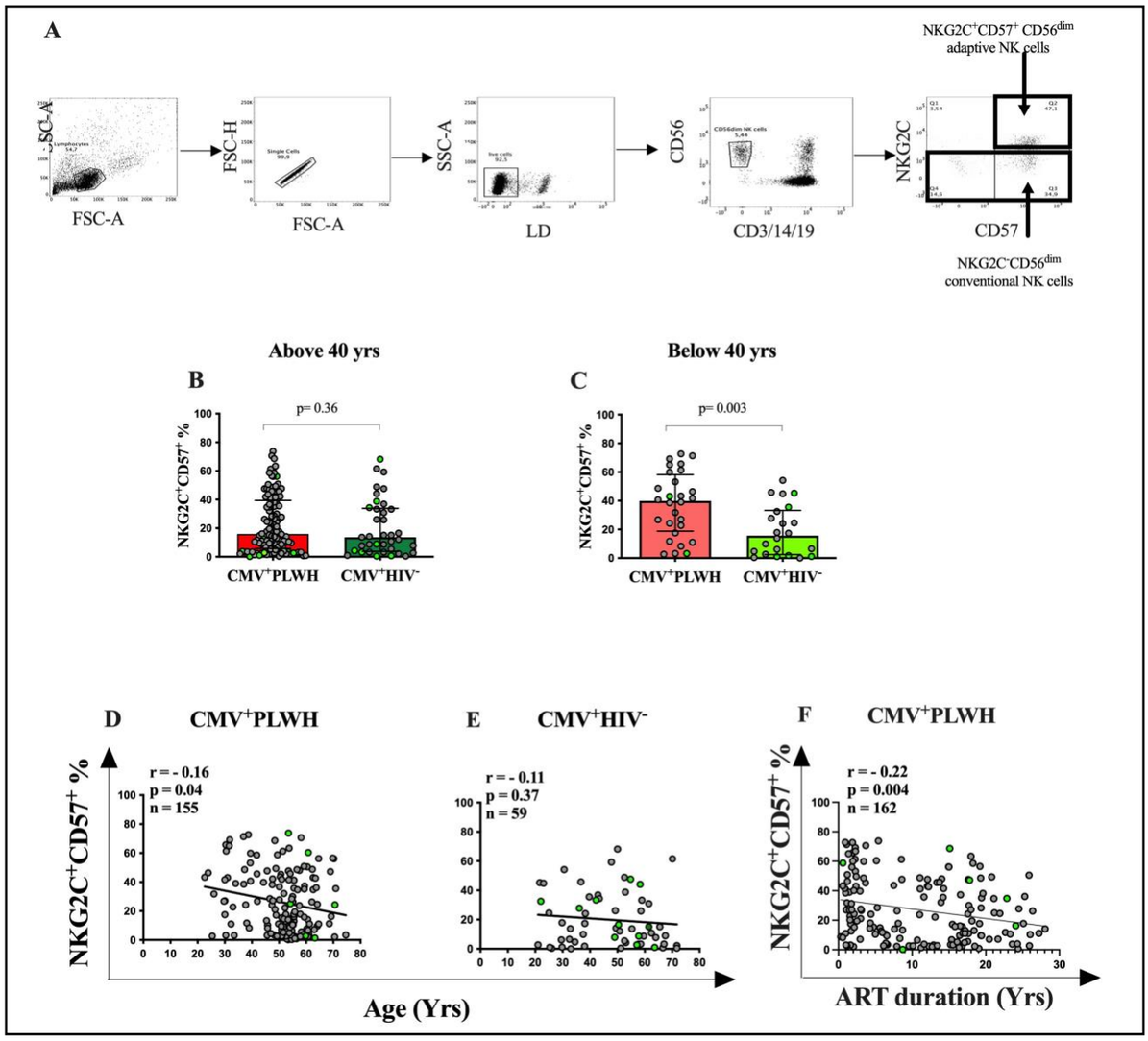

Figure 1.4. The frequency of adaptive NK cells in CMV$^+$PLWH and CMV mono-infected persons declines with age and time on antiretroviral therapy (ART)

(**A**) Gating strategy to detect adaptive and conventional NK cell frequencies. Live, singlet lymphocytes were gated on from peripheral blood mononuclear cells. From these, NK cells were identified as CD3$^-$CD14$^-$CD19$^-$CD56^{dim} cells. The frequency of adaptive and conventional NK cells was determined as the proportion of NKG2C$^+$CD57$^+$CD56^{dim} NK cells (upper right-hand quadrant) and NKG2C$^-$CD56^{dim} NK cells (combined lower left- and right-hand quadrants) in the CD56^{dim} NK cell gate, respectively. (**B**, **C**) The y-axes show the frequency of NKG2C$^+$CD57$^+$ adaptive NK cells in CMV$^+$PLWH and CMV mono-infected persons aged >40 yrs (**B**) and <40 yrs (**C**). Each data point represents results for a single individual. Bar heights and error bars show the median and interquartile ranges (IQR) of the frequency of adaptive NK cells for each group. Mann—Whitney tests were used to assess the statistical significance of between-group differences for the frequency of adaptive NK cells. *p*-values indicating the significance of between group differences are shown over the lines linking the groups being compared. Bars for

CMV+PLWH individuals are colored in red whereas bars for CMV+HIV- individuals are colored in green. Dark red and dark green represent subjects above 40 yrs, while light red and light green represent subjects below 40 yrs. (**D**–**F**) Correlations between the frequency of adaptive NK cells and study subject age (**D**, **E**) or duration of time on ART (**F**). The y-axes show the frequency of NKG2C^{+}CD57^{+} adaptive NK cells while the x-axes show the age in years of CMV^{+}PLWH (**D**), CMV mono-infected persons (**E**) or time on ART in years for CMV^{+}PLWH. Spearman correlation tests were used to assess the statistical significance of these correlations. The correlation coefficients (r), *p*-values, and the number of subjects tested in each panel are shown in the top left corner of each graph. Data points corresponding to results attributed to females are distinguished from those attributed to males by being shown in light green.

Figure 2 shows that the frequency of adaptive NK cells was significantly higher in CMV^{+}PLWH who were <40 than compared to ≥40 yrs of age (39.8% [18.8, 58.2] versus 16.1% [6.53, 39.8], respectively, p = 0.002, Mann-Whitney test). In CMV mono-infected participants the frequency of adaptive NK cells did not differ in those <40 versus >40 yrs of age (15.6% [2.4, 33.3] versus 13.7% [3.79, 33.9], respectively, p = 0.69, Mann-Whitney test). These results show that the absence of a significant differences in the frequency of adaptive NK cells in CMV^{+}PLWH compared to CMV mono-infected persons aged ≥40 yrs was due to a decline in the frequency of these cells with age in CMV^{+}PLWH. It is notable that length of time on ART is likely to increase with duration of infection. The presumed date of infection was not available for CHACS participants precluding the possibility of estimating the duration of infection. However, duration of time on ART was available for CMV^{+}PLWH who were enrolled in the CHACS and PI cohorts. There was a significant negative correlation between the frequency of adaptive NK cells and time on ART (r = -0.22, p = 0.004, Spearman correlation) (Figure 1F).

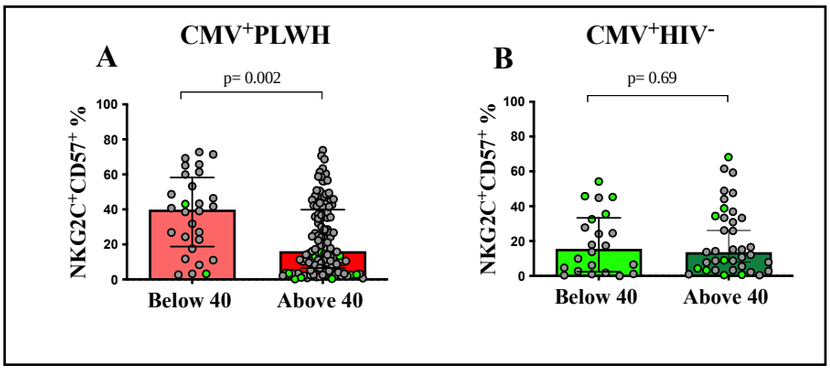

Figure 2.4. CMV$^+$PLWH who are older than 40 yrs of age have lower frequencies of adaptive NK cells than those who are younger than 40 yrs of age

The y-axes show the frequency of NKG2C$^+$CD57$^+$CD56^{dim} NK cells in (A) CMV$^+$PLWH and (B) CMV mono-infected (CMV$^+$HIV$^-$) subjects who are <40 and >40 yrs of age. Each data point represents a single individual. Bars graph heights and error bars show the median and IQR for the frequency of adaptive NK cells for each group. Mann-Whitney tests were used to assess the statistical significance of between-group differences for the frequency of adaptive NK cells. P-values indicating the significance of between group differences are shown over the lines linking the groups being compared. Data points corresponding to result attributed to females are distinguished from those attributed to males by being shown in light green.

To address whether age or time on ART was a more important determinant of the frequency of adaptive NK cells, we examined their frequency in 15 PI cohort participants who were >40 yrs of age and on ART for 1.94 (1.8, 2.0) yrs. The older PI subjects and CHACS participants were similarly aged and older than the PI cohort participants who were <40 yrs old (Figure 3A). In terms of time on ART, the two PI groups did not differ from each other and both were younger than the CHACS participants (Figure 3B). The frequency of adaptive NK cells did not differ in the PI cohort subjects who were less than versus greater than 40 yrs of age. The percent of these cells was higher in younger PI subjects than in CHACS participants and showed a non-significant trend toward being higher in older PI subjects than in CHACS participants (Figure 3C). Together these results support the interpretation that increasing length of time on ART, rather than increasing age is associated with declining frequencies of adaptive NK cells. Furthermore, the absence of an effect of age on the frequency of adaptive NK cells in CMV

mono-infected individuals (Figure 1E and 2B) would support this interpretation as they are ART naïve.

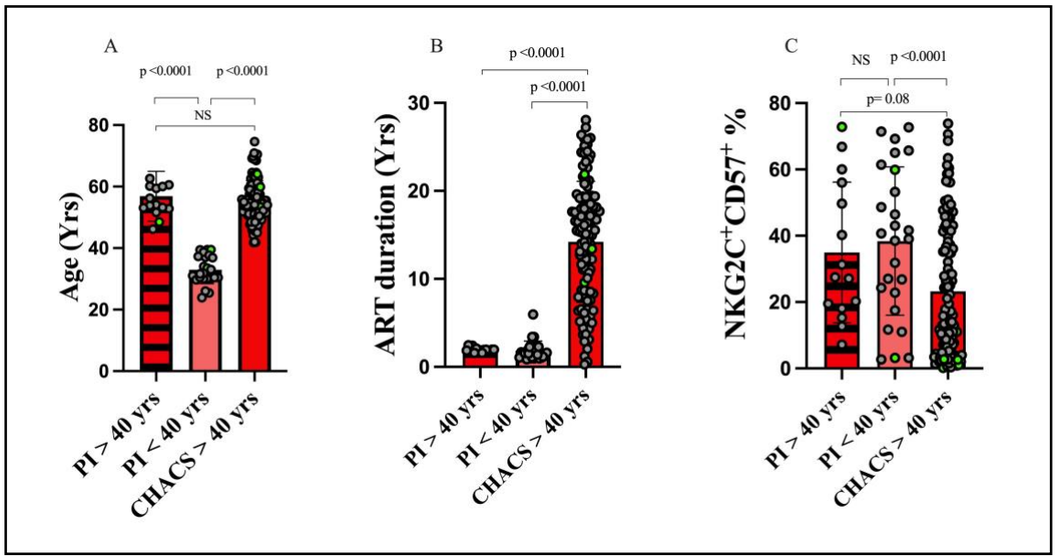

Figure 3.4. Time on ART rather than age is associated with lower frequencies of adaptive NK cells

(**A**–**C**) The y-axes show the age (**A**), time on ART (**B**) and the frequency of adaptive NK cells (**C**) for 15 CMV^+PLWH from the HIV Primary Infection (PI) cohort who were > 40 yrs of age and on ART for a median of 1.9 yrs, 26 CMV^+PLWH from the HIV PI cohort who were < 40 yrs of age and on ART for a median of 1.4 yrs and 126 participants of the Canadian HIV and Aging Cohort Study (CHACS) who were > 40 yrs of age and on ART for a median of 16 yrs. Each data point represents results for a single individual. Bar heights and error bars show the median and interquartile ranges (IQR) for each data set. Kruskal—Wallis tests with Dunn's post tests were used to assess the significance of between-group differences. *p*-values indicating the significance of between group differences are shown over the lines linking the groups being compared. Data points corresponding to results attributed to females are distinguished from those attributed to males by being shown in light green.

Adaptive and conventional NK cell function in CMV$^+$PLWH and CMV mono-infected subjects

We next compared the ability of adaptive and conventional NK cells from CMV$^+$PLWH and CMV mono-infected subjects to respond to an AD stimulus. Figure 1A shows the gating strategy used to determine the frequency of adaptive NK (right hand panel, upper right-hand quadrant) and conventional NK (right hand panel, combined lower left- and right-hand quadrants) cells that degranulated or secreted CCL4, IFN-γ or TNF-α following stimulation with HIVIG opsonized siCEM cells.

The frequency of adaptive NK (Figure 4B-E) and conventional NK (Figure 3F-I) cells producing CD107a, IFN-γ and TNF-α did not differ significantly when from CMV$^+$PLWH or CMV mono-infected subjects (Mann-Whitney). Although the frequency of adaptive NK, but not conventional, NK cells producing CCL4 was higher or showed a trend towards being higher in CMV-mono-infected persons compared to CMV$^+$PLWH, the difference fell below the level of significance when a Bonferroni correction was applied. These results show that the frequency of functional adaptive NK cells persists in the setting of treated HIV infection.

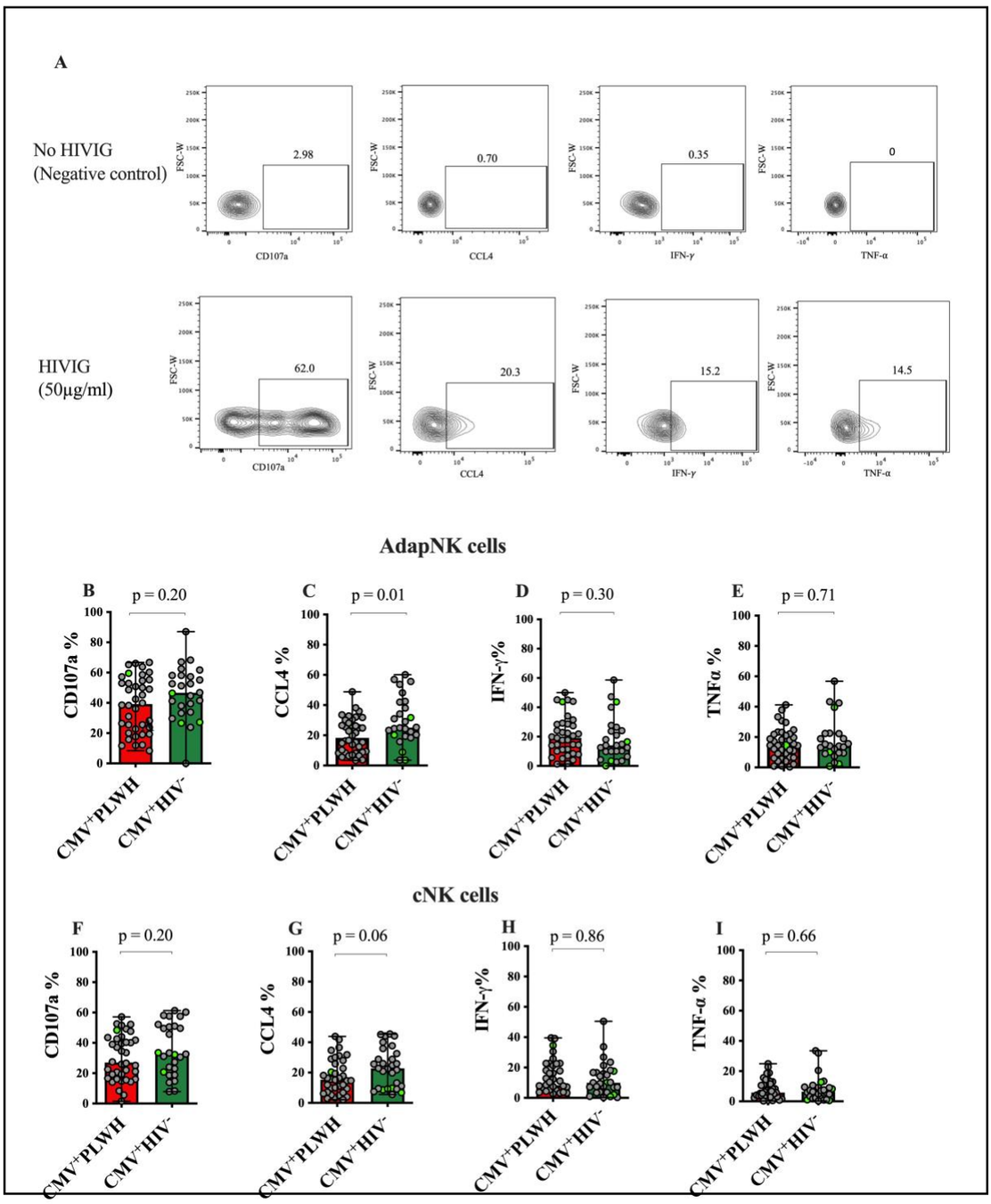

Figure 4.4. HIV infection does not compromise the functionality of adaptive and conventional NK cell responses to antibody opsonized HIV infected cells

(**A**) Gating strategy to detect the frequency of functional adaptive and conventional NK cells. After stimulating peripheral blood mononuclear cells for 6 hrs with anti-HIV Envelope-specific antibody opsonized or un-opsonized HIV infected cells (siCEM) and anti-CD107a specific antibody, cells were surface stained and stained intracellularly with antibodies to CCL4, IFN-γ and TNF-α. Adaptive and conventional NK cells were gated on as shown in Figure 1A. The frequency of adaptive NK cells (**B**–**E**) and conventional NK cells (**F–I**) producing CD107a (**B**, **F**), CCL4 (**C**, **G**), IFN-γ (**D**, **H**) and TNF-α (**E**, **I**) was assessed. The condition in which PBMC were stimulated with un-opsonized siCEM cells was used as a negative control to set the gating for functional marker expression (**A**, top panels). (**B**–**I**) the y-axes show the frequency of adaptive NK cells (**B**–**E**) and conventional NK cells (F-I) from CMV^+PLWH (red bars) versus

CMV mono-infected (CMV^+HIV^-) individuals (green bars) expressing CD107a (**B**, **F**), and secreting CCL4 (**C**, **G**), IFN-γ (**D**, **H**) and TNF-α (**E**, **I**). Bar colors refer to the same study groups as defined in Figure 1 panels B and C. Each data point represents results for a single individual. Bar graph heights and error bars show the median and interquartile ranges (IQR) for each data set. The significance of between-group differences was assessed using Mann—Whitney tests. *p*-values indicating the significance of between group differences are shown over the lines linking the groups being compared. Data points corresponding to results attributed to females are distinguished from those attributed to males by being shown in light green.

A comparison of within-individual frequencies of functional adaptive and conventional NK cells responding to stimulation with HIVIG opsonized siCEM cells showed that a higher frequency of adaptive, than conventional, NK cells from CMV^+PLWH expressed CD107a, secreted IFN-γ or TNF-α ($p < 0.0001$ for all, Wilcoxon matched- pairs test) (Figure S1 A, C, D). As well, a higher frequency of adaptive, than conventional, NK cells from CMV mono-infected individuals secreted CCL4, IFN-γ and TNF-α, though the statistical significance of between-group differences in the frequency of CCL4 secreting cells fell below the level of significance when a Bonferroni correction was applied (Figure S1 F-H).

Effect of age and time on ART on $NKG2C^+CD57^+CD56^{dim}$ adaptive NK cell functions

Figure 5 compares the frequency of adaptive NK cells expressing CD107a and secreting CCL4, IFN-γ and TNF-α in response to stimulation with HIVIG opsonized siCEM cells in participants aged <40 versus ≥40 yrs of age. There were no significant differences in the frequency of antibody opsonized target cell stimulation of adaptive NK cells expressing CD107 or secreting CCL4 from either CMV^+PLWH or CMV mono-infected subjects who were <40 versus ≥40 yrs of age (Figure 5A, B). A significantly higher frequency of adaptive NK cells from younger than older CMV^+PLWH secreted IFN-γ and TNF-α, ($p \leq 0.005$ for both, Mann-Whitney tests, Figure 5C, D). These differences remained statistically significant following the application of Bonferroni corrections. On the other hand, the frequency of adaptive NK cell producing each of the functions tested did not differ significantly in CMV mono-infected subjects (Figure 5G, H).

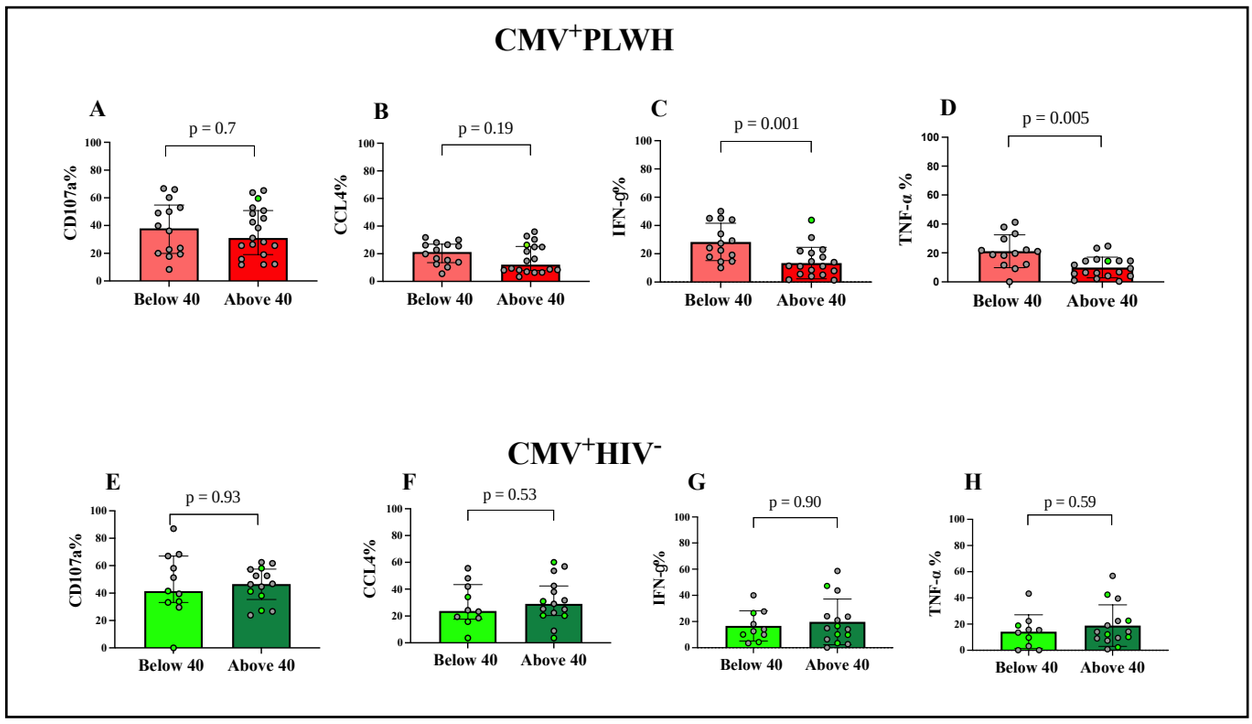

Figure 5.4. A higher frequency of adaptive NK cells from younger than older CMV$^+$PLWH responded to anti-HIV Envelope-specific antibody opsonized HIV-infected cells by secreting IFN-γ and TNF-α.

The y-axes show the frequency of adaptive NK cells (A-H) from CMV$^+$PLWH (A-D) and CMV mono-infected (CMV$^+$HIV$^-$) individuals (E-H) aged <40 yrs (light red and green bars) or >40 yrs (dark red and green bars) producing CD107a (A, E), CCL4 (B, F), IFN-γ (C, G) and TNF-α (D, H). Each data point represents results for a single individual. Bars graph heights and error bars show the median (IQR) for each data set. The significance of between-group differences was assessed using Mann-Whitney tests. P-values for the comparison of adaptive NK cell frequencies between those who were <40 versus >40 yrs of age are shown over the lines linking the 2 populations being compared. Data points corresponding to results attributed to females are distinguished from those attributed to males by being shown in light green.

Figure S2 shows how the frequency of functional adaptive NK cells correlates with age (Figure S2 A-D) and duration of ART (Figure S2 E-H) in CMV$^+$PLWH and with age in CMV$^+$HIV$^-$ individuals (Figure S2 K-L). The frequency of adaptive NK cells secreting IFN-γ and TNF-α are inversely correlated with age (Figure S2 C, D) and with secretion of CCL4 as well with duration of time on ART (Figure S2 F, G, H) in CMV$^+$PLWH. No significant correlations were seen

between the frequency of functional adaptive NK cells and age in CMV mono-infected persons following AD stimulation.

4.4. DISCUSSION

We showed here that the frequency of adaptive NK cells in individuals younger than 40 yrs of age was significantly higher in CMV^+PLWH than in CMV mono-infected individuals, while in participants who were 40 yrs of age or older there was no significant between-group difference in the frequency of adaptive NK cells. We also observed a negative correlation between the frequency of adaptive NK cells with increasing age in CMV^+PLWH. As age and time on ART are positively correlated with each other we investigated whether age or time on ART could account for changes in the frequency of adaptive NK cells. In two groups of study subjects on ART for a similar duration we found no differences in the frequency of adaptive NK cells despite significant differences in age. This finding suggested that increasing time on ART, rather than increasing age, was associated with declining frequencies of adaptive NK cells. We observed no significant differences in the frequency of adaptive and conventional NK cells from CMV^+PLWH and CMV mono-infected individuals responding to AD stimulation by externalizing CD107a, or secreting CCL4, IFN-γ or TNF-α. Thus, HIV infection was not associated with a reduced frequency of functional adaptive, compared to conventional, NK cells in CMV seropositive persons responding to AD stimulation. Indeed, a within-individual comparison of the AD functionality of adaptive versus conventional NK from CMV^+PLWH showed higher frequencies of functional adaptive than conventional NK cells for all functions tested, except CCL4 secretion. This was also the case for adaptive NK cells from CMV mono-infected individuals for secretion of IFN-γ and TNF-α. The frequency of adaptive NK cells from CMV^+PLWH responding to AD stimulation by secreting IFN-γ or TNF-α was higher in younger than older individuals who also differ in their length of time on ART. On the other hand, the frequency of functional adaptive NK cells from CMV mono-infected persons responding to AD stimulation did not differ significantly in participants from these two age groups. This finding is consistent with time on ART influencing adaptive NK cell frequency and functionality since CMV mono-infected individuals are HIV uninfected and thus not on ART.

We initially studied CHACS participants who were all ≥40 yrs of age. Comparing the frequency of adaptive NK cells in CMV$^+$PLWH and CMV mono-infected persons revealed no significant between-population differences. The frequency of adaptive NK cells in CMV$^+$PLWH versus CMV mono-infected persons had been examined by others and found to be higher in some (35-37), but not in all (23) reports. This prompted us to recruit CMV$^+$PLWH and CMV mono-infected persons who were <40 yrs of age so that we could compare results with those who were ≥40 yrs of age. In work reported by others, either the median (IQR or range) or mean ± standard deviation of the ages of the PLWH and HIV uninfected study populations examined was often not provided. In only one instance was the age distribution reported for the subset of study participants who were CMV$^+$PLWH [38]. Heath et al. showed that the frequency of adaptive NK cells was higher in CMV$^+$PLWH than in CMV mono-infected individuals who had a median (IQR) age of 49 (45, 55) for CMV$^+$PLWH and 48 (39, 61) for CMV mono-infected persons [38]. This observation differs from the results reported here where CHACS participants who were also ≥40 yrs of age and whose adaptive NK cell frequency did not differ significantly between CMV$^+$PLWH and CMV mono-infected participants. The reasons underlying this discrepancy may be due to differences in time on ART rather than age being a more important determinant of the frequency of adaptive NK cells. In Heath et al. most PLWH were reported to be on ART though neither the duration of HIV infection nor the time on ART was specified. In the PLWH included in this study those aged ≥ 40 yrs had been on ART for a median (IQR) of 16 (8.6, 19.1) yrs while those who were <40 yrs of age were more recently infected and therefore on ART for a shorter duration of 1.4 (1.0-2.2) yrs. By including individuals of younger age and length of time on ART in this study, we built upon previously reported results by showing that there was an inverse correlation between the frequency of adaptive NK cells and both age and time on ART in CMV$^+$PLWH. The correlation between adaptive NK cell frequency and age for CMV mono-infected persons was more modest and did not achieve statistical significance. While this could be due to the smaller samples size of the CMV mono-infected population compared to the CMV$^+$PLWH group, if ART duration is important in influencing adaptive NK cell frequency, the absence of a correlation between this variable and age in CMV mono-infected persons would be consistent with their HIV seronegative status. A limitation of this analysis is its cross-sectional nature. Work done by others showed that in CMV$^+$PLWH starting ART in chronic phase infection, the frequency of adaptive NK cells, as defined by being positive for cell surface

NKG2C or negative for intracellular FcεRIγ remained stable for up to 24 months on ART [31, 39]. The frequency of NKG2C$^+$ adaptive NK cells in a small sample of 5 individuals examined longitudinally in untreated early infection, established viremic infection and after 1 yr on ART was stable over these three time points [34]. In a fourth study, ART treatment of CMV$^+$PLWH for 48 weeks was accompanied by an increase in the frequency of NKG2C$^+$CD57$^+$ adaptive NK cells from pre-ART initiation to 48 weeks on ART time points [53]. While these longitudinal studies provide information on the within-individual changes with time in the frequency of adaptive NK cells over time intervals of up to 2 yrs on ART, they do not address changes that may occur over longer periods of time such as after a decade or more on ART and are limited by the small number of subjects studied. To our knowledge, this is the first study to examine frequency of adaptive NK cells in persons on ART for various lengths of time and in some cases beyond 20 years on ART.

CMV infection is accompanied by several epigenetic modifications [32, 33]. For example, adaptive NK cells lack the intracellular signaling adaptor molecule FcεRIγ, due to hypermethylation of the *FCER1G* promoter, which silences this gene encoding FcεRIγ [32, 33, 35, 54-56]. FcεRIγ is a molecule that contains 2 immunoreceptor tyrosine-based activation motifs (ITAMs) while the intracellular signaling adaptor molecule, CD3ζ has 3 ITAMs. Both molecules are present in conventional NK cells, where they usually form heterodimers or FcεRIγ homodimers, which participate in the transmission of signals from the activating NKR CD16 [57-59]. When FcεRIγ is absent as occurs in many adaptive NK cells, the CD16 signaling cascade uses CD3ζ homodimers whose 6 ITAMs support more robust CD16 Fc receptor mediated effector responses, favoring adaptive NK cells achieving broader and more potent antigen specificity through AD functions compared to conventional NK cells [57, 60]. The adaptive NK cell subsets characterized as NKG2C$^+$ and FcεRIγ$^-$ are largely overlapping, though not identical [61]. It should be noted that NKG2C expression appears to not be essential for conferring adaptive NK cells with superior AD responsiveness compared to conventional NK cells [57]. In CMV infected individuals homozygous for a deletion mutant of *NKG2C* who fail to express this receptor, there exist NK cells having the same features as adaptive NK cells including exhibiting a terminal differentiation phenotype, functional reprogramming and

epigenetic modifications similar to those seen in NKG2C^{+} or FcεRIγ^{-} adaptive NK cells [57]. The NKR CD2 can synergize with NKG2C in typical adaptive NK cells and plays an important role in the AD responses of adaptive NK cells even when NKG2C is absent on these cells [57].

To investigate the effect of HIV infection on the AD function of adaptive NK cells, we enumerated the frequency of adaptive NK and conventional NK cells externalizing CD107a and secreting CCL4, IFN-γ and TNF-α following AD stimulation. While the frequency of functional adaptive and conventional NK cells tended to be modestly lower in CMV^{+}PLWH than in CMV mono-infected individuals, between-population differences did not achieve statistical significance. We also performed a sub-analysis after separating study subjects into groups who were <40 and ≥40 yrs of age. In both cases, between-population differences in the frequency of adaptive NK and conventional NK cells producing the 4 functions tested did not differ significantly (not shown). This suggests that treated HIV infection has a minimal effect on the responsiveness of adaptive, compared to conventional, NK cells to AD stimulation.

This contrasts with a study conducted in rhesus macaques (RM). Both adaptive NK cells, which were characterized as FcεRIγ^{-} and conventional NK cells from rhesus CMV^{+} (rhCMV^{+}) RMs responded robustly and similarly to AD stimulation by externalizing CD107a and secreting CCL4, IFN-γ or TNF-α. The adaptive NK cells were shown to preferentially use the CD3ζ/ZAP70 CD16 signaling pathway rather than the FcεRIγ /Syk signaling pathway for adaptive NK cell activation. However, adaptive and conventional NK cells in rhCMV^{+} RMs co-infected with SIV had compromised AD functionality [60]. When the CD16 signaling pathway of conventional NK and adaptive NK cells from rhCMV^{+} and rhCMV^{+}SIV^{+} NK cells was compared, the CD3ζ/ZAP70 CD16 signaling pathway in rhCMV^{+}SIV^{+} animals was compromised compared to that of rhCMV^{+} RMs [60]. While it is possible that host species and pathogen differences could account for compromised adaptive NK cell function in RMs that was not observed in adaptive NK cells from CMV^{+}PLWH, it is also possible that in the human participants studied here, ART, which suppressed viral load preserved adaptive and conventional NK cell functionality in the setting of HIV infection.

A higher frequency of within-individual NKG2C^{+}CD57^{+} adaptive than conventional NK cells from CMV^{+}PLWH and CMV-mono-infected individuals responded to stimulation through CD16 by secreting IFN-γ and TNF-α. This was also the case for the frequency of adaptive NK cells externalizing CD107a in CMV^{+}PLWH. The superior ability of adaptive, compared to conventional, NK cells to respond to AD stimulation by secreting IFN-γ and TNF-α has been reported by others [57, 62, 63]. The mechanism underlying this phenomenon is likely due to the promoter regions of *IFNG* and *TNF*, the genes encoding IFN-γ and TNF-α, respectively, being hypomethylated in adaptive NK cells allowing for greater cytokine production upon AD stimulation through ITAM-coupled receptors such as CD16 expressed on adaptive NK cells [32, 62, 63]. Taken together, epigenetic modification is responsible for several specific phenotypes of adaptive NK cells. This is likely to be the mechanism underlying the elevated within-individual capacity of adaptive, compared to conventional, NK cells to release these cytokines following activation via CD16. These results provide further support for the interpretation that HIV infection had little impact on the AD function of adaptive NK cells over that seen for conventional NK cells.

This study had some limitations. The population size, particularly for CMV mono-infected subjects was small, which may have precluded achieving significance for some the analyses performed in which only CMV-mono-infected subjects were included. The results presented have distinguished data generated by cells originating from males and females by using different colors in the plotted figures. The majority of study subjects were male and Caucasian as would be expected given that study subjects recruited to both the CHACS and the PI cohorts were predominantly male and Caucasian. This precluded performing analyses on women only or on persons whose race/ethnicity was other than Caucasian due to the small size of these groups. Overall, it appears that data attributed to women are similarly distributed to that of males.

Adaptive NK cells likely play a role in CMV control through interactions between the activating receptor NKG2C and HLA-E CMV encoded UL40 peptide complexes on CMV infected cells that activate these cells. Adaptive NK cells may also exert control over other HIV and other infections that induce pathogen specific antibodies through the ability of these NK cells to be

activated by ADNKA. Our observation that time on ART decreases the frequency of adaptive NK cells and their functionality may have implications in terms of reduced control of CMV, HIV and other pathogens. The results presented in this manuscript would be relevant for HIV infected individuals who are treated with ART long term though the effect may not be limited to HIV. Further investigations are warranted to explore how the reduced frequency and function of adaptive NK cells with time on ART impacts human health and pathogen control.

4.5. Conclusion

These results provide evidence supporting time on ART-associated changes in the frequency and function of NKG2C^{+}CD57^{+} adaptive NK cells in participants who were CMV^{+}PLWH. These ART-related changes were evident in CMV^{+}PLWH. Our results showed, for the first time, a reduction in both NKG2C^{+}CD57^{+} adaptive NK cell frequency and function accompanied with time on ART in CMV^{+}PLWH. Adaptive and conventional NK cell function was not impaired in the setting of treated HIV infection. Furthermore, a decreased frequency of functional adaptive NK cells was associated with increasing age and time on ART, and an augmented population of NKG2C^{+}CD57^{+} adaptive NK producing IFN-γ and TNF-α was found in younger CMV^{+}PLWH.

Author contributions

Conceptualization, Franck Dupuy and Nicole Bernard; Data curation, Khlood Alsulami, Louise Gilbert and Marc Messier-Peet; Formal analysis, Khlood Alsulami and Franck Dupuy; Funding acquisition, Madeleine Durand and Cécile Tremblay; Investigation, Khlood Alsulami, Franck Dupuy and Nicole Bernard; Methodology, Khlood Alsulami; Project administration, Nicole Bernard; Resources, Madeleine Durand, Cécile Tremblay, Jean-Pierre Routy, Julie Bruneau and Jean-Guy Baril; Software, Benoit Trottier; Supervision, Franck Dupuy and Nicole Bernard; Validation, Franck Dupuy and Nicole Bernard; Visualization, Franck Dupuy and Nicole Bernard; Writing – original draft, Khlood Alsulami and Nicole Bernard; Writing – review & editing, Khlood Alsulami, Franck Dupuy, Louise Gilbert, Marc Messier-Peet, Madeleine Durand, Cécile Tremblay, Jean-Pierre Routy, Julie Bruneau, Jean-Guy Baril, Benoit Trottier and Nicole Bernard.

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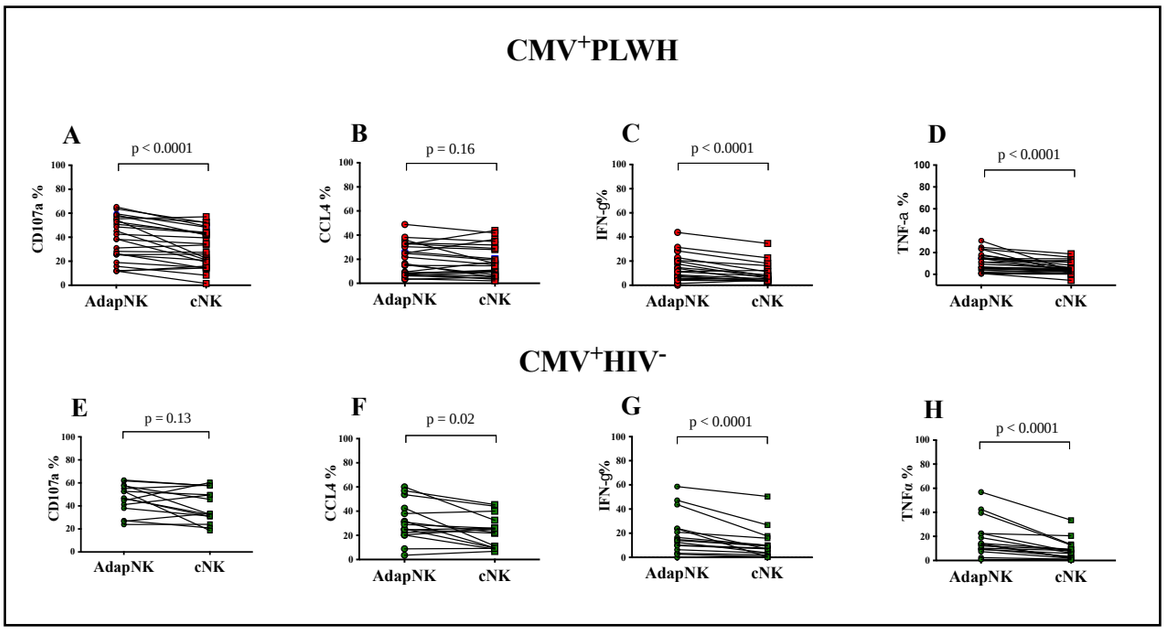
Conflict of interest

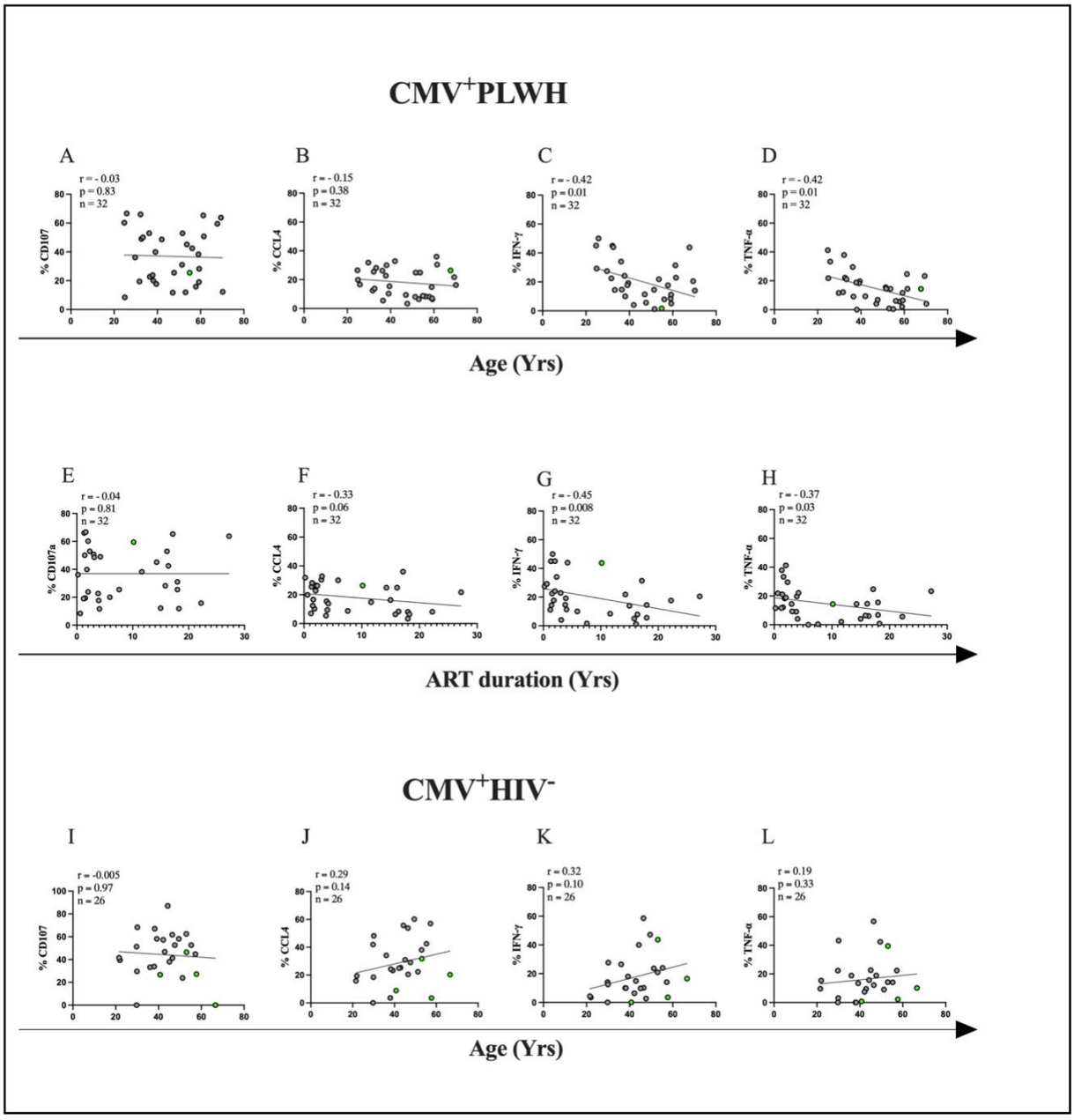
The authors declare no conflict of interests. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

4.6. SUPPLEMENTAL FIGURES AND LEGENDS



Supplemental Figure 1. 4. Differences in the frequency of functional adaptive versus conventional NK cells responding to stimulation with anti-HIV Envelope-specific antibody opsonized HIV-infected cells in CMV$^+$PLWH and CMV mono-infected (CMV$^+$HIV) subjects.

(A-H) The y-axes show the frequency of adaptive NK (adapNK) cells and conventional NK (cNK) cells from CMV$^+$PLWH (A-D) and CMV mono-infected (CMV$^+$HIV$^-$) persons (E-H) producing CD107a (A, E), CCL4 (B, F), IFN-γ (C, G) and TNF-α (D, H). Data points joined by a line show within- individual results. The significance of within-person differences in the frequency of functional adapNK versus cNK cells was assessed using Wilcoxon matched-pairs tests. P-values corresponding to these comparisons are shown over the lines linking the 2 data sets being compared.



Supplemental figure 2.4. Correlations between the frequency of functional adaptive NK cells stimulated by antibody dependent NK cell activation with age and time on ART.

(A-L) The y-axes show the frequency of functional adaptive NK cells responding to stimulation with anti-HIV Envelope-specific antibody opsonized HIV-infected cells. The x-axes show the ages (A-D, I-J) and duration of time on ART (E-H) for CMV^+PLWH (A-H) and CMV mono-infected (CMV^+HIV^-) (I-L) participants. The responsiveness of adaptive NK cells to antibody dependent stimulation was assessed by measuring the frequency of these cells externalizing CD107a (A, E, I) or secreting CCL4 (B, F, J), IFN-γ (C, G, K) or TNF-α (D, H, L). The significance of correlations between the frequency of stimulated functional adaptive NK cells and age/duration of infection was assessed using Spearman correlation tests. The correlation coefficients (r), the p-values, and the number of subjects tested for each correlation are shown in the top left corner of the graphs. Data points corresponding to results attributed to females are distinguished from those attributed to males by being shown in light green.

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CHAPTER V

5. GENERAL DISCUSSION

In chapter II of this thesis, I investigated whether any *NKG2C* genotypes were associated with protection from HIV infection or with HIV VL control in those infected. The rationale underlying this project arose from a publication reporting that carriage of an *NKG2C*$^{-}$ allele was more frequent in PLWH than in controls unexposed to HIV (338). The frequency of NKG2C^{+} NK cells was found to be positively correlated with VL in this study and negatively correlated with VL in others (283, 338, 339). When I compared the *NKG2C* genotype distribution in PLWH enrolled in the Montreal PI cohort with HESN subjects who remained HIV uninfected despite multiple high-risk HIV exposures, I found that the *NKG2C*$^{-/-}$ genotype was more frequent among PLWH than HESN participants (340). In fact, none of the HESN carried this genotype. However, neither the *NKG2C*$^{+/+}$ nor the *NKG2C*$^{+/-}$genotypes alone or combined was associated with HIV susceptibility. The absence of *NKG2C*$^{-/-}$ genotype in HESN but not PLWH suggested that this genotype was associated with HIV susceptibility.

The Montreal PI cohort has enrolled and followed recently infected PLWH since the late 1990s. At certain times during the history of this cohort, the standard of care regarding ART initiation was to delay treatment in order to spare the limited number of drug combinations available at that time. This resulted in some individuals in the PI cohort remaining treatment naïve for a certain length of follow up. This situation allowed for a VL set point to be established for individuals who remained treatment naïve on at least three occasions at least 6 months after their presumed date of infection. I observed no differences in the VL set point between PLWH who carried the three possible *NKG2C* genotypes (340). Also, there was no correlation between the VL set point and the frequency of NKG2C^{+} NK cells or the intensity of NKG2C expression on these NK cells. My results differed from those of others. Thomas et al. found a positive correlation between the VL of the PLWH taken at the same time that PBMCs were assessed for the frequency of their NKG2C^{+} cells (338). However, this analysis was done only on subjects who were *NKG2C*$^{+/+}$ carriers and only on a total of 7 subjects. Two other studies demonstrated a negative correlation between the frequency of NKG2C^{+} NK cells and VL at the same time point

in 21 (283) and 17 (339) persons, respectively, in early HIV infection. My results contribute to new knowledge in three ways: 1) Untreated VL setpoint was calculated on at least three treatment naïve time points that were beyond the spike in VL seen in acute HIV infection for all PLWH included in this analysis. This reduced between time point variations in VL measurements compared to VL measurements made on a single time point as was done by others. 2) A larger number of PLWH were included in this analysis than in those reported by others, i.e. 160 *NKG2C*$^{+/+}$, 83 *NKG2C*$^{+/-}$ and 6 *NKG2C*$^{-/-}$ carriers. This allowed me to make the novel observation that VL setpoint did not differ according to *NKG2C* genotype. 3) I assessed the correlation between the frequency of NKG2C^{+} NK cells, the MFI of NKG2C expression on NK cells, and the fold over background MFI of NKG2C expression on NK cells from PLWH who were CMV seropositive with HIV VL setpoint for all of the *NKG2C* genotypes together and for each NKG2C genotype separately. In none of these cases were correlations between measures of NKG2C and VL setpoint statistically significant. Together, these results support the interpretation that *NKG2C* genotype, NKG2C frequency and NKG2C expression levels are not associated with HIV VL in recently infected PLWH.

The *NKG2C* genotype distributions in PLWH and in HESN persons I described in chapter II of this thesis to assess the role of NKG2C in susceptibility to HIV infection differed from the one reported by Thomas et al. (338, 340). Thomas et al. compared the *NKG2C* genotype distribution in 433 PLWH with that in 280 controls who had no history of HIV exposure (338). They found *NKG2C*$^{-/-}$ carriers among their HIV-uninfected population, while I did not. They reported a significant association between carriage of an *nkg2c*$^{-}$ allele (i.e., combined carriers of the *NKG2C*$^{+/-}$ and *NKG2C*$^{-/-}$ genotypes) with HIV infection and that there was a higher proportion of *NKG2C*$^{+/+}$ carriers among uninfected controls than among PLWH. The main difference between the HIV uninfected population reported by Thomas et al. and the one described in chapter II was that in Thomas at al., the control population was not HIV exposed and thus was at a low risk for HIV infection while HESN persons are HIV exposed and therefore at risk for HIV infection. While it is possible that some of the people at high risk for HIV exposure in the population I studied remained HIV uninfected by chance, they represent a group that is likely to have a higher level of resistance to HIV infection than the HIV-uninfected population described by Thomas et

al. The inclusion of HESN participants allowed me to explore more directly whether *NKG2C* genotypes were associated with HIV susceptibility. This may account for the discrepancy between my results and those reported by Thomas et al. regarding which *NKG2C* genotypes were associated with HIV susceptibility.

I was interested in investigating whether there was evidence that carriage of the $NKG2C^{-/-}$ genotype was linked to HIV susceptibility by mucosal and/or parenteral exposure. To address this, I stratified both PLWH and HESN subjects into those whose route of HIV infection/exposure was mucosal (sexual exposure [SE]) versus parenteral (i.e. through injection drug use). I observed that the frequency of the $NKG2C^{-/-}$ genotype was significantly higher in IDU PLWH than in IDU HESN subjects, while this frequency did not differ significantly between SE PLWH and SE HESN. The reason why the frequency of $NKG2C^{-/-}$ genotype did not differ significantly between SE PLWH and SE HESN is unknown. However, the level of exposure to HIV may be a factor if a higher proportion of HIV transmitting partners of SE than IDU HESN populations were on ART. This information was not available as the HESN persons I studied were not investigated within the context of HIV serodiscordant couples. Additionally, in the context of $NKG2C^{+}$ cells, the biology of HIV transmission by parenteral versus sexual exposure may be a factor. For example, parental exposure involves the introduction of needles contaminated with HIV-infected cells and/or virions into the circulation. Subsequently, these HIV-infected cells will express HLA-E, which is the ligand for $NKG2C^{+}$ NK cells, and downmodulate HLA-A, B, C, the ligands for inhibitory KIRs that are also present on $NKG2C^{+}$ NK cells. The integration of these signals promotes $NKG2C^{+}$ NK cells activation that may contribute to HIV clearance prior to the establishment of a productive infection. A possible weakness of this analysis may be related to the classification of routes of exposure. While SE individuals may find it easier to exclude that they were exposed parenterally, it may be more difficult to exclude possible sexual exposure in persons classified as being parenterally exposed through injection drug use. While this information was collected, in many cases the HIV serostatus of sexual partners of IDU was unknown. Overall, more work needs to be done to better understand differences in the SE versus IDU routes of HIV transmission that could explain

why the *NKG2C*$^{-/-}$ genotype is associated with HIV susceptibility in parenteral but not in sexual HIV transmission.

In the context of CMV infection, I observed that the frequency of NKG2C^{+} NK cells in CMV^{+}PLWH and in CMV mono-infected subjects differed according to *NKG2C* genotype. CMV infection drives the expansion of NKG2C^{+} NK cells (299, 477, 478). This was the rationale for confining this analysis to PLWH and HIV-uninfected subjects who were CMV seropositive. In results reported by others, the frequency of NKG2C^{+} NK cells, the MFI of NKG2C expression and the fold change over the background in the MFI of NKG2C expression was done on a study population that was not tested for CMV serostatus, which, if negative, would preclude the expansion of a population of NKG2C^{+} NK cells (338). I found that in CMV mono-infected subjects, differences in the frequency and intensities of NKG2C expression between *NKG2C*$^{+/+}$ and *NKG2C*$^{+/-}$ carriers were not significantly different. However, these values among CMV^{+}PLWH compared to CMV mono-infected persons were higher for cells from carriers of both *NKG2C*$^{+/+}$ and *NKG2C*$^{+/-}$ genotypes, as has been seen by others (478-480). Overall, my results support that carriage of the *NKG2C*$^{-/-}$ genotype is associated with higher susceptibility to HIV infection, particularly by the parenteral infection route. However, NKG2C^{+} NK cells did not influence VL set point, which is a determinant of the rate of HIV disease progression(134, 481).

In chapter III of this thesis, I assessed the frequency of adapNK cells in CHACS participants who were stratified according to their HIV and CMV infection status into four groups: CMV^{+}PLWH, CMV^{+}HIV^{-}, CMV^{-}PLWH and CMV^{-}HIV^{-} participants. The rationale for doing this arose from the observation that PLWH develop CVD at higher rates than age-matched HIV uninfected persons (350-353). Aside from TCRF, co-infection with CMV may have a substantial effect on the progression of AS. In my study, coronary AS was defined by the presence of AS plaque in the coronary arteries, which was measured by CT, to obtain a TPV, which represents the total burden of coronary AS for every CHACS participant. In this analysis, TPV was dichotomized as 0 and >0, i.e. absence or presence of coronary AS, respectively. My objective

was to determine whether the frequency of adapNK cells was associated TPV in CMV^+PLWH and CMV mono-infected (CMV^+HIV^-) subjects.

I found that the frequency of $NKG2C^+CD57^+$ adapNK cells was higher in CMV^+PLWH subject who had a TPV=0 compared to TPV>0. While in CMV^+HIV^- persons $NKG2C^+CD57^+$ adapNK cell frequency did not differ significantly in persons with a TPV=0 versus TPV>0. To the best of my knowledge, this is the first study to report that a high frequency of $NKG2C^+CD57^+$ adapNK cells was associated with protection from CVD. In contrast to my findings, Martinez-Rodriguez et al. reported that a CMV driven expansion of $NKG2C^+$ NK cells were associated with carotid atherosclerotic plaque (CAP) instability (482). They found a higher frequency of peripheral blood $NKG2C^+$ NK cells in persons with a high risk of CAP compared to those without high-risk CAP. Furthermore, peripheral $NKG2C^+$ NK cell frequency was directly associated with hs-CRP, which is consistent with higher subclinical systemic inflammation (482) To the best of my knowledge, this is the only study that has reported that the frequency of $NKG2C^+$ NK cells in subjects with CAP was associated with an increased risk of plaque destabilization. There is no clear explanation for the discrepancy between my results and those reported by Martinez-Rodriguez et al. regarding the association between the frequency of $NKG2C^+$ or $NKG2C^+CD57^+$ NK cells and markers of CVD. Some possibilities could be explored to explain this discrepancy. It may be that the frequency of adapNK cells is associated with a protective versus a pathogenic role at different stages of the AS process. The population studied by Martinez-Rodriquez et al. was at a more advanced stage of CVD as evidenced by their requiring an endarcterectomy, which was used to classify their CAP as stable or unstable (482). In contrast, the CVD in the CHACS population I studied was at a pre-clinical stage of CVD (482).

My study had some limitations. Using measures of peripheral NK cell subsets may not be the ideal marker to use for measures of CVD manifestations. However, in most cases, it is not possible to obtain coronary and carotid artery tissue to examine the cellular composition of plaque in these tissues and the extent of other CVD manifestations. The study population size, particularly for CMV mono-infected subjects was small, which may have precluded achieving

statistical significance for some of the analysis in which only CMV mono-infected individuals were included. As most HIV infected persons are also CMV co-infected, it was not possible to dissect the contribution of each viral infection to clinical outcomes. I performed a multivariable Poisson regression analysis to test the link between the frequency of adapNK cells with TPV in CMV seropositive participants. For this analysis, I determined the cut off for having an expanded population of adapNK cells in CMV seropositive individuals from the average of adapNK cells in CMV seronegative persons plus two standard deviations, which was 4.6%. CMV seropositive donors having a frequency of adapNK cells <4.6% were classified as having a "low" level of adapNK cell expansion, while those with a frequency of adapNK cells of between 4.6% and < 20% and ≥ 20% were classified as having "intermediate" and "high" levels of adapNK cells, respectively. I observed that after adjusting for TCFRs, the Poisson regression analysis showed that having a high frequency of adapNK cells was associated with absence of TPV and thus was protective in the context of coronary AS. The model had only four independent variables due to the small size of the sample in this study. These independent variables were the most common classic TCFRs that included age and smoking intensity, and adapNK cells as the main exposure variable. Sex and diabetes were not included into the final model due to small cell issues (i.e. too few observations for female sex and diabetes). The HIV variable was included in the model because it is an important variable in CHACS. CMV infection is also an important variable in my study but was not included in the model because it can be considered as a confounding factor that can cause bias in the results. Thus, I could conclude that a high frequency of adapNK cells was associated with a reduced prevalence of coronary AS in CMV seropositive individuals, both PLWH and CMV mono-infected groups. Future investigation should focus on determining the directional causality of this link in the setting of pre-clinical AS and in other stages in the development of AS and other manifestations of CVD. Another future direction for investigation could be to establish a collaboration with autopsy studies in which coronary and carotid tissue is collected. From such tissue it may be possible to assess the cellular make up of plaque in these tissues, whether adapNK cells are present at these sites, in what quantity and the locations of such cells in terms of what other cell types they may be contiguous to and possibly be interacting with. While such an approach would be of interest, there would be many challenges to overcome in participating in such a research direction. In conclusion, my findings showed that a high

frequency of circulating NKG2C^{+}CD57^{+}adapK cells was associated with a reduced prevalence of coronary AS plaque in PLWH co-infected with CMV.

In Chapter IV, I compared the ADNKA activity of adapNK and cNK cells from CMV^{+}PLWH and CMV^{+}HIV^{-} subjects. My rationale for doing this arose from a study conducted in RMs showing that the function of adapNK cells was higher in RMs infected with rhesus CMV (rhCMV^{+}SIV^{-}) only than in RMs infected with both rhCMV and SIV (483). RhCMV infection improved adapNK cell activity stimulated by ADNKA, while this activity in rhCMV infected RMs was compromised by co-infection with SIV. My objective was to answer the question whether HIV infection in humans compromised the capacity of adapNK cells expanded by CMV infection to respond to an ADNKA stimulus by degranulating and producing cytokines/chemokines in response to anti-HIV Env specific Ab opsonized HIV infected cells. The results described in chapter IV showed that adapNK cells from CMV^{+}PLWH were not compromised in their capacity to respond to an ADNKA stimulus, at least compared with the ability of cNK cells from the same individuals to do so. This observation differed from what was reported by Shah et al. in rhCMV^{+}SIV^{+} RMs. They found that in rhCMV^{+}SIV^{+} RMs, the frequency of adapNK cells expressing CD107a and secreting CCL4, IFNγ and TNF-α was lower than the frequency of adapNK cells with these functions present in rhCMV^{+}SIV^{-} RMs (483). The discrepancy between the results described by Shah et al., and mine could have several explanations. First, the discrepancies could be due to differences in the species used in these experiments, i.e., humans versus RMs or in the pathogens infecting these study subjects, i.e. rhCMV versus human CMV and HIV versus SIV. Second, there were differences in the experimental strategies used in the two studies. For example, SIV infected RMs were left untreated whereas CMV^{+}PLWH from the CHACS were on ART and had been on ART for a median of 16 yrs. Such long-term treatment would have controlled HIV VL to below detectable levels and maintained low levels of IA thus preserving adapNK cell functionality.

Next, I assessed the influence of age on the frequency of NKG2C^{+}CD57^{+} adapt NK cells in CMV^{+}PLWH and CMV^{+}HIV^{-} participants. I was interested in understanding why the frequency

of adapNK cells did not differ between CMV^+PLWH and CMV^+HIV^- subjects who were above 40 yrs of age (shown in chapter III). I found that in subjects below 40 yrs of age, the frequency of adapNK cells was higher in CMV^+PLWH compared to CMV^+HIV^- subjects. I also showed that the frequency of adapNK cells was significantly negatively correlated with age in CMV^+PLWH but not in CMV^+HIV^- participants. I concluded that the absence of a significant difference in the frequency of adapNK cells in CMV^+PLWH subjects from the CHACS aged above 40 yrs was due to a lower frequency of adapNK cells in CMV^+PLWH subjects aged above 40 versus below 40 yrs. I also examined the effect of age on the frequency of adapNK cells responding to HIVIG opsonized siCEM cells by expressing CD107a and secreting CCL4, IFNγ and TNF-α. There were no significant differences in the frequency of stimulated adapNK cells expressing CD107 or secreting CCL4 from subjects who were older compared to younger than 40 yrs of age when CMV^+PLWH and CMV^+HIV^- subjects were considered. I found that a lower frequency of adapNK cells from older, compared to younger, CMV^+PLWH secreted IFN-γ and TNF-α.

However, the older CMV^+PLWH enrolled in the CHACS were treated for longer than their younger counterparts enrolled in the Montreal PI cohort who were followed for a maximum of 4 yrs. Therefore, it was possible that duration of ART rather than age determined the frequency of adapNK cells. To address this, I compared the frequency of adapNK cells in three groups of CMV^+PLWH: CHACS cohort participants who were >40 yrs of age and on ART for a median of 16 yrs, PI cohort participants who were >40 yrs of age and on ART a median of 1.94 yrs and PI cohort participants who were <40 yrs of age and on ART a median of 1.4 yrs. The 2 CMV^+PLWH groups from the PI cohort were on ART for similar durations that were shorter than the CMV^+PLWH CHACS participants. The age of the older PI cohort group and the CMV^+PLWH CHACS participants did not differ significantly. The frequency of adapNK cells was similar in the 2 PI cohort groups and higher than in the CMV^+PLWH CHACS participant implicating that time on ART was as a determinant of adapNK cell frequency. How duration of treatment affected the frequency of adapNK cells has been reported in several other studies (318, 484-486). However, in all the other studies, the frequency of adapNK cells was followed over a duration of treatment that did not go beyond 1.9 yrs on ART. Therefore, my observation that

long term ART was negatively associated the frequency of adapNK cells was novel. A rigorous way to confirm this observation would have been to assess the frequency of adapNK cells in long term ART treated CMV^+PLWH longitudinally. It may be possible in the future to determine whether some of the participants in the PI cohort study that started ART soon after infection, remained on ART and were later recruited into the CHACS. To do this would require obtaining Research Ethics Board approval reconsenting participants meeting these criteria to permit a link to be made between the data and biobanks established for the two cohorts. This would be a worthwhile avenue to explore in future studies to confirm whether the frequency of adapNK cells does indeed decline with time on ART,

The results suggesting that time on ART had an influence on the frequency of adapNK cells is supported by the lack of a significant negative correlation between the frequency of adapNK cells and age in CMV^+HIV^- individuals who were never on ART and the absence of differences in the frequency of adapNK cells in these individuals aged less than versus greater than 40 yrs.

I investigated the functionality of adapNK cells from CMV^+PLWH and CMV^+HIV^- subjects following stimulation with antibody opsonized stimulatory cells. While the frequency of IFN-γ and TNF-α secreting cells was higher in younger than in older CMV^+PLWH, this was not the case for CMV^+HIV^- persons. Since time on ART is significantly and positively correlated with the age of study subjects the older CMV^+PLWH would be expected to be on ART for longer than their younger counterparts. I speculate, therefore, that longer time on ART is likely to be responsible for the loss in the frequency of adapNK cells secreting these two cytokines in older CMV^+PLWH. For the CMV^+HIV^- subjects, HIV infection would play no role in contributing to the expansion of functional adapNK cells numbers nor would time on ART. A future direction for these investigations would be to perform longitudinal analyses on not only adapNK cell frequency but also on adapNK cell functionality in CMV^+PLWH to confirm that within the same individuals, over differing age ranges, that time on ART is associated with declining NK cell functionality.

5.1. What markers best characterize adapNK cells?

Several studies characterized adapNK cells as NKG2C^{+} (299, 318, 477). Other studies have used both NKG2C and CD57 to identify adapNK cells (312, 486). Still other studies have characterized adapNK cells as being negative for the intracellular FcRγ adaptor molecule; one of these used absence of intracellular Syk as a marker for adapNK cells (269, 487, 488). In this thesis, I used co-expression of NKG2C and CD57 on CD3^{-}CD56^{dim} NK cells to identify adapNK cells. I did include a polyclonal antibody specific for FcRγ in my antibody panel, and in some cases antibodies for both FcRγ and Syk were included in the antibody panel that was used to identify NKG2C^{+}CD57^{+} NK cells. The antibody detecting FcRγ is a polyclonal rabbit serum. In my experience, this antibody often detected a smear of cells with a wide range of intensities that made it challenging to distinguish between positive versus negative NK cell populations. Including antibodies specific for FcRγ and Syk did not improve my ability to distinguish cells that were positive versus negative for these markers. Different anti-FcRγ specific antibody concentrations were tried without success. In a sub-analysis, I focused on the subset of experimental results where positive cells could be distinguished from negative cells. I found that the frequency of NKG2C^{+}FcRγ^{-} NK cells did not differ significantly between CMV^{+}PLWH and CMV^{+}HIV^{-} participants. A study done by Kim et al., used positivity for NKG2C and absence of FcRγ to identify adapNK cells in CMV infected subjects regardless of HIV status. They found that the NKG2C^{+}FcRγ^{-} NK cell subpopulation had unique features compared to the other possible subsets of NK cells identified by these markers, i.e. NKG2C^{-}FcRγ^{+}, NKG2C^{+}FcRγ^{+} and NKG2C^{-}FcRγ^{-} NK cells. For example, NKG2C^{+}FcRγ^{-} NK cells were also CD57^{+} (218). Kim et al. measured the frequency of cells producing IFN-γ, TNF-α and CD107a in each of NK cell subpopulation induced by anti-CD16 coated P815 cells. They found that the highest frequency of TNF-α producing cells were the NKG2C^{+}FcRγ^{-} NK cell subset. This subset also produced a higher frequency of IFN-γ and CD107a positive cells than did NKG2C^{-}FcRγ^{+} NK cells. This suggests that using absence of the FcRγ marker may better characterize adapNK cells. However, since I was not successful in reliably distinguishing FcRγ^{+} from FcRγ^{-} cells I was unable to include this marker in the identification of adapNK cells.

5.2. Are NKG2C^{+}CD57^{+} adapNK friends or foes in the context of CVD?

There is controversy concerning the number of peripheral blood NK cells in AS. Certain authors demonstrated a decline in circulating of NK cells numbers (345-347), while others showed an elevated number of NK cells in participants with AS compared to healthy controls (348, 349). Collectively, the results in this thesis, particularly those described in chapter III, showed that a high frequency of adapNK cells were associated with no TPV and therefore protection from AS. In this analysis, I looked at the frequency of peripheral adapNK cells in CMV^{+}PLWH versus CMV^{+}HIV^{-} subject enrolled in the CHACS who were followed more intensively with annual visits and on whom CVD imaging studies were performed. All subjects were above 40 yrs of age and were receiving ART as part of the current standard of care for HIV infection. TPV was measured for all participants by using advanced software and summed to obtain the TPV. My findings showed that a high frequency of peripheral adapNK cells was associated with a TPV = 0 in persons with sub-clinical AS. One other study showed the opposite result that the frequency of adapNK cells was associated with more advanced CVD (482). Therefore, the issue as to whether adapNK cells are beneficial or detrimental in the development of AS in CMV^{+}PLWH remains an open question. One possibility that may explain these discrepant results is that I identified adapNK cells as NKG2C^{+}CD57^{+}, while Martinez-Rodriguez et al. used only NKG2C to identify adapNK cells. As well, in my study I investigated the frequency of adapNK cells in coronary plaques while Martinez-Rodriguez et al. examined adapNK cells in carotid plaques. My findings were in line with previous studies that demonstrated that the frequency of NK cells, and their function were lower in patients with AS than in a control group (345, 347). However, CMV infection was not considered in this study and the NK cells that were stained for were not identified as cNK or adapNK cells. These observations would support the proposal that either NK cells or adapNK cells are friend in the context of AS. At least in my hands, the presence of a high frequency of adapNK cells is associated with absence of AS and may prevent the development of AS. The limitations of the work presented in chapter III should be acknowledged. Although a high frequency of the adapNK cells was associated with a TPV=0, the mechanism by which these adapNK cells are associated with a TPV=0 is presently unknown. There are possible mechanisms that could be explored. NK cells have been identified within atherosclerotic plaques in humans (344). CMV infection not only drives the expansion of adapNK cells but also drives the expansion of CMV-specific CD8^{+} T cells that are associated

with higher levels of carotid intima-media thickness in PLWH. Up to 30% of all $CD8^+$ T cells in CMV^+ subjects can be CMV-specific (489). When these cells are activated, they can play a role in CVD pathogenesis by recognizing CMV epitopes that are reported to be present within AS plaque complexed with MHC-I antigens (490, 491). AdapNK cells can control CMV infection, and they may regulate CMV specific $CD8^+$ T cells, which express higher levels of HLA-E, the ligand for NKG2C (492).

5.3. Can $NKG2C^+CD57^+$ adapNK cells be used as a biomarker predicting CVD?

The use of specific biomarkers in basic research, clinical research and practice has been well characterized and repeatedly shown to correctly predict relevant clinical outcomes. In the CVD context, there exists a broad range of biomarkers associated with CVD risk, which were mentioned in chapter I such as CRP, CK and troponin. Although these biomarkers are important diagnostic tools in clinical practice, none of these biomarkers has significantly improved the distinction between health and CVD. There remain challenges in the discovery of novel biomarkers that may improve risk prediction of CVD, monitor disease progression and that can be used as biomarkers before clinical signs and symptoms appear. NK cells harbor great potential as biomarkers for several autoimmune diseases, cancer, and chronic inflammatory diseases. It has been shown that the activating and inhibitory NK receptors are essential for the regulation of NK cell activity and some of these receptors are strongly associated with autoimmunity. For instance, the KIR3DS1, KIR2DS1 and KIR2DS2 activating receptors are associated with an increased risk of in rheumatoid arthritis (RA) (493), while the inhibitory NK cell receptors, KIR2DL1 and KIR3DL1 were reported to be associated with protection from the development of RA (493). NK cell activity has been used as a biomarker for lung cancer detection in participants who were diagnosed either at an early or late stage of lung cancer. NK cell activity was significantly higher in late-stage lung cancer than in healthy controls (494). Little is known regarding NK cells as CVD biomarkers. NK cells play an important role in immune responses by producing IFN-γ (495), which is known as a pro-inflammatory cytokine. IFN-γ is likely to play both pro and anti-inflammatory roles in AS. IFN-γ is involved in multiple stage of AS such as in foam cell formation, immune responses, and plaque development (496). This observation may suggest that the IFN-γ production by NK cells can be used as a CVD marker in AS progression. In the context of adapNK cells as a biomarker for the CVD detection,

it is unclear whether the expansion of adapNK cells can be used at this stage of our knowledge as a CVD biomarker because we first need to understand the relationship between the biological process of CVD development with regard to adapNK cell numbers and function.

5.4. Does CMV co-infection increase CVD in PLWH?

It is well known that CMV infection is a risk factor associated with AS (491, 497, 498). CMV prevalence increases with age and has a high prevalence approaching 95% among PLWH. Thus, the effect of CMV infection and aging are difficult to dissociate from each other. The exact mechanisms by which CMV infection contributes to CVD remains unclear. In chapter III of this thesis, I was unable to examine the association between CMV infection and an elevated TPV in CMV^{+}PLWH compared to the $CMV^{+}HIV^{-}$ participants because of the collinearity issue that can exist between CMV serostatus and the frequency of $NKG2C^{+}CD57^{+}$ adapNK cells. This collinearity in regression analysis occurs when two of exposure variables are highly correlated to each other, such as CMV infection driving the expansion of $NKG2C^{+}CD57^{+}$ adapNK cells. This can cause problems in fitting and interpreting the results of regression models. Because of the collinearity between CMV and adapNK cells, it was impossible to investigate the impact of CMV infection in PLWH co-infected with CMV in CHACS participants.

5.5 The potential for $NKG2C^{+}CD57^{+}$ adapNK cells in cancer immunotherapies

Chimeric antigen receptor (CAR) T cells have achieved some success in the clinic, particularly as immunotherapies for hematologic cancers. This has prompted the development of CAR-NK cells for cancer immunotherapy. Although CAR-NK cells are at an earlier stage of development, they have several unique features that support a better safety profile and off-the-shelf properties than their CAR-T cell counterparts. CAR-NK cells may work in situations where CAR-T cells fail. NK cells have spontaneous cytotoxic activity against infected cells, tumor cells and stressed cells that have downmodulated MHC-I antigens. This allows them to kill target cells independently of tumor antigen recognition. In contrast, CAR-T cells only kill their targets following CAR-specific antigen recognition (499). Not only can NK cells become activated and kill target cells that have lost MHC-I, but they are also active against allogeneic cells, allowing a batch of CAR-NK cells to be used to treat several cancer patients, a feature bringing the concept

of off-the-shelf therapy closer to reality. Unlike T cells, NK cells can serially kill multiple target cells (500). The use of CAR-T cells is associated with significant toxicities including cytokines release syndrome (CRS) and neurotoxicity. CRS is the most serious side effect of CAR-T cells that leads to multiple organ dysfunctions (501). Other major advantages of CAR-NK cells is that their use is associated with a lower risk of graft-versus host disease (GVHD) and lower CRS and neurotoxicity than CAR-T cells (499). CAR-NK cells can also be used with infusion of antibodies specific for tumor antigens in order to induce ADCC activity directed towards tumor specific antigens.

Although only a few pre-clinical and clinical studies have been conducted using CAR-NK cells, data collected thus far has been encouraging and supports the use of CAR-NK cells to treat hematological and solid tumors expressing CD19, CD20, CD244 (2B4) and HER2. For example, CAR-NK cells recognizing CD19 and CD20 target B cells malignancies (502-504). Another attractive example of CAR-NK cells is their use to treat HER2 expressing tumors, which showed a high level of cytotoxicity against breast and ovarian cancer expressing HER2 (505).

$NKG2C^{+}CD57^{+}$ adapNK cells have been implicated in anti-tumor activity. It has been reported by Merion et al, that in lymphoid malignancy patients who were CMV seropositive and who underwent allogeneic hematopoietic cell transplantation (AHCT), a high absolute number of adapNK cells were generated after AHCT. This suggests that adapNK cell expansion was associated with low acute myeloid leukemia (AML) relapse (506). Multiple myeloma cells are sensitive to NK cell mediated killing but less is known about the role of adapNK cells against myeloma. Interestingly, myeloma cells expressed high level of HLA-E, which is the ligand for the activating receptor NKG2C that is expressed on adapNK cells. The interaction between NKG2C and its HLA-E ligand on the surface of myeloma cells leads to adapNK cell activation that may explain the anti-myeloma effect of adapNK cells (477, 507). The role of expression of CD57 NK cells is implicated in cancer immunosurveillance. The inhibition of GSK3 kinase in NK cells in the presence of IL-15 leads to CD57 upregulation that is associated high level of IFN-γ and TNF-α secretion and increased ADCC activity in response to target cells and solid tumor cell lines (508). In sum, there is a great deal of enthusiasm directed towards developing CAR-NK and CAR-adapNK cell-based therapies for cancer immunotherapy.

5.5. Final conclusion and future direction

In conclusion, data presented in this thesis characterized the frequency of adapNK cells and their functionality in CMV^+PLWH and CMV^+HIV^- subjects among those who were above versus below 40 yrs and with known duration of time on ART. When CMV^+PLWH and CMV^+HIV^- subjects were compared, HIV infection did not compromise the capacity of adapNK cells to produce cytokines and chemokines, at least compared to within individual cNK cell, in response to anti-HIV Env specific Ab opsonized HIV infected cells. Results presented here provide new insight into the role of adapNK cells in the context of AS in PLWH co-infected with CMV. High level of adapNK cell frequencies can be a good marker for prediction of CVD outcomes in PLWH. We found that there was no association either between *NKG2C* genotypes and protection from HIV infection in HESN persons at risk for HIV exposure. Neither *NKG2C* genotypes nor the frequency of adapNK cells was associated with HIV VL control in PLWH.

With regard to adapNK cells in CVDs, our findings alone cannot definitely prove that adapNK cells are associated with protection from CVD because our *in vitro* studies focused solely on peripheral adapNK cells. What needs to be done in the future is more research to characterize adapNK cells and their functionality in AS plaques to complement what has been described here. See figure 5.1. Such information could serve as a springboard toward further defining protective mechanisms used by adapNK cells to modulate HIV, other infectious diseases, and cancer.

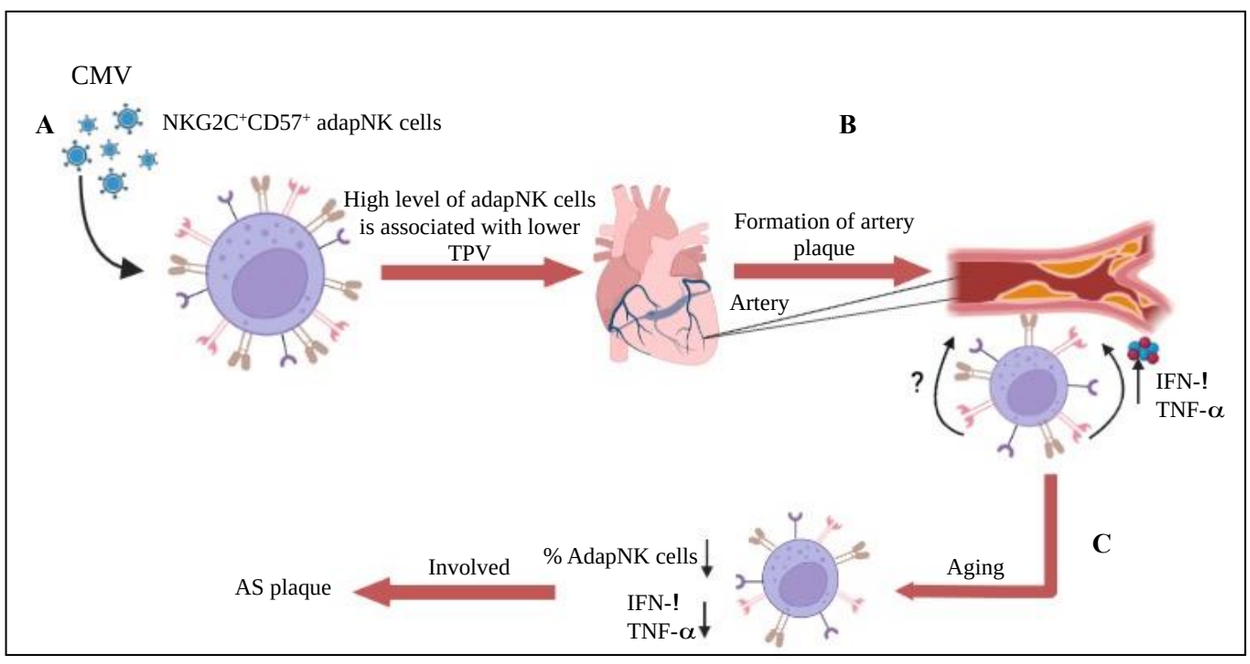

Figure 1.5. Mechanism regulated by peripheral blood adapNK cells in AS plaque. (Made by K Alsulami using BioRender.com).

A) CMV infection drives the expansion of adapNK cells in CMV infected individuals. High frequencies of adapNK cells are associated with a TPV=0. B) In the AS plaque area, CMV viremia activates adapNK cells expressing NKG2C receptors by binding to their CMV derived epitope complexed to HLA-E present in AS plaques, which induced adapNK cells to produce cytokines such as IFN-γ and TNF-α to lyse AS plaque. C) With aging or an extended duration of time on ART, adapNK cell frequency and their functionality decline, a phenomenon that may be associated with AS plaque formation. However, the presence of adapNK cells and the function of these cells within AS plaques still needs to be elucidated.

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